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Document downloaded from:

<http://hdl.handle.net/10459.1/63058>

The final publication is available at:

<https://doi.org/10.1007/s12035-017-0710-4>

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# Regulation of Survival Motor Neuron Protein by the Nuclear Factor-Kappa B Pathway in Mouse Spinal Cord Motoneurons

Saravanan Arumugam,<sup>1</sup>

Stefka Mincheva-Tasheva,<sup>1</sup>

Ambika Periyakaruppiyah,<sup>1</sup>

Sandra de la Fuente,<sup>1</sup>

Rosa M. Soler,<sup>1</sup>

Phone +34 973 70 22 64 Email rosa.soler@cmb.udl.cat

Ana Garcera,<sup>1</sup>

<sup>1</sup> Unitat de Senyalització Neuronal, Dep. Medicina Experimental, Universitat de Lleida-IRBLLEIDA, Rovira Roure 80, 25198 Lleida, Spain **AQ1**

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## Abstract

Survival motor neuron (SMN) protein deficiency causes the genetic neuromuscular disorder spinal muscular atrophy (SMA), characterized by spinal cord motoneuron degeneration. Since SMN protein level is critical to disease onset and severity, analysis of the mechanisms involved in SMN stability is one of the central goals of SMA research. Here, we describe the role of several members of the NF- $\kappa$ B pathway in regulating SMN in motoneurons. NF- $\kappa$ B is one of the main regulators of motoneuron survival and pharmacological inhibition of NF- $\kappa$ B pathway activity also induces mouse survival motor neuron (Smn) protein decrease. Using a lentiviral-based shRNA approach to reduce the expression of several members of NF- $\kappa$ B pathway, we observed that IKK and RelA knockdown caused Smn reduction in mouse-cultured motoneurons whereas IKK or RelB knockdown did not. Moreover, isolated motoneurons obtained from the severe SMA mouse model showed reduced protein levels of several NF- $\kappa$ B members and RelA phosphorylation. We describe the alteration of NF- $\kappa$ B pathway in SMA cells. In the context of recent studies suggesting regulation of altered intracellular pathways as a future pharmacological treatment of SMA, we propose the NF- $\kappa$ B pathway as a candidate in this new therapeutic approach.

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## Keywords

SMN

NF-kappaB

Motoneurons

Spinal muscular atrophy

RelA

Rosa M. Soler and Ana Garcera last co-authors should be regarded as joint corresponding authors

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## Introduction

The NF- $\kappa$ B (nuclear factor- $\kappa$ B) intracellular pathway is involved in physiological processes of the nervous system such as regulation of apoptosis, neurite outgrowth and synaptic plasticity [1, 2, 3, 4]. A ubiquitously expressed transcription factor system, NF- $\kappa$ B consists of homodimers and heterodimers of five structurally related proteins: RelA/p65, RelB, c-Rel, p50 and p52, of which the p50/RelA heterodimer is the most abundant [5, 6]. In the absence of stimuli, NF- $\kappa$ B homo- or heterodimers are present in the cytoplasm and form inactive complexes with their inhibitors, the I $\kappa$ B (Inhibitor  $\kappa$ B) protein family. When the pathway is activated, phosphorylation of I $\kappa$ B induces p50/RelA release and translocation to the nucleus. I $\kappa$ B is phosphorylated by the kinases of the inhibitor (I $\kappa$ B kinases), IKK $\alpha$  and IKK $\beta$  [7]. RelA can be phosphorylated at several sites inducing conformational changes which impact RelA ubiquitination, stability and protein-protein interactions. Phosphorylated RelA in S536 has lower affinity for I $\kappa$ B $\alpha$ , which results in increased nuclear translocation and accumulation of p65 [8]. In the nervous system, NF- $\kappa$ B activation regulates genes and proteins involved in several neuronal processes, such as Bcl-x<sub>L</sub> [9], BDNF [10], NCAM [11] and Cu/Zn-SOD [12]. The canonical NF- $\kappa$ B activation regulates neuronal and motoneuron (MN) cell survival [13, 14], and evidences are emerging about its role in neurodegenerative disorders and neuronal injury [6, 15]. Based on NF- $\kappa$ B functions, it has been suggested that pharmacological regulation of NF- $\kappa$ B activity could be beneficial in some of these diseases [16].

Spinal muscular atrophy (SMA) is a genetic neuromuscular disorder characterized by the degeneration of MNs in the anterior horn of the spinal cord, together with muscular atrophy and weakness. SMA results from survival motor neuron (SMN) protein deficiency. In humans, SMN is encoded by the *SMN* gene, which is present in multiple copies, one telomeric copy of *SMN1* and several centromeric copies of *SMN2*. SMA is caused by homozygous disruption of the *SMN1* gene by deletion, conversion, or mutation [17]. *SMN1* expresses a full-length transcript, and *SMN2* expresses primarily a truncated isoform that is unable to compensate *SMN1* deficiency. SMN protein level is critical to disease onset and severity and is determined in part by the *SMN2* copy numbers [18]. One of the central

objectives of developing SMA therapeutics is to identify mechanisms that increase SMN protein levels [19], which require a basic understanding of SMN protein regulation. In the present study, we characterized the contribution of several members of the NF- $\kappa$ B pathway in regulating mouse survival motor neuron (Smn) protein level in cultured spinal cord MNs. Pharmacological inhibition of NF- $\kappa$ B pathway activity induced Smn protein decrease. Using a lentiviral-based short-hairpin RNA (shRNA) approach to reduce the expression of several members of the NF- $\kappa$ B pathway, we observed that IKK $\alpha$  and RelA knockdown caused Smn protein reduction whereas IKK $\beta$  or RelB knockdown did not. Isolated and cultured MNs from severe SMA mouse model showed reduced protein levels in several members of NF- $\kappa$ B and RelA phosphorylation. Our results suggest that NF- $\kappa$ B pathway activation regulates SMN protein level in spinal cord MNs.

## Materials and Methods

### Mouse MN Isolation and Culture

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MN cultures were prepared from embryonic 12.5-day (E12.5) CD1 male and female mouse spinal cords essentially as described [20, 21]. Isolated cells were pooled in a tube containing culture medium and plated [21]. Cultured MNs enriched by Iodixanol were clearly identified by morphological criteria. All of the procedures were in accordance with the Spanish Council on Animal Care and approved by the University of Lleida Advisory Committee on Animal Services.

Isolated MNs were plated in four-well tissue culture dishes (Nunc, Thermo Fisher Scientific) in a density of 15,000 cells per well for survival and immunofluorescence experiments, 70,000 cells per well for western blot analysis and 300,000 cells per well for qRT-PCR. Complete culture medium was Neurobasal (Gibco, Invitrogen) supplemented with B27 (Gibco, Invitrogen), horse serum (2% v/v), L-glutamine (0.5 mM), and 2-mercaptoethanol (25  $\mu$ M). Cells were plated and maintained with complete medium containing a cocktail of recombinant NTFs: 1 ng/ml brain-derived neurotrophic factor, 10 ng/ml glial cell line-derived neurotrophic factor, 10 ng/ml cardiotrophin-1, and 10 ng/ml hepatocyte growth factor (Peprotech).

Survival evaluation was performed using photomicrographs of different microscopic areas from each dish (4 central areas per well, 3 wells for each condition), and counting the number of large-phase bright neurons with long neurite processes present in the photomicrographs. Survival was expressed as the percentage of cells counted 3 days after treatment with respect to the initial value (3 h after plating, 100%).

### SMA Animals

Severe SMA mice FVB.Cg-Tg(SMN2)<sup>89Ahmb</sup>Smn1<sup>tm1Msd</sup>/J were kindly provided by Dr.

Josep E Esquerda (IRBLLEIDA-Universitat de Lleida). Heterozygous animals were crossed to obtain homozygous *Smn*<sup>-/-</sup>;SMN2<sup>+/+</sup> (mutSMA). Littermates mutSMA and *Smn*<sup>+/+</sup>;SMN2<sup>+/+</sup> (WT) were used for the experiments. For MN purification, E13 embryos were removed from the uterus and a piece was snipped from the head for genotyping. The REDExtract-N-Amp Tissue PCR Kit (Sigma) was used for genomic DNA extraction and polymerase chain reaction setup, with the following primers: WT forward 5' CTCCGGATATTGGGATTG 3', SMA reverse 5' GGTAACGCCAGGGTTTTCC 3' and WT reverse 5'TTTCTTCTGGCTGTGCCTTT 3'. After genotyping, WT and mutSMA embryos were submitted to spinal cord dissection, MN isolation and culture as described above.

## Immunofluorescence of RelA Nuclear Translocation

The determination of cellular localization of RelA into MNs was performed as previously described [13]. Briefly, cultures were fixed 5 days after lentivirus transfection in 4% paraformaldehyde and 100% methanol, incubated overnight at 4 °C with a polyclonal anti-RelA antibody (1:100; Santa Cruz Biotechnology), and consecutively with an anti-rabbit secondary antibody conjugated with Alexa Fluor 488 (Invitrogen). Hoechst 33,258 staining was used for nuclear localization. The images were obtained using a FluoView 500 Olympus confocal microscope.

## Western Blot Analysis

Western blot analysis was performed as previously described [22]. Cells were rinsed in ice-cold PBS (pH 7.2) after stimulation or lentiviral transduction. Total cell lysates were collected and resolved in SDS-polyacrylamide gels and transferred onto polyvinylidene difluoride Immobilon-P transfer membrane filters (Millipore, Billerica, MA) using an Amersham Biosciences (Piscataway, NJ, USA) semidry Trans-Blot according to manufacturer instructions. Membranes were blotted with the specific antibodies anti-RelA (1:1000), anti-phosphoRelA (Ser536) (1:1000) or anti-CREB (1:1000) from Cell Signaling Technology; anti-SMN (1:5000) from BD Transduction Laboratories; anti-IKK $\beta$  (1:1000) or anti-IKK $\alpha$  (1:1000) from Calbiochem or anti-RelB (1:1000) (C-19) from Santa Cruz, following the instructions of the providers. To control the specific protein content per lane, unless stated otherwise, the membranes were re-probed with a monoclonal anti- $\alpha$ -tubulin (Tub) antibody (1:50,000, Sigma), as described by the provider. Blots were developed using the Super Signal chemiluminescent substrate (Pierce, Rockford, IL, USA) or the ECL Advance Western Blotting detection kit (Amersham Biosciences).

NF- $\kappa$ B inhibitor SN50, the inactive control peptide SN50M, and the proteasome inhibitor MG132 were purchased from Calbiochem.

## RNA Isolation and Quantitative RT-PCR

For qRT-PCR analysis, MNs (300,000 cells/condition) were plated and transduced with

shRNA. Total RNA was extracted using the NucleoSpin® RNA columns (Machery-Nagel, Düren, Germany) according to manufacturer instructions. One microgram of total RNA from each condition was reverse-transcribed to complementary (cDNA), and 25 ng was used for each individual RT-PCR reaction. The assays were performed in a CFX96 Real-Time System (Bio-Rad) using iTaq™ Universal SYBR® Green Supermix from Bio-Rad. Real-Time was performed using SMN-specific primers: SMN exon 1-forward (5'-GATGATTCTGACATTTGGGATG-3') and SMN Exon 2-reverse (5'-TGGCTTATCTGGAGTTTCAGAA-3') and specific primers of glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*): forward (5'-TGCACCACCAACTGCTTAG-3') and reverse (5'-GGATGCAGGGATGATGTTC-3') as internal control. Quantification was completed using iCycler IQ real-time detection system software (version 2.3, Bio-Rad). Relative expression ratios were calculated on the basis of  $\Delta C_p$  values with efficiency correction based on multiple samples.

## Plasmids and Production of Lentiviral Particles

Lentiviral-based vectors for RNA interference-mediated gene silencing (FSVi) were performed as described [23]. FSVi consisted of a U6 promoter for expression of short-hairpin RNAs (shRNAs) and the Venus variant of green fluorescent protein (GFP) under the control of an SV40 promoter to monitor transduction efficiency. Lentiviruses were propagated in HEK293T cells using the polyethylenimine (PEI, Sigma) cell transfection method. Twenty micrograms of the above plasmids containing the shRNAs or the empty vector (FSV), 13  $\mu$ g of pSPAX2 and 7  $\mu$ g of pM2G were transfected to HEK293T cultures. Cells were allowed to produce lentivirus for 4 days. Then, the medium was centrifuged at 1200 $\times$ g for 5 min and the supernatant was filtered using a 45  $\mu$ m filter. The medium containing the lentiviruses was stored at 4 °C. Biological titres of the viral preparations, expressed as the number of transducing units per ml (TU/ml), were determined by transducing HEK293T cells in limiting dilutions. After 48 h, the percentage of GFP-positive cells was measured and viruses at  $4 \times 10^5$ – $1 \times 10^6$  TU/ml were used for the experiments. For lentiviral transduction, MNs were plated in four-well dishes and after 2 h, the medium containing lentivirus (2 TU/cell) was added. The medium was changed 20 h later and infection efficiency was monitored in each experiment by direct-counting GFP-positive cells. The frequency of infection rose 99%. RNA interference efficiency was monitored by western blot analysis using specific antibodies.

The following sequences were used for generation of shRNAs: IKK $\alpha$ : 5'-CAGGCTCTTTCAGGGCAT-3'; IKK $\beta$ : 5'-GCTGCACATTTGAATCTGTAT-3'; RelA: 5'-AGATCTTGAGCTCGGCAGTG-3'; RelB: 5'-GCATGCGCTTCCGCTACGAGT-3'; CREB: 5'-GTGGATAGTGTAAGTACTGATT-3'. shRNAs of IKKs and RelA have been previously described [24].

## Statistical Analysis

All experiments were performed at least three times. Values were expressed as mean  $\pm$  SEM.

The data obtained from the independent experiments were used for statistical analysis.

We used one-way analysis of variance (ANOVA) to assess differences between groups for variable treatment. If the ANOVA test was statistically significant, we performed “post-hoc” pairwise comparisons using the Bonferroni test. We used the Student *t* test to compare the means of two groups. *P* values < 0.05 were considered significant.

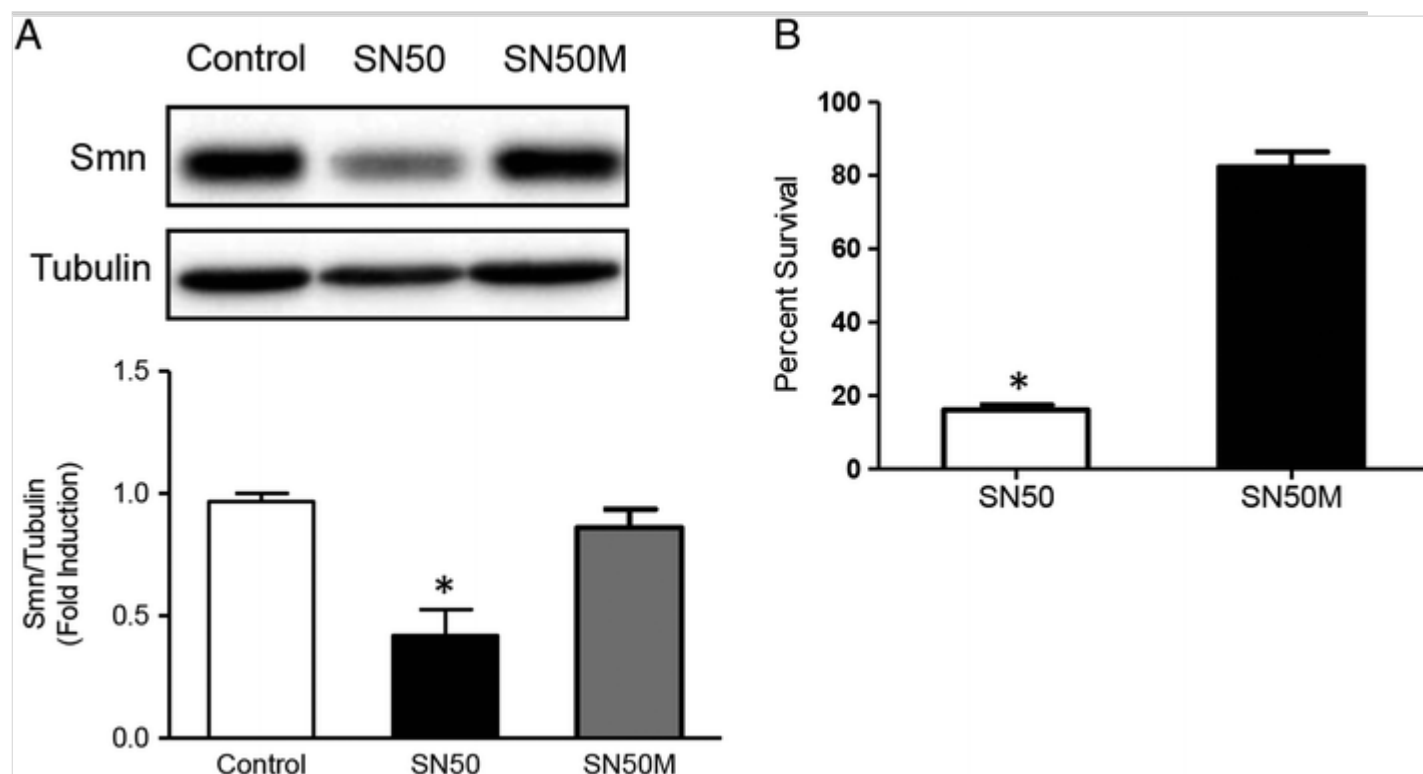
## Results

### NF- $\kappa$ B Inhibitor SN50 Reduces Smn Protein Level in Cultured Spinal Cord MNs

Previous studies have shown that NF- $\kappa$ B pathway contributes to neurotrophic factor (NTF)-mediated MN survival [13]. To further investigate the role of NF- $\kappa$ B in MN physiology and Smn regulation, we pharmacologically inhibited the NF- $\kappa$ B pathway using the SN50 inhibitor peptide and analysed Smn levels in protein extracts of cultured MNs. SN50, a cell-permeable peptide that blocks the nuclear localization sequence of p50, inhibits the translocation of NF- $\kappa$ B active complex to the nucleus [25]. MNs were isolated from E12.5 mouse embryos and maintained in the presence of the NTF cocktail (1 ng/ml BDNF, 10 ng/ml GDNF, 10 ng/ml CNTF and 10 ng/ml HGF). Cultures were left untreated (control) or treated with 30  $\mu$ M SN50 or its inactive control peptide, SN50M. Two days later, cell extracts were collected and analysed by western blot using a specific antibody against Smn (Fig. 1). The presence of the NF- $\kappa$ B inhibitor in the culture medium significantly decreased Smn protein level, whereas its negative control SN50M did not induce significant changes in Smn ( $0.42 \pm 0.1$  SN50 vs control  $p < 0.05$  and  $0.86 \pm 0.07$  SN50M vs control). Survival evaluation after 2 days of treatment showed a significant decrease from baseline in the number of MNs in SN50-supplemented cultures, compared with SN50M ( $16.1 \pm 1.3\%$  SN50 and  $82.3 \pm 4.1\%$  SN50M,  $p < 0.0001$ ) and an increase of apoptotic nuclei (data not shown). These results indicate that the pharmacological inhibition of the NF- $\kappa$ B pathway reduces survival and Smn protein level in cultured mouse spinal cord MNs.

#### Fig. 1

Effect of NF- $\kappa$ B inhibitor SN50 on Smn protein level. **In the text and figure legends the labels "a, b, c" are lower case, but in the images are capital. Is it correct?** **a** Representative Western blots for Smn and  $\alpha$ -tubulin (loading control) in protein extracts of MNs treated during 2 days with the NF- $\kappa$ B inhibitor SN50 (30  $\mu$ M) and its inactive control SN50M (30  $\mu$ M). Graph values indicate the expression of Smn vs tubulin and correspond to the quantification of three independent experiments  $\pm$  SEM (error bars). Asterisk indicates significant differences using one-way ANOVA test and Bonferroni post hoc multiple comparisons ( $p < 0.05$ ). **b** MNs were cultured in the presence of SN50 (30  $\mu$ M) or SN50M (30  $\mu$ M). Cell survival was evaluated 2 days after treatment. Graph values are the mean of the percentage of cell survival for each condition from three independent experiments  $\pm$  SEM. Asterisk indicates significant differences using Student *t* test ( $p < 0.001$ )



## RelA, But Not RelB, Inhibition Causes Smn Reduction in MNs in the Presence of NTFs

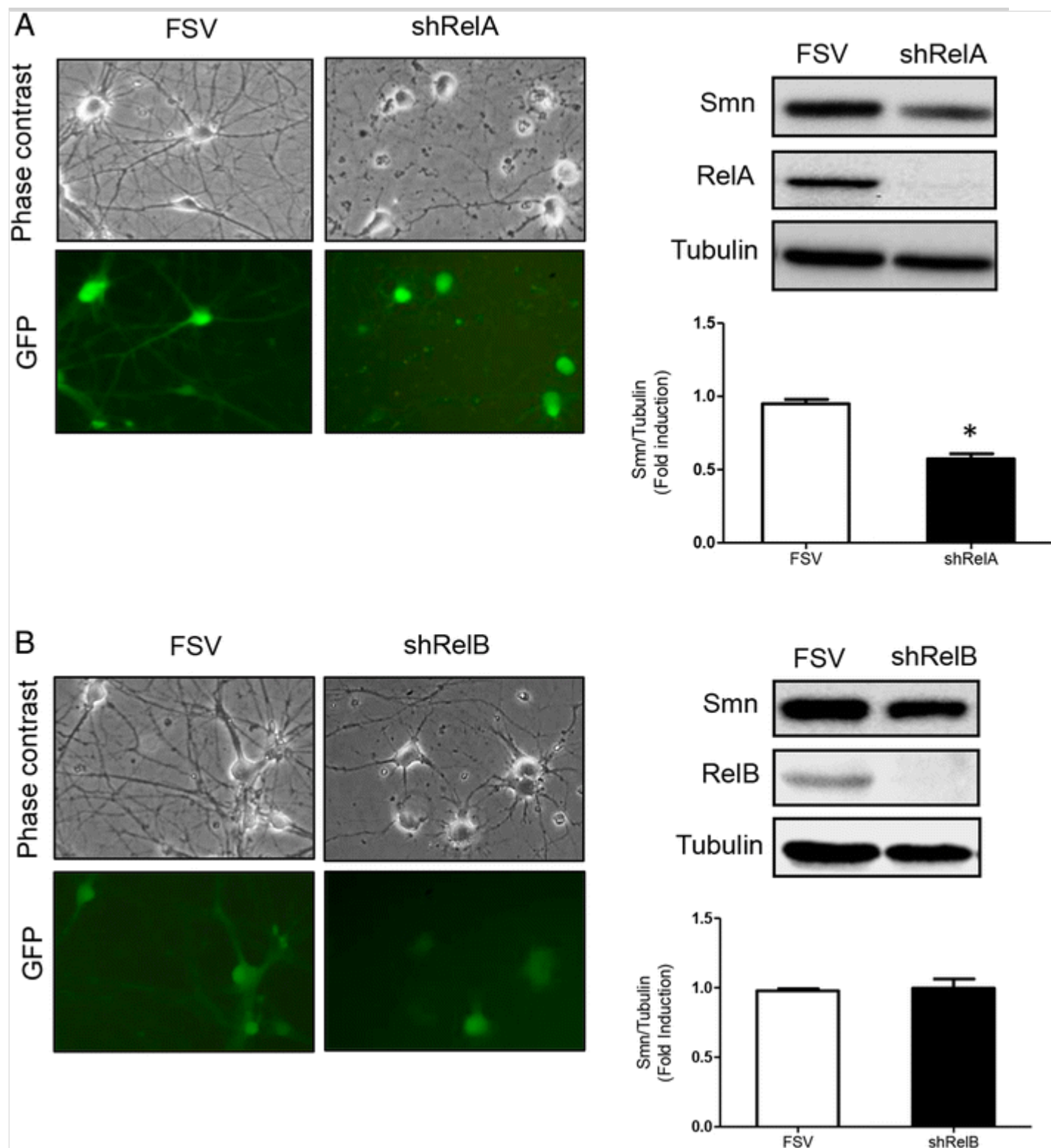
To determine whether the canonical or non-canonical NF- $\kappa$ B pathways are relevant in Smn protein regulation in MNs, we analysed the effect of RelA or RelB knockdown. The RelA/p50 heterodimer is activated in the canonical and RelB/p52 in the non-canonical pathway [7]. Therefore, we used shRNA targeting specific sites of mouse RelA (shRelA) or RelB (shRelB) sequences to block the canonical or non-canonical pathways, respectively [13]. MNs were isolated from E12.5 mouse embryos and maintained in the presence of NTFs. Two hours after plating, culture medium was changed and medium containing NTFs plus lentivirus of shRelA or shRelB or shRNA empty vector (FSV) was added. Twenty hours later, the lentivirus-containing medium was washed and replaced by fresh medium supplemented with NTFs. Fluorescence microscopy of GFP-positive cells showed nearly 100% transduction (Fig. 2). After 5 days, western blot analysis of cultures transduced with the lentivirus carrying the shRelA exhibited a significant reduction in Smn protein ( $0.57 \pm 0.03$ ;  $p < 0.05$ ) whereas cultures transduced with shRelB showed no change ( $0.99 \pm 0.06$ ) (Fig. 2a, b, respectively). Control western blots of RelA or RelB protein revealed significant reductions in both cases, under shRelA or shRelB conditions, respectively. These results showed NF- $\kappa$ B pathway regulating Smn protein level by the canonical pathway, but not by the non-canonical pathway.

### Fig. 2

RelA, but not RelB, knockdown reduced Smn protein level in cultured spinal cord MNs. Mouse MNs were transduced with lentivirus containing the shRelA, shRelB or empty vector (FSV) constructs. **a** Representative microscopy images of 5-day shRelA- or FSV-transduced



cells: phase contrast (top) and GFP (bottom). Total cell lysates were probed by western blot with anti-SMN or anti-RelA antibodies and reprobed using an antibody against  $\alpha$ -tubulin. Graph values represent the expression of Smn vs  $\alpha$ -tubulin and correspond to the quantification of three independent experiments  $\pm$  SEM. Asterisk indicates significant differences using Student *t* test ( $p < 0.05$ ). **b** Representative microscopy images of 5 days shRelB- or FSV-transduced cells: phase contrast (top) and GFP (bottom). Total cell lysates were probed by western blot with anti-SMN or anti-RelB antibodies and reprobed using an antibody against  $\alpha$ -tubulin. Graph values represent the expression of Smn vs  $\alpha$ -tubulin and correspond to the quantification of three independent experiments  $\pm$  SEM

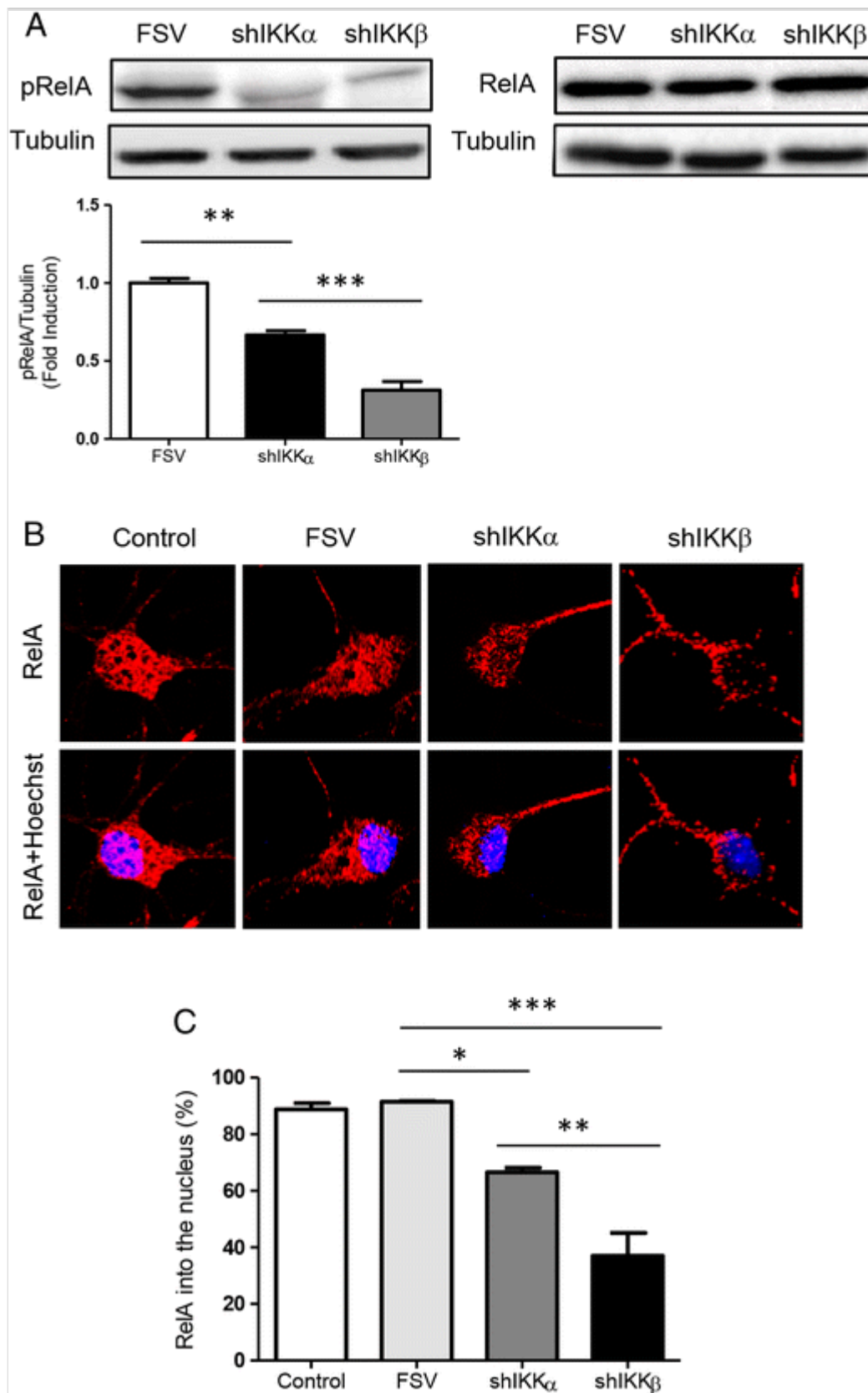


## Both IKK $\alpha$ and IKK $\beta$ Knockdown Prevent RelA Phosphorylation and Nuclear Translocation in Cultured MNs

RelA phosphorylation and nuclear translocation is a key event in canonical NF- $\kappa$ B activation and target gene regulation. To analyse the effect of IKK $\alpha$  or IKK $\beta$  reduction on RelA phosphorylation and translocation to the nucleus, MNs were transduced with shIKK $\alpha$ , shIKK $\beta$  or empty vector (FSV) or were left non-transduced (control). After 5 days, cultures were submitted to western blot analysis using anti-phosphoRelA (pRelA) or anti-RelA antibodies or were fixed to determine RelA localization by immunofluorescence using an anti-RelA antibody. RelA phosphorylation was significantly reduced in shIKK $\alpha$  ( $0.66 \pm 0.02$ ,  $p < 0.001$ ) and shIKK $\beta$  ( $0.31 \pm 0.05$ ,  $p < 0.0001$ ) cultures compared with the FSV control (Fig. 3a). It was also evident that pRelA level was significantly reduced in shIKK $\beta$  compared with shIKK $\alpha$  cultures ( $p < 0.001$ ). However, no differences were observed in total RelA level, indicating that the reduction of RelA phosphorylation was not due to RelA protein decrease.

### Fig. 3

Effect of IKK knockdown on RelA phosphorylation and nuclear translocation. Mouse MNs were transduced with lentivirus containing the shIKK $\alpha$ , shIKK $\beta$  or empty vector (FSV) constructs. **a** Representative western blot for phospho-Ser536 RelA (pRelA) and total RelA in protein extracts of 5-day transduced cultures. Membranes were reprobed using an antibody against  $\alpha$ -tubulin. Graph values represent the expression of p-RelA or RelA vs  $\alpha$ -tubulin and correspond to the quantification of three independent experiments  $\pm$  SEM. **b** Five-day transduced cells were fixed and immunofluorescence was performed using an anti-RelA antibody. Representative confocal images of p65 (red) and Hoechst nuclear staining (blue). Graph represents the percentage of cells with nuclear RelA in each condition and corresponds to the quantification of three independent experiments  $\pm$  SEM. In **a** and **b**, asterisks indicate significant differences using one-way ANOVA test and Bonferroni's post hoc multiple comparisons ( $***p < 0.0001$ ,  $**p < 0.001$ ,  $*p < 0.01$ )



In control cultures, immunofluorescent confocal images of cultured MNs using an anti-RelA antibody showed RelA localization clearly distinguishable into the nucleus (Fig. 3b). Hoechst33258 was used as a nuclear marker. We measured the percentage of MNs with nuclear RelA in control, FSV, shIKK $\alpha$  and shIKK $\beta$  cultures. Cellular distribution showed a reduced percentage of MNs with nuclear RelA in shIKK $\alpha$  ( $66.5 \pm 1.6\%$ ) and shIKK $\beta$  ( $37.1 \pm 8.1\%$ ) conditions, compared to FSV ( $91.5 \pm 0.2\%$ ;  $p < 0.01$  and  $p < 0.0001$ , respectively). No significant differences in nuclear RelA were observed in non-lentivirus

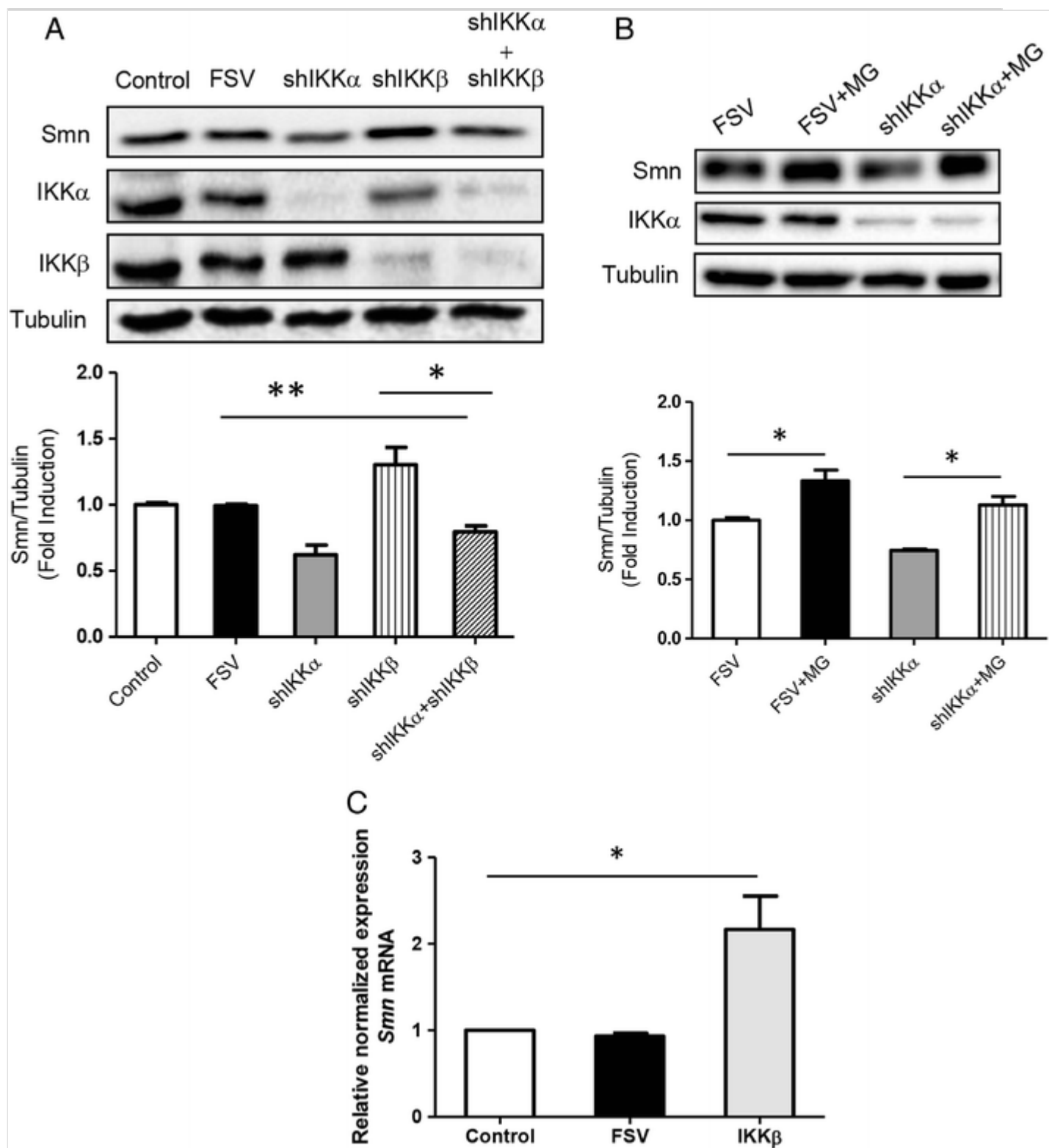
treated (control) and FSV conditions ( $88.8 \pm 2.2$  and  $91.5 \pm 0.4\%$ , respectively). These results also indicate a decreased percentage of nuclear RelA in IKK $\beta$  knockdown cells, compared with shIKK $\alpha$  cultures ( $p < 0.001$ ), supporting the hypothesis that IKK $\beta$  is the main kinase responsible for RelA phosphorylation.

## Inhibiting IKK $\alpha$ Vs IKK $\beta$ has Opposite Effect on Smn Protein Level in MNs

Because the activation of the canonical NF- $\kappa$ B pathway depends upon the phosphorylation status of the two kinases IKK $\alpha$  and IKK $\beta$ , we analysed the effect of IKK knockdown on the Smn level of cultured MNs. To this end, we produced lentivirus containing the shRNA targeting specific sites of mouse IKK $\alpha$  (shIKK $\alpha$ ) or IKK $\beta$  (shIKK $\beta$ ), as previously described [13]; MNs were isolated and transduced following the same protocol. After 5 days, cultures transduced with shIKK $\alpha$  or shIKK $\beta$  exhibited a strong reduction in IKK $\alpha$  or IKK $\beta$  protein, respectively, compared with the empty vector condition (FSV). Western blot analysis of protein extracts of shIKK $\alpha$  condition showed a significant reduction in Smn protein ( $0.62 \pm 0.07$ ,  $p < 0.001$ ) compared to the FSV or non-transduced (control) conditions (Fig. 4a). To discard changes in Smn after lentiviral transduction, protein extracts from non-transduced cultures were submitted to western blot; no differences were observed when compared with the FSV condition. When cells were transduced with the shIKK $\beta$ , Smn protein level was significantly increased ( $1.3 \pm 0.13$ ,  $p < 0.005$ ) compared to the controls (Fig. 4a). These findings demonstrated that both IKK $\alpha$  and IKK $\beta$  regulate Smn level in MNs. Nevertheless, IKK $\alpha$  reduction decreases Smn level whereas IKK $\beta$  knockdown increases Smn count.

### Fig. 4

Smn protein and mRNA regulation by IKK knockdown. MNs were transduced with lentivirus containing the shIKK $\alpha$ , shIKK $\beta$ , shIKK $\alpha$  + shIKK $\beta$  or empty vector (FSV) constructs. **a** Protein extracts of 5-day transduced cells were submitted to western blot using anti-SMN, anti-IKK $\alpha$  and anti-IKK $\beta$  antibodies. **b** Five-day FSV and shIKK $\alpha$  transduced cultures were treated (12 h) with 2.5  $\mu$ M MG132. Protein extracts were submitted to western blot using anti-SMN and anti-IKK $\alpha$  antibodies. In **a** and **b**, membranes were reprobated with an anti- $\alpha$ -tubulin antibody. Graph values represent the expression of Smn vs  $\alpha$ -tubulin and correspond to the quantification of 5 (**a**) or 3 (**b**) independent experiments  $\pm$  SEM. Asterisks indicate significant differences using one-way ANOVA test and Bonferroni's post hoc multiple comparisons ( $*p < 0.05$ ). **c** Total RNA was extracted from 5-day FSV-, shIKK $\alpha$ - and non-transduced cultures and reverse-transcribed to cDNA. *Gapdh* gene was used as a control. Graph values are the mean of *Smn* gene expression for each from three independent experiments  $\pm$  SEM. Asterisks indicate significant differences using one-way ANOVA test and Bonferroni's post hoc multiple comparisons ( $*p < 0.05$ )



Because IKK $\alpha$  and IKK $\beta$  knockdown produced opposite effects on Smn levels, we analysed the effect of shIKK $\alpha$  and shIKK $\beta$  co-transduction on Smn protein level. Five days after, shIKK $\alpha$  and shIKK $\beta$  co-transduction protein lysates were obtained and submitted to western blot analysis (Fig. 4a). Results revealed that Smn protein was not significantly altered in co-transduced cultures compared with FSV or non-transduced controls. However, Smn level in the co-transduced condition ( $0.82 \pm 0.08$ ) was significantly reduced ( $p < 0.05$ ) in comparison with shIKK $\beta$  ( $1.03 \pm 0.13$ ). Therefore, IKK $\alpha$  knockdown clearly prevents Smn increase caused by IKK $\beta$  reduction.

To further analyse *Smn* regulation by IKK $\alpha$  knockdown, we evaluated the effect of proteasome inhibition. Previous studies have shown that SMN degradation is mediated via the ubiquitin/proteasome pathway in SMA patient-derived fibroblasts [26, 27]. Cells were transduced with FSV or shIKK $\alpha$ . Five days after transduction, cultures were treated for 12 h with 2.5  $\mu$ M of the proteasome inhibitor MG132. Protein extracts were submitted to western blot using the anti-SMN antibody. Under both FSV and shIKK $\alpha$  conditions, *Smn* protein level was increased in MG132-treated cells compared with the non-treated FSV or shIKK $\alpha$  condition ( $1.33 \pm 0.09$ -fold induction and  $1.13 \pm 0.07$ -fold induction, respectively,  $p < 0.05$ ) (Fig. 4b). This result suggests the *Smn* decrease caused by IKK $\alpha$  knockdown occurs as a result of the proteasome activity.

Next, to determine whether the *Smn* increase caused by IKK $\beta$  knockdown was associated with activation of *Smn* gene expression, we quantified *Smn* messenger RNA (mRNA) by quantitative RT-PCR (qRT-PCR). *Gapdh* gene was used as a control. Cultured MNs were transduced with FSV or shIKK $\beta$  lentiviral constructs. After 4 days, total RNA was extracted and reverse-transcribed to cDNA, used as a template to quantify *Smn* transcript level. IKK $\beta$  reduction was related to an increase in *Smn* mRNA expression ( $2.17 \pm 0.38$ -fold induction,  $p < 0.05$ ) compared with control conditions (non-transduced and FSV), showing that IKK $\beta$  knockdown regulates *Smn* at the transcriptional level (Fig. 4c).

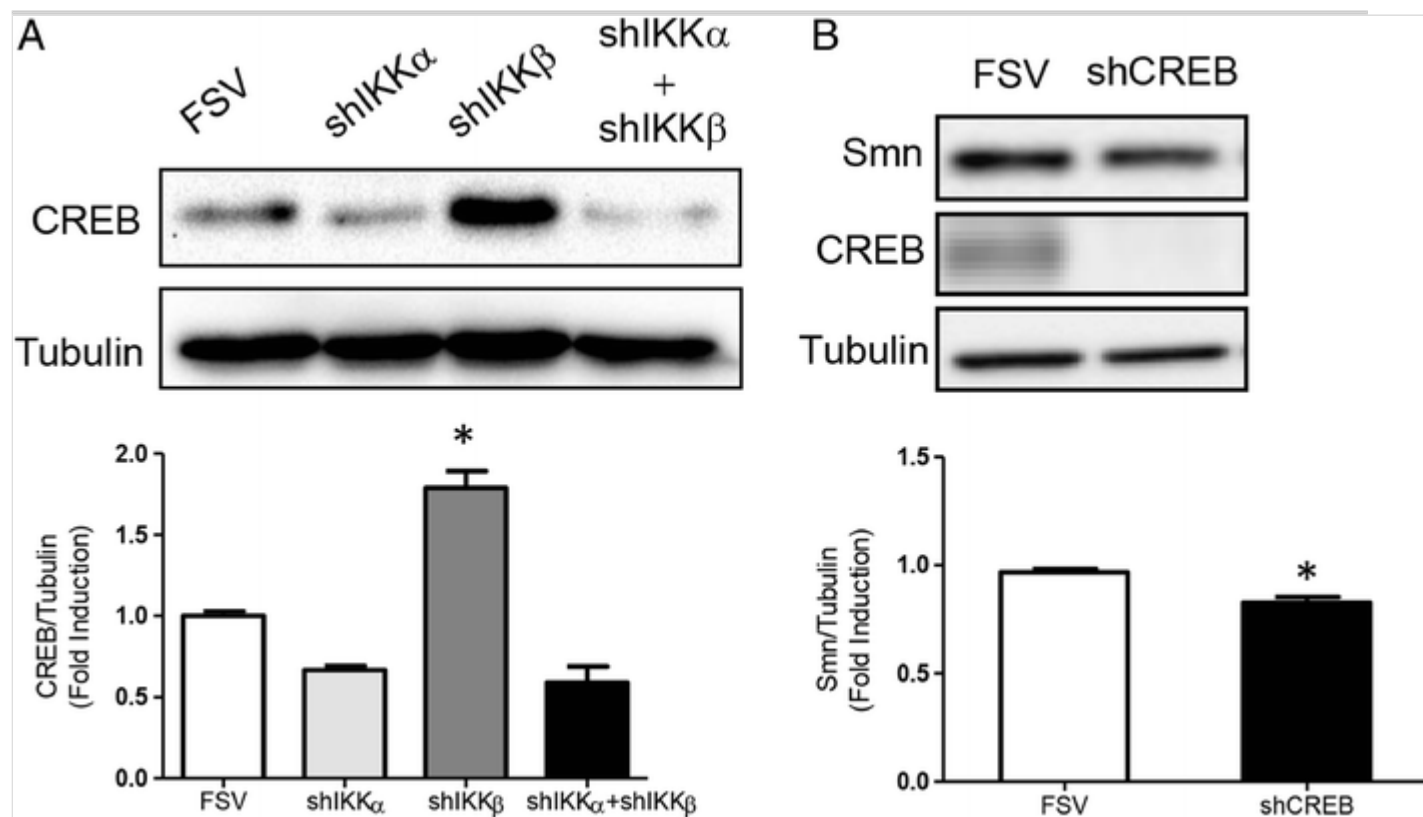
## IKK $\alpha$ or IKK $\beta$ Knockdown Affects CREB Protein Levels in MNs

Previous evidence suggests that cAMP-response element-binding (CREB) may regulate *SMN* expression [28]. In turn, NF- $\kappa$ B signalling pathway controls CREB protein level in MNs [13, 28]. In this context, we decided to analyse CREB protein level in IKK knockdown cultures. MNs were isolated and transduced with empty vector (FSV) or shIKK $\alpha$ , shIKK $\beta$  or shIKK $\alpha$  plus shIKK $\beta$ . Twenty-four hours after transduction, medium containing lentiviral particles was replaced with fresh medium supplemented with NTFs. Five days later, cells were lysed and protein extracts were submitted to western blot analysis. The CREB protein level was significantly reduced in shIKK $\alpha$ -transduced MNs ( $0.66 \pm 0.02$ ,  $p < 0.001$ ), compared to the FSV control. In contrast, IKK $\beta$  knockdown significantly increased the level of CREB protein ( $1.79 \pm 0.1$ ,  $p < 0.005$ ), compared to FSV control. When cultures were co-transduced with shIKK $\alpha$  plus shIKK $\beta$ , the level of CREB protein ( $0.59 \pm 0.09$ ,  $p < 0.05$ ) was comparable to that observed in shIKK $\alpha$ -only cultures (Fig. 5a). These results show that the IKK complex regulates the intracellular level of the CREB protein with a pattern similar to that observed in *Smn* experiments.

### Fig. 5

CREB protein regulation by IKK knockdown. **a** MNs were transduced with lentivirus containing the shIKK $\alpha$ , shIKK $\beta$ , shIKK $\alpha$  + shIKK $\beta$  or empty vector (FSV) constructs. Five days after transduction, protein lysates were submitted to western blot using an anti-CREB antibody. **b** MNs were transduced with shCREB or FSV and 5 days later, protein extracts

were submitted to western blot analysis using an anti-SMN antibody. In **a** and **b**, membranes were reprobbed with an anti- $\alpha$ -tubulin antibody. Graph values represent the expression of CREB or Smn vs  $\alpha$ -tubulin and correspond to the quantification of three independent experiments  $\pm$  SEM. Asterisks in **a** indicate significant differences using one-way ANOVA test and Bonferroni's post hoc multiple comparisons ( $p < 0.005$ ). Asterisk in **b** indicates significant differences using Student  $t$  test ( $p < 0.05$ )



To analyse whether CREB reduction causes changes in Smn protein level, we generated lentivirus shCREB, isolated the MNs, and transduced the cells with the empty vector (FSV) or shCREB. After 24 h, the medium was replaced with NTF-supplemented medium and cells were maintained in this medium for 5 days. To measure the level of Smn protein, total protein extracts were submitted to western blot analysis. The Smn protein level was significantly reduced in CREB knockdown condition ( $0.82 \pm 0.02$ ,  $p < 0.05$ ), compared with the FSV control (Fig. 5b). These experiments suggest that the CREB transcription factor regulates Smn protein level in MNs.

## NF- $\kappa$ B Levels in Cultured MNs Differ between Wild-Type and SMA Mutant Mice

To analyse whether levels of NF- $\kappa$ B pathway proteins were altered in SMA-mutant MNs, E13 embryos of SMA type I mice were genotyped and spinal cords of WT (*Smn*<sup>+/+</sup>;SMN2<sup>+/+</sup>) and mutSMA (*Smn*<sup>-/-</sup>;SMN2<sup>+/+</sup>) were dissected. MNs were isolated following the same protocol described in the "Materials and Methods" section. Cells were maintained in the presence of NTFs for 6 days before cell lysates were obtained. Western blot analysis of total protein extracts demonstrated that IKK $\alpha$  and IKK $\beta$  were significantly reduced in mutSMA

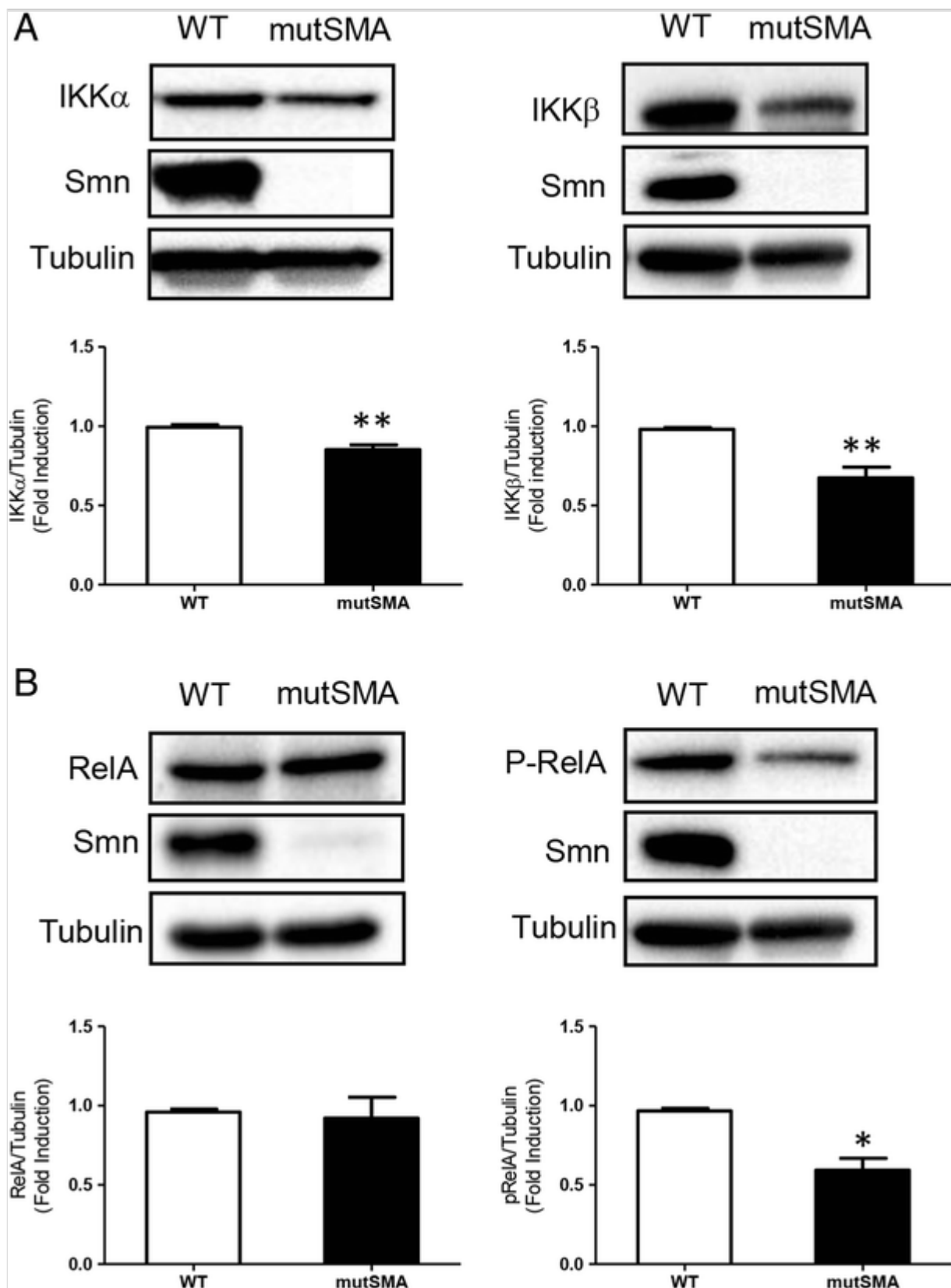
( $0.85 \pm 0.03$ ,  $p < 0.005$ ;  $0.67 \pm 0.06$ ,  $p < 0.005$ , respectively) compared to the WT condition (Fig. 6a). A time-course experiment demonstrated that IKK $\alpha$  and IKK $\beta$  reduction was maintained from day 2 to day 8 in culture (data not shown). The level of RelA protein ( $0.92 \pm 0.13$ ) did not differ significantly from the WT control. Nevertheless, we observed that RelA phosphorylation was reduced after 6 days in culture, compared to the WT control ( $0.59 \pm 0.07$ ,  $p < 0.01$ ) (Fig. 6b). Together, these results show that the protein level of some members of the NF- $\kappa$ B pathway is reduced and RelA phosphorylation is compromised in cultured SMA MNs.

### Fig. 6

NF- $\kappa$ B members are modified in SMA-cultured mice spinal-cord MNs. WT and mutSMA isolated MNs were cultured in the presence of NTFs. Six days later, protein extracts were submitted to western blot analysis using the following antibodies: anti-IKK $\alpha$ , anti-IKK $\beta$  (**a**), anti-RelA, anti-phosphoRelA (P-RelA) (**b**) and anti-SMN. Membranes were re probed with an anti- $\alpha$ -tubulin antibody as a loading control. Graph values represent the expression of IKK $\alpha$ , IKK $\beta$ , RelA or P-RelA vs  $\alpha$ -tubulin and correspond to the quantification of at least three independent experiments  $\pm$  SEM. Asterisks indicate significant differences using Student  $t$  test ( $*p < 0.05$ ,  $**p < 0.005$ )

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## Discussion

In the present work, we show that the canonical NF- $\kappa$ B pathway regulates Smn protein in a primary culture of isolated mouse embryonic MNs. Reducing protein levels of several members of the NF- $\kappa$ B signalling pathway revealed that these proteins modulate Smn. RelA knockdown was associated with a significant decrease in Smn, but RelB reduction was not. The NF- $\kappa$ B pathway can be activated by two main routes: canonical (or classical) and non-

canonical. The canonical pathway is characterized by the activation and translocation of p50/RelA heterodimers to the nucleus, whereas the non-canonical pathway relies on p52/RelB heterodimers [7]. It is known that RelA and RelB heterodimers regulate separate subsets of NF- $\kappa$ B target genes and might therefore regulate diverse cellular functions [29, 30]. Previous studies on MN survival describe this differential effect: RelA knockdown caused MN cell death; RelB knockdown did not [13]. Our present results clearly indicate that canonical NF- $\kappa$ B pathway inhibition reduces Smn levels in cultured MNs, supporting the involvement of this pathway in the regulation of Smn in these cells. This hypothesis is also supported by the results observed after pharmacological inhibition of NF- $\kappa$ B using SN50, a cell-permeable peptide that blocks the nuclear localization sequence of p50 and prevents its nuclear translocation [25]: SN50 treatment clearly reduced Smn level in cultured MNs; therefore, pharmacological and knockdown experiments have provided evidence that NF- $\kappa$ B activation is involved in Smn stability in MNs.

Inhibition of I $\kappa$ B kinases regulates Smn differentially. Smn protein decreased with IKK $\alpha$  knockdown and increased with IKK $\beta$  knockdown. IKK $\beta$  is the predominant kinase responsible for I $\kappa$ B $\alpha$  and p105 phosphorylation [31]. In cultured MNs, we described a significant reduction in RelA nuclear translocation and phosphorylation in IKK $\beta$  knockdown compared with IKK $\alpha$  knockdown cells, supporting the predominant role of IKK $\beta$  in activating the canonical NF- $\kappa$ B pathway in these cells. Previous studies using IKK $\beta$  knockout mice demonstrate a clear phenotype similar to RelA knockout, suggesting that IKK $\beta$  causes canonical NF- $\kappa$ B pathway activation [31, 32]. Although IKK $\alpha$  and IKK $\beta$  share structural and biochemical similarities, different phenotypes in IKK $\alpha$  and IKK $\beta$  knockout mice imply distinct physiological roles of the IKK isoforms [33]. They phosphorylate distinct substrates in the cytoplasm and their sub-cellular distribution differs: IKK $\alpha$  can be detected in both the cytoplasm and the nucleus whereas IKK $\beta$  is detected predominantly in the cytoplasm [34]. Studies of the nuclear role of IKK $\alpha$  provide evidences for the essential role of this kinase in NF- $\kappa$ B-dependent transcription. Our results are also in accordance with earlier studies of the roles of IKK $\alpha$  and IKK $\beta$  in other cellular models, such as chondrocytes and fibroblasts [35, 36], indicating that the differential requirements for IKK in regulating proteins can be an extended process.

The addition of the proteasome inhibitor MG132 to the shIKK $\alpha$  condition prevented Smn reduction. This finding suggests that proteasome activity may be increased in IKK $\alpha$  knockdown cells, which could be the main reason for Smn decrease in this culture condition. It has been previously reported that SMN protein degradation is mediated via the ubiquitin/proteasome pathway, which regulates its cytoplasmic level [27]. In MNs, treatment with proteasome inhibitors increases SMN protein level [37], suggesting that this degradation pathway is involved in SMN stability in these neurons. Therefore, proteasome activity and SMN level may constitute a key point in MN degeneration in SMA. Increased proteasome activity can exacerbate SMN reduction, leading to deterioration in MN function.

On the other hand, the effect of IKK $\beta$  knockdown on *Smn* level occurs through increased *Smn mRNA*. Thus, the absence of IKK $\beta$  stimulates *Smn* transcription. One candidate to produce this effect is CREB. Previous experiments described CREB protein binding to the CRE-II site in the *SMN* promoter as positively regulating *SMN* expression, and treatment with cAMP-elevating agents increased expression of both the full-length and exon $\Delta$ 7SMN transcript in HeLa cells [28]. Moreover, when activated in SMA spinal cords, CREB binding to its response elements is significantly increased at the level of the SMN2 gene promoter and is correlated with an increase in SMN expression [38]. We measured CREB protein level in IKK $\alpha$ - and IKK $\beta$ -reduced cells, and our results showed the increase of CREB in shIKK $\beta$  condition and the reduction of CREB in shIKK $\alpha$ . Furthermore, CREB-reduced cells showed a slight, but significant, *Smn* decrease, indicating that CREB may be regulating SMN protein level in MNs. In conclusion, our results are in accordance with previous reports describing CREB involvement in SMN regulation.

The contribution of IKKs in neurodegenerative disorders has been proposed as a direct interaction of the IKK complex with huntingtin [39] and as regulating the pathogenesis of Huntington disease by inducing cleavage of mutated huntingtin [40]. IKKs phosphorylate huntingtin residues, activating their clearance by proteasomes and lysosomes. SMN complex and SMN itself can be phosphorylated in serine/threonine and lysine residues that regulate the subcellular localization of the SMN complex and its accumulation into the nucleus [41]. PKA can phosphorylate SMN, reducing its degradation in cultured cells [26]. These evidences may lead to a further analysis of the role of SMN phosphorylation, proteasome degradation, and NF- $\kappa$ B pathway. A comprehensive analysis of post-translational modifications such as phosphorylation and increase of proteasome degradation can explain some of the events involved in SMA pathogenesis and the specific vulnerability of spinal cord MNs to SMN protein level. However, studies using SMA patient-derived fibroblasts have not detected alterations in proteasome activity [26], suggesting that proteasome function in SMA patient cells is preserved; nevertheless, pharmacological inhibition of proteasome activity, together with an upregulation of *SMN* gene transcription, increases SMN levels and improves lifespan of SMA mice [42, 43]. Additionally, treatment with sodium butyrate-based compounds ameliorates SMA pathology, possibly by regulating intracellular pathways altered in SMN deficiency and not by regulating SMN protein expression [44]. These observations should help to further define the pathways and allow the identification of more specific targets for therapeutic approaches.

Our results provide new evidences that the NF- $\kappa$ B pathway is modified in MNs of a mouse SMA model. Changes in RelA protein levels were not statistically significant, but the phosphorylation of RelA was reduced in SMA samples. Given recent results supporting the hypothesis that the regulation of altered intracellular pathways may be the future pharmacological treatment of SMA [44, 45], we propose the NF- $\kappa$ B pathway as a candidate to this new therapeutic model.

## Acknowledgements

This work was supported by grants from Ministerio de Economía y **e**Competitividad, Instituto de Salud Carlos III, Fondo de Investigaciones Sanitarias (PI14/00060), Unión Europea, Fondo europeo de Desarrollo Regional (FEDER) “Una manera de hacer Europa”, and Generalitat de Catalunya (SGR740). AP holds a fellowship from Comissionat d’Universitats i Recerca, Departament d’Innovació, Universitats i Empresa de la Generalitat de Catalunya i Fons Social Europeu, SA and SdF hold a fellowship from Universitat de Lleida. We thank Elaine Lilly, Ph.D., for the English language revision of the manuscript.

## Compliance with Ethical Standards

*Conflict of Interest* The authors declare that they have no competing financial interests.

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