



Transcriptional regulation of CRMP5 controls neurite outgrowth through Sox5

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Abstract Transcriptional regulation of proteins involved in neuronal polarity is a key process that underlies the ability of neurons to transfer information in the central nervous system. The Collapsin Response Mediator Protein (CRMP) family is best known for its role in neurite outgrowth regulation conducting to neuronal polarity and axonal guidance, including CRMP5 that drives dendrite differentiation. Although CRMP5 is able to control dendritic development, the regulation of its expression remains poorly understood. Here we identify a Sox5 consensus binding sequence in the putative promoter sequence upstream of the *CRMP5* gene. By luciferase assays we show that Sox5 increases *CRMP5*

promoter activity, but not if the putative Sox5 binding site is mutated. We demonstrate that Sox5 can physically bind to the *CRMP5* promoter DNA in gel mobility shift and chromatin immunoprecipitation assays. Using a combination of real-time RT-PCR and quantitative immunocytochemistry, we provide further evidence for a Sox5-dependent upregulation of *CRMP5* transcription and protein expression in N1E115 cells: a commonly used cell line model for neuronal differentiation. Furthermore, we report that increasing Sox5 levels in this neuronal cell line inhibits neurite outgrowth. This inhibition requires CRMP5 because *CRMP5* knock-down prevents the Sox5-dependent effect. We confirm the physiological relevance of the Sox5–CRMP5 pathway in the regulation of neurite outgrowth using mouse primary hippocampal neurons. These findings identify Sox5 as a critical modulator of neurite outgrowth through the selective activation of CRMP5 expression.

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Introduction

Controlling neurite outgrowth is a key element for the establishment and maintenance of neuronal polarity which are critical processes for the coordination between neuronal morphology and function. Processes that underlie the initial specification of axons and dendrites are tightly controlled through the interplay of extrinsic signals and cell-autonomous intrinsic mechanisms. Among the extracellular signals that are relevant for neuronal polarity, guidance cues such as Semaphorin 3A (Sema3A) can sequentially exert repulsive and attractive effects on cortical axons and dendrites, respectively [1]. Interestingly, Collapsin Response Mediator

Proteins (CRMP), which were first identified as intracellular mediators in Sema3A signaling [2] have been shown to control axon–dendrite protein sorting in *C. elegans* [3].

CRMP is a family of 5 cytosolic proteins that regulates several neurobiological processes in the developing brain. They control neural stem cell differentiation, neurite outgrowth, neuronal polarity, and axonal guidance in normal conditions, whereas altered CRMP levels or posttranslational modifications in the adult brain are linked to psychiatric and neurodegenerative diseases [4]. Strong evidence indicates that CRMPs act as cytoskeleton regulators, first by promoting microtubule polymerization, but also by reorganizing the actin network, which is essential for endocytosis [5–7]. Two members of the CRMP family, CRMP2 and CRMP5, have been shown to exert differential roles in mediating neurite outgrowth and neuronal polarity. CRMP2 not only participates in axonal guidance by inducing axonal growth cone collapse [2], but also instructs axon initiation and specification in hippocampal neurons [8]. The exquisite regulation of CRMP2 levels controls the specification of a single axon because supernumerary axons are formed when CRMP2 is overexpressed, whereas knockdown of CRMP2 results in neurons that do not form an axon [9]. The function of CRMP5 has been examined after overexpression in hippocampal neurons and promotes filopodial growth and the formation of growth cones. CRMP5 is also able to block Sema3A-mediated growth cone collapse suggesting that it might regulate filopodial actin dynamics in growth cones during development [10]. Furthermore, CRMP5 exerts a negative regulatory effect on neurite extension [8], and our recent findings demonstrated that it specifically targets dendritic growth in hippocampal neurons. Consistently, whereas CRMP2 promotes axonal growth through tubulin polymerization, CRMP5 requires an interaction with tubulin and MAP2 to block dendritic growth. CRMP5 binding to tubulin could be dominant over CRMP2 binding in hippocampal dendrites and antagonizes the CRMP2 promotional activity on growth [8]. In vivo, CRMP5 deletion has been reported to prevent the normal dendritic development of output projection neurons from the cerebellar cortex [11]. Beyond neuronal differentiation, the establishment of cell polarity is considered as a key process underlying cell proliferation and maintenance of the proliferative pool [12]. Interestingly, CRMP5 has been shown to impact on neurogenesis in the adult brain [13] and elevated CRMP5 levels results in the amplification of cell proliferation in primary brain tumors [14]. Thus, given the implication of CRMP5 dysregulation in brain disorders, it is critical to better understand the molecular mechanisms responsible for CRMP5 gene expression control.

Sex determining region Y-box 5, also called Sox5, belongs to the Sox-containing gene family, which encodes highly conserved transcription factors during evolution.

The function of Sox5 has been characterized in chondrogenesis, oligodendrogenesis and in the generation of neural crest and cortical projection neurons [15, 16]. Two Sox5 isoforms are produced including a long (L-Sox5) isoform expressed in chondrocytes and postmitotic neurons [17, 18] and a short (S-Sox5) isoform, mainly testicular [19]. Gain- and loss-of-function approaches indicate that Sox5 controls the specification of corticofugal neurons in the developing cortex and plays a key role in the establishment of early neocortical circuits [16]. In addition, haploinsufficiency of *Sox5* causes mild intellectual disability in patients such as speech delays and behavioral anomalies [20]. Thus, given the common defects in neurodevelopment resulting from CRMP5 and Sox5 dysregulation, it raises the possibility that Sox5 transcription factor might regulate CRMP5 expression level.

To characterize potential transcription factors modulating *CRMP5* expression, we investigated DNA sequence motifs upstream of the transcription starting site (TSS) and identified a Sox5 consensus binding site. Examination of gene expression data highlighted the strong correlation between *Sox5* and *CRMP5* mRNA in embryonic telencephalic vesicles. Next, we demonstrated that the wild-type *CRMP5* promoter activity is upregulated by Sox5, whereas its mutant form in the Sox5 binding site is not. We further confirmed a physical interaction between Sox5 and its consensus sequence in the *CRMP5* promoter. Finally, we revealed a Sox5-dependent increase in CRMP5 expression that is responsible for the inhibition of neurite outgrowth in a cell line model mimicking a neuronal phenotype and in hippocampal primary neurons. Taken together, the findings of this study uncovered that Sox5 controls downstream *CRMP5* gene expression and acts as a key regulator of neurite growth.

Methods

In silico analysis

The Allen Developing Mouse Brain Atlas (Website: ©2013 Allen Institute for Brain Science, available from: <http://developingmouse.brain-map.org>) is a genome-wide database that gathers in situ hybridization data from mouse of more than 2000 genes at several developmental stages and assembled into the five main brain structures (neural plate, telencephalic vesicle, diencephalon, midbrain and hindbrain). We used the correlation search tool available on the results page of gene search at E13.5 and E15.5 in the telencephalic vesicle to find the most correlated expressed gene with *CRMP5*.

PromoterInspector [21] and MatInspector [22] softwares (Genomatix, Germany) were used firstly to identify the putative murine *CRMP5* promoter, and secondly to search for potential transcription factor regulatory-binding sites

(RBS). A comparison between 6 species (*Homo sapiens*, *Macaca mulatta*, *Pan troglodytes*, *Canis familiaris*, *Rattus norvegicus* and *Mus musculus*) was performed to identify evolutionary conserved RBS in the 5' DNA sequence of their *CRMP5* gene.

Plasmids

pGL4.18 and pRL-SV were bought (Cat#E2231, Promega, Madison, WI). pGL4.18 was used to prepare CRMP5-wt-Fluc: the putative promoter sequence of the murine *CRMP5* promoter [nucleotides from -2005 to +135 with +1 corresponding to the transcription start site (TSS), NCBI Reference Sequence: NC_000071.6] was amplified with 5'-ACC TGA GCT CGC TAG CAC GTG AAA CCT CAG GCT TGG GT-3' and 5'-TAT CCT CGA GGC TAG CGA TGC GAT TGT GCG GGT CCG T-3' primers, then inserted in pGL4.18 before firefly luciferase coding sequence using In-Fusion[®] cloning kit (Cat#011614, Clontech Laboratories, Inc., Mountain View, CA, USA). CRMP5-mut-Fluc was obtained by alteration of the Sox core sequence in CRMP5 promoter GCATTGTTTC into GCT TTA ATT AA to introduce a Pac1 restriction site, using the QuikChange II XL Site-Directed Mutagenesis Kit (Cat#200521, Agilent Technologies, Santa Clara, CA), 5'-GAG AGA CCC CGC CAC CGC TTA ATT AAA GCC CCT CGG GTG GGG TC-3' and 5'-GAC CCC ACC CGA GGG GCT TTA ATT AAG CGG TGG CGG GGT CTC TC-3' primers. pCAGEN was a gift from Connie Cepko [23] (Addgene, Cat#11160, Cambridge, MA, USA); CAG-L-Sox5 was developed from pCAGEN and enables expression of murine L-Sox5 protein under the CAG promoter, it was generously gifted by Neñad Sestan [18]. We also used vectors with CAG promoter containing *IRE5-GFP*, alone: CBig, a generous gift of C. Lois (MIT, USA) [24] that allows GFP expression or with murine L-Sox5 sequence: CBig-L-Sox5, a generous gift of Jeffrey Macklis [24] that allows independent L-Sox5 and GFP overexpression. Murine L-Sox5 coding sequence was amplified by PCR using 5'-AAG GAT CCC ATG CTT ACT GAC CCT GAT TTA CC-3' and 5'-TAA AGC TTG TTG GCT TGT CCC GCA AT-3' primers and cloned into pET21b (Cat#69741, Merck-Millipore, Darmstadt, Germany) between *Bam*HI and *Hind*III restriction sites to produce pET21b-L-Sox5, that allows production of recombinant L-Sox5 with a polyhistidine tail. Home-made and gifted plasmids were verified by Sanger sequencing (Eurofins, Germany).

Cell culture and transfection

GL15, a human glioblastoma cell line [25] was propagated in DMEM (Dulbecco's modified Eagle medium) containing low glucose (1 g/L), pyruvate and Glutamax[®] (Cat#21885, ThermoFisher Scientific, Waltham, MA) supplemented with

10% fetal calf serum (FCS, Cat#26140079, Gibco, Waltham, MA, USA), 100 U/mL penicillin and 100 µg/mL streptomycin (P/S, Cat#15070063, Gibco). N1E115 (Cat#CRL-2263, ATCC, Teddington, UK), a murine neuroblastoma cell line was chosen because of its ability to develop a neuronal phenotype with differentiated neurites in culture. N1E115 cells were maintained in DMEM, high glucose (4.5 g/L, Cat#11960, Gibco), supplemented with 10% FCS and P/S or differentiated in high glucose DMEM supplemented with 0.5% FCS, 1.5% DMSO and P/S to study neuritogenesis [26]. Primary cultures of mouse hippocampal neurons were obtained from 18-day-old embryos (C57BL/6; Charles River Laboratories, Saint-Germain-Nuelles, France) and prepared as previously described [27]. Briefly, hippocampal neurons were seeded on petri dishes (first day in vitro; DIV1) coated with poly-L-lysine (Sigma-Aldrich) in a neurobasal media supplemental with B-27 reagent (Cat#17504044, Gibco, Waltham, MA, USA).

For luciferase assays, GL15 or N1E115 cells were seeded into 96-well clear bottom microplate at 10,000 cells per well in a volume of 100 µL of their respective media. Then, 16 h later, cells were transfected with 0.3 µL Lipofectamine LTX (Cat#15338100, Life technologies, Carlsbad, CA, USA) using 150 ng of nucleic acids per well, following manufacturer instructions. For L-Sox5 overexpression studies, N1E115 cells were seeded at 200,000 per well in 6-well plates in 2 mL of media; 16 h later, cells were transfected with 6 µL Lipofectamine LTX using 2.5 µg of nucleic acids per well. For neurite outgrowth assays, N1E115 cells were seeded at 30,000 cells per well in 6-well plates in differentiation media; 16 h later, cells were transfected with 6 µL of Lipofectamine 2000 (Cat#11668027, Life technologies) using 2.5 µg of nucleic acids per well; with primary cultures, embryonic hippocampal cells were plated on poly-L-lysine-coated glass coverslips at a density of 150,000 cells/cm² and incubated in 12-well plates in 1 mL medium, 16 h later, cells were transfected with 3 µL Lipofectamine 3000 (Cat#L3000-008, Life Technologies) using 1.25 µg of nucleic acids per well. All cells were incubated at +37 °C under 5% CO₂ atmosphere.

Luciferase assay

Experiments conducted on GL15 cells to explore the promoting activity of the 5' DNA sequence of the murine *CRMP5* gene were realized by co-transfection of pGL4.18 or CRMP5-wt-Fluc in presence of pRL-SV (ratio 1:1) as a standard. Luciferase activities were quantified by luminescence assays, they were revealed with Dual Glo kit (Cat#E2920, Promega), according to the manufacturer's protocol, by reading the firefly luciferase activity, normalized on the Renilla luciferase activity. Experiments conducted on N1E115 cells to explore L-Sox5 overexpression

on murine native or mutated *CRMP5* promoter were realized by co-transfection of CRMP5-wt-Fluc or CRMP5-mut-Fluc in presence of pCAGEN or CAG-L-Sox5 (ratio 1:1), firefly luciferase activity was revealed with Bright Glo kit (Promega, USA) according to manufacturer's protocol, by incubating cells 5 min with lysis buffer, transferring to a 96-well plate and adding an equal volume of Bright Glo buffer. All samples were duplicated and experiments were conducted in triplicate. All luminescence measures were done with a Luminoskan Ascent reader (Cat#5300160, ThermoFisher).

Electrophoretic mobility shift assay (EMSA)

Escherichia coli BL21-DE3 bacteria (Cat#200131, Agilent, Santa Clara, CA, USA) were transformed with pET21b-L-Sox5. The bacterial culture was grown at 37 °C to OD = 0.6 at 600 nm, then plasmid expression was induced with 1 mM isopropyl-D-thiogalactopyranoside (IPTG). After overnight incubation at 16 °C, cells were harvested by centrifugation and recombinant L-Sox5 was purified with Ni-NTA Agarose beads (Cat#30210, QIAGEN, Hilden, Germany) by adding 2 mL of agarose beads and incubating 30 min at 4 °C, washing three times with TBST and adding 200 mM of imidazole to elute the protein. As a control, non-induced bacteria culture was processed in parallel. Production or absence of recombinant L-Sox5 was checked with blue coomassie staining after gel electrophoresis separation (Online resource 1).

5' biotinylated, or not, complimentary oligonucleotides 5'-GAG AGA CCC CGC CAC CGC ATT GTT TCA GCC-3' and 5'-GGC TGA AAC AAT GCG GTG GCG GGG TCT CTC-3' mimicking the putative Sox family binding site of murine *CRMP5* promoter were synthesized (Eurogentec, Liège, Belgium). Annealing was performed by slow cooling (1 °C/min) after heat denaturation at 95 °C. Biotinylated probe was incubated with recombinant Sox5 protein using LightShift Chemiluminescent EMSA kit (Cat# 20148, ThermoFisher) following manufacturer instructions: reactions were performed in presence or absence of 1 µg of protein extract from induced or non-induced bacteria culture, 1 µg of poly dI/dC, with or without a 200-fold excess of unlabeled probe as a competitor. After incubation, reactions were electrophoresed on a non-denaturing 10% polyacrylamide gel. After transfer on 0.2 µm Protran nitrocellulose membrane (Cat#10600004, GE Healthcare Lifesciences, Little Chalfont, UK), biotinylated probes were revealed by HRP-conjugated streptavidin (Cat# GERPN1231, Sigma-Aldrich, Saint Louis, MO, USA) diluted at 1/5000 and revealed by chemiluminescence using Amersham ECL Western Blotting Detection Kit (Cat# RPN2106, GE Healthcare).

Chromatin immunoprecipitation (ChIP)

ChIP assays were performed by Pierce™ Agarose ChIP kit (ThermoFisher Scientific, Rockford, USA). Transfected N1E115 cells with CAG-L-Sox5 plasmid were fixed with 1% formaldehyde to induce DNA-protein cross-links. Cell lysates were then randomly digested with micrococcal nuclease in order to generate chromatin fragments, then immunoprecipitated with anti-Sox5 antibody abID#26041 (Abcam, Cambridge, UK) or anti-rabbit IgG as control. Precipitated chromatin was analyzed by PCR with primers surrounding the putative Sox5 RBS in murine *CRMP5* promoter (5'-ACC TCA GGC TTG GGT AGG AT-3' and 5'-GGC CTC TGA TGA ACT TTT GC-3'). Positive control was checked on input unprecipitated cell lysate. Processed DNAs were migrated on 2% gel agarose.

Semi-quantitative RT-PCR

After 48 h of L-Sox5 overexpression, N1E115 cells were lysed with 1 mL of TriReagent (Cat#T9424, Sigma-Aldrich, Saint Louis, MO, USA) in Phase Lock tubes (Cat# 2302830, 5 Prime, Hilden, Germany). Total RNA extractions were carried out following manufacturer guidelines. Samples were assayed by optical density at 260 and 280 nm with a spectrophotometer: 1 µg RNA was transcribed into complementary DNA (cDNA) by reverse transcription assay using the iScript™ Reverse Transcription Supermix (Cat#1708840, Biorad, Hercules, CA, USA) in a final volume of 20 µL. Relative quantification of *CRMP5* mRNAs was realized by semi-quantitative PCR (qPCR). Each reaction was performed onto 0.5 µL cDNA in a final volume of 20 µL using 5× HotPol EvaGreen qPCR Mix Plus kit (Cat#08-25-00001, Euromedex, Souffelweyersheim, France) and 500 ng of each primer. For *CRMP5*, primers used were 5'-CCC AGA AGG AAT CGA GAT CA-3' and 5'-ACA CGG TGA GTG GCT TCT G-3'. The transcription-repair coupling factor (*TRCF*) gene was used as an endogenous control and was checked to be insensitive to L-Sox5 overexpression, its amplification was carried out using the following primers: 5'-GTC CAG TGT GGG AAC AGG TC-3' and 5'-CAG TCC AGC TGG CAA AGA TT-3'. A reference sample was used as a calibrator, allowing us to pool results from different experiments. After an initial step of 3 min at 95 °C to activate the Taq enzyme, qPCR was carried out for 40 cycles at 95 °C for 10 s, 60 °C for 20 s and 72 °C for 20 s in a Master Cycler Ep Realplex (Eppendorf, Germany). All samples were duplicated and experiments were conducted in triplicate. Comparison of the threshold cycle for *CRMP5* versus *TRCF* and calibrator sample allowed us to calculate the relative level of *CRMP5* transcription with or without L-Sox5 overexpression.

Immunocytochemistry and CRMP5 protein level measure

After 48 h of L-Sox5 overexpression, proliferating N1E115 cells were fixed for 5 min with 4% paraformaldehyde. For CRMP5 immunolabeling, cells were preincubated for 90 min with blocking solution [0.3% Triton, 5% FCS in phosphate buffer saline (PBS, Cat#14190094, Gibco)] then incubated overnight at 4 °C in the same buffer with rabbit anti-CRMP5 antibody (2 µg/mL) previously manufactured in our laboratory [28]. After rinsing with washing buffer (0.3% Triton in PBS), finally, the cells were incubated for 2 h in a goat anti-rabbit secondary antibody conjugated to Alexa 555 (Molecular Probes, Invitrogen, Eugene, OR) diluted at 1/2000 in washing buffer, for red immunofluorescence labeling. Plates were observed and imaged using an Axiovert 200 M microscope (Zeiss, Germany). Five 8-bit pictures were randomly taken from each well. CRMP5 quantification was realized on GFP positive-transfected cells by measuring the grey level of green and red fluorescences with ImageJ software (NIH, USA) [29], with a value from 0 in absence of signal to 255 for maximal fluorescence. A threshold of 200 defined as a fluorescence signal being fourfold above average cell green autofluorescence was applied to clearly select transfected (GFP expression) from untransfected (no GFP expression) cells. CRMP5 quantification was assessed by red fluorescence level on these transfected cells. Experiment has been conducted three times in duplicates.

Quantification of neurite outgrowth

To quantify neurite outgrowth on N1E115, cells were transfected with CBig-Sox5 or control CBig plasmid in presence of a fluorescent ATTO-TEC double-stranded phosphorothioate RNA homologous to CRMP5 RNA (CRMP5-siRNA: 5'-GGA CUU CAU GUA CAA UCG A-3', Eurogentec, Liege, Belgium) or not (scrambled siRNA) (ratio 1:0.03). Forty-eight hours later, plates were imaged using an Axiovert 200 M microscope (Zeiss, Oberkochen, Germany). Five pictures were randomly taken from each well. Neurite lengths (>10 µm) were manually analyzed for green (L-Sox5 overexpression or not) and red (CRMP5 silencing or not) fluorescent cells with ImageJ software. Experiments have been conducted three times in duplicates.

To quantify neurite outgrowth on primary hippocampal cells, cells were transfected at DIV2 with CBig-Sox5 or control CBig plasmid in presence of CRMP5-siRNA or scrambled siRNA. At DIV4, plates were observed and imaged. Neurite length (>10 µm) were manually measured with ImageJ software. The experiment was conducted twice.

Statistical analysis

Statistical significance of differences of luciferase, RT-PCR and quantitative immunocytochemistry assays was evaluated with non-parametric tests: Mann–Whitney test between pairs, or Kruskal–Wallis followed by Dunn's post hoc test (Graphpad prism 7). For analysis of neurite length on large samples obtained with N1E115 cells, one-way ANOVA followed by Tukey's post hoc test was realized and z test for their distribution analysis (SigmaPlot 12.5). For analysis of neurite length on hippocampal primary neurons, a Kruskal–Wallis followed by Dunn's post hoc test with p values adjusted with the Bonferroni method (Fisheries Stock Analysis. R package version 0.8.12) [30] was used and z test for their distribution analysis. $p < 0.05$ was considered as a proof of significant difference.

Results

L-Sox5 as a candidate for CRMP5 regulation

To identify molecular mechanisms controlling CRMP5 gene transcriptional activation in neurons, we investigated CRMP5 mRNA expression pattern during mouse brain development with the Allen Developing Mouse Brain Atlas. We focused our study on neocortical development at stages when projection neurons from deep cortical layers first initiate their axon, between E13.5 and E15.5 [31]. Correlation analysis indicated that among more than 2000 genes the transcription factor Sox5 showed the strongest spatio-temporal correlation with CRMP5 gene expression (Pearson's correlation coefficient $r = 0.687$). In situ hybridization revealed a significant overlap between CRMP5 and Sox5 mRNA expression at both E13.5 and E15.5 (Fig. 1a–d). We found both genes to be coexpressed in the dorsal and medial pallium strata with a common gradient of expression from the ventricular, with the lower level, to the superficial zones, with the higher level (Fig. 1a–d). Our data mining reveals the concomitant and overlapping gene expression patterns of Sox5 and CRMP5 during mouse brain development at the regional level, suggesting a role for Sox5 in the transcriptional control of CRMP5 gene expression at the cellular level.

To further assess the relevance of the transcription factor Sox5 in driving CRMP5 gene expression, we first used the PromoterInspector[®] software [21] to identify the putative promoter region of the murine CRMP5 gene (Celera[®] accession number AC_000027) from positions 28188434 to 28190822 on mouse chromosome 5. Considering the transcription start site (TSS), located at position 28190733, an analysis with MatInspector[®] software [22] was conducted on the 2300 base pairs upstream from the TSS. This allowed us to identify a putative Sox regulatory-binding site (RBS)

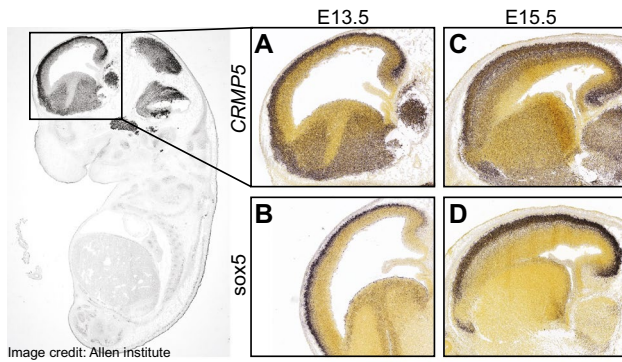


Fig. 1 Concomitant *CRMP5* and *sox5* expression in telencephalic vesicles during mouse embryonic development. Pictures extracted from the Allen Developing Mouse Brain Atlas. On the left, in situ hybridization of whole mouse embryo, zoomed at 281%, and probed for *CRMP5* (slice number 7/20 at E13.5). *CRMP5* was expressed in dorsal (DPall) and medial (MPall) pallium. Insets are enlarged pictures at the telencephalic vesicle level **a** slice 7/20 at E13.5 probed with *dpsyl5* antisense and zoomed at 563% <http://developingmouse.brain-map.org/experiment/show/100083357>; **b** slice 7/16 at E13.5 probed with *sox5* antisense and zoomed at 563% <http://developingmouse.brain-map.org/experiment/show/100076419>; **c** slice 7/20 at E15.5 probed with *CRMP5* antisense and zoomed at 625% <http://developingmouse.brain-map.org/experiment/show/100083366>; **d** slice 7/19 at E15.5 probed with *sox5* antisense and zoomed at 625% <http://developingmouse.brain-map.org/experiment/show/100085310>. Concomitant expression of *CRMP5* and *sox5* was observed in dorsal (DPall) and medial (MPall) pallium at embryonic stages E13.5 and E15.5

located in the -435 to -411 bp region upstream from the *CRMP5* gene TSS (Online resource 2). A phylogenetic analysis of orthologous sequences across 6 species (*Homo sapiens*, *Macaca mulatta*, *Pan troglodytes*, *Canis familiaris*, *Rattus norvegicus* and *Mus musculus*) revealed the high conservation of this regulatory site (Online resource 3). These analyses support the hypothesis that *CRMP5* transcriptional activation could be mediated by Sox5.

Sox5 binding site controls *CRMP5* gene expression

To confirm that the putative promoter region upstream of the *CRMP5* gene (-2000 bp from the TSS) is sufficient to promote transcription, we cloned and fused it upstream of the firefly luciferase reporter gene (Fig. 2a, *CRMP5*-wt-Fluc). This novel plasmid construct allowed us to directly link the amount of luciferase activity, within a cell, to *CRMP5* promoter activity. Glioblastoma cells (GL15), previously reported to express *CRMP5* [14], were transiently transfected with the *CRMP5*-wt-Fluc plasmid. We observed a significant increase (Mann–Whitney, $p < 0.01$) in the firefly/Renilla luciferase activities ratio (firefly luciferase activity was normalized to Renilla luciferase activity), in cells transfected with *CRMP5*-wt-Fluc plasmid compared with

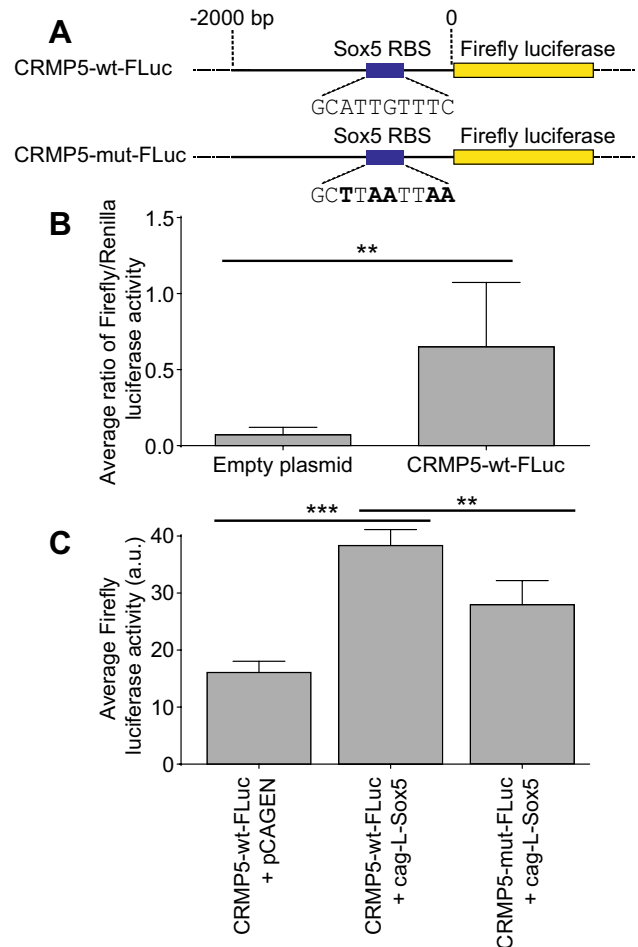


Fig. 2 Sox5 expression activates *CRMP5* promoter. **a** Schematic representation of mouse *CRMP5* promoter sequence [-2000 bp to 0 compared to the transcription starting site (TSS)], cloned in the pGL4.18 plasmid (*CRMP5*-wt-Fluc), upstream of the firefly luciferase coding sequence (in yellow). *CRMP5* promoter contains a putative Sox5 regulatory-binding site (RBS, in blue) located -435 and -411 bp upstream of the TSS. Sox5 RBS core sequence was mutated to prevent Sox5 binding (bolded amino acids, *CRMP5*-mut-Fluc). **b** Bar graph showing the average ratio of firefly/Renilla luciferase activity in GL15 cells transfected with either empty pGL4-18 plasmid or *CRMP5*-wt-Fluc described in **a**. pRL-SV plasmid was co-transfected to normalize firefly luciferase activity with Renilla luciferase activity. Results are from three independent experiments. Values represent the average \pm SEM, $**p < 0.01$ (Mann–Whitney test). **c** Bar graph showing the average firefly luciferase activity in GL15 cells transfected with the indicated plasmids. pCAGEN is the empty plasmid serving as a control compared to Cag-L-Sox5 which allows for the L-Sox5 overexpression. *CRMP5* promoter was activated by L-Sox5. Mutating Sox5 RBS prevented *CRMP5* promoter activation by L-Sox5. Results are from three independent experiments. Values represent the average \pm SEM, $**p < 0.01$, $***p < 0.001$ (One-way ANOVA)

the empty plasmid (Fig. 2b). The validation of this assay allowed us to investigate the role of the previously identified Sox regulatory-binding site (Online resource 2) in *CRMP5* transcriptional activation. Thus, we inactivated the Sox RBS by mutating the core sequence from GCATTG

TTTC to GCTTAATTA (Fig. 2a, CRMP5-mut-Fluc). First, GL15 cells transfected with a plasmid allowing for L-Sox5 overexpression (Cag-L-Sox5) together with CRMP5-wt-Fluc showed a significant increase in firefly luciferase activity ($p < 0.001$, Kruskal–Wallis, Dunn’s post hoc test) compared to cells transfected with the empty plasmid control (pCAGEN) (Fig. 2c). Cells co-transfected with CRMP5-mut-Fluc (where Sox RBS has been mutated) and Cag-L-Sox5 displayed a tendency toward an increase, however not significant, in firefly luciferase activity compared to the empty plasmid control (pCAGEN). In contrast, the firefly luciferase activity was significantly lower in this condition ($p < 0.01$, Kruskal–Wallis, Dunn’s post hoc test) than with the construct encoding for the wild-type CRMP5 promoter (Fig. 2c). These results demonstrate the role of L-Sox5 as a transcriptional activator of *CRMP5*, through the Sox RBS located from -422 to -413 bp upstream of the *CRMP5* TSS.

L-Sox5 binds *CRMP5* promoter

Prompted by the interaction between L-Sox5 and *CRMP5* promoter activity, we next assessed whether L-Sox5 has the ability to physically bind the Sox RBS. In a first step, we used an electrophoretic mobility shift assay (EMSA) approach to study binding of recombinant L-Sox5 on Sox RBS. Bacteria were transformed with pET21b-L-Sox5 plasmid, which allows the expression of a 6xHis tagged L-Sox5 under IPTG induction. Protein extracts were controlled by SDS-PAGE

and coomassie blue staining (Online resource 1). Extracts were incubated with double-stranded synthetic biotinylated Sox RBS (biot-Sox^{RBS}) and then separated by electrophoresis on a non-denaturing 10% polyacrylamide gel. The biot-Sox^{RBS} probe migrated freely when incubated alone (Fig. 3a, lane 4) or with protein extract from uninduced bacteria (Fig. 3a, lane 1). In contrast, incubating purified L-Sox5 with biot-Sox^{RBS} resulted in a clear mobility shift (Fig. 3a, lane 2), which identifies a major L-Sox5-Sox^{RBS} DNA complex. An excess of unlabeled-Sox^{RBS} probe successfully competed with biot-Sox^{RBS} and clearly reduced this mobility shift. In a second step, we used chromatin immunoprecipitation (ChIP) approach to study in situ binding of L-Sox5 to Sox RBS. As a positive control, we have been able to amplify the *CRMP5* promoter sequence in the total input sample (Fig. 3b, lane 1). Also a clear DNA amplification was observed after immunoprecipitation with Sox5 antibody (Fig. 3b, lane 3) but not with irrelevant IgG antibody (Fig. 3b, lane 2). These findings highlighted the physical interaction between L-Sox5 and the Sox RBS consensus sequence in the *CRMP5* promoter and provide a molecular mechanism underlying L-Sox5-dependent *CRMP5* transcriptional activation.

L-Sox5 stimulates endogenous *CRMP5* expression in vitro

To further examine the biological relevance of this physical interaction, we investigated endogenous *CRMP5* mRNA

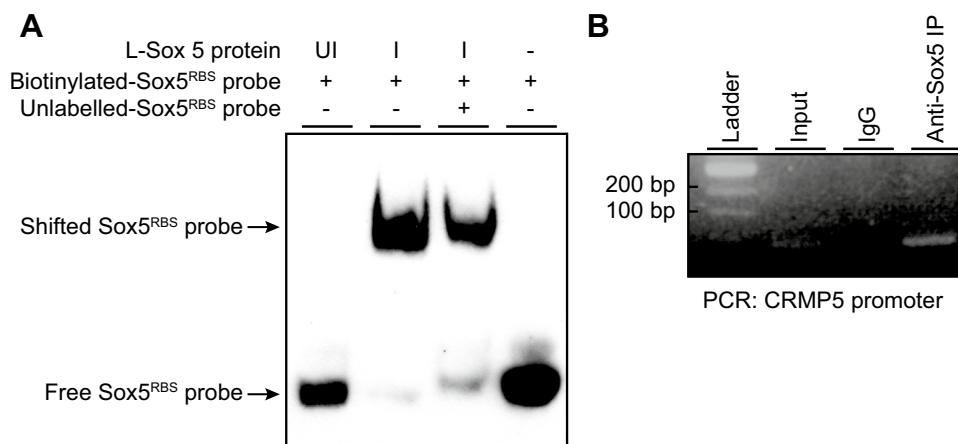


Fig. 3 L-Sox5 can bind to Sox5 RBS from *CRMP5* promoter sequence. **a** Biotinylated double-stranded oligonucleotide (Sox^{RBS} probe), representing nucleotides -435 to -411 of the *CRMP5* promoter was incubated with purified protein extracts from uninduced bacteria (“UI”: lane 1), induced bacteria (“I”: lanes 2 and 3) or without protein extract (lane 4). A “shift” in electrophoretic mobility indicated an interaction between the L-Sox5 protein extract and the biotinylated Sox^{RBS} probe. Using an unlabeled-Sox^{RBS} probe reduced the intensity of the shifted Sox^{RBS} probe showing the specificity of binding with L-Sox5 protein extract. Representative blot from three independent experiments. **b** Chromatin immunoprecipitation assay

of Sox5 with the *CRMP5* promoter. Chromatin was isolated from N1E115 cells transfected with CAG-L-Sox5 plasmid and fixed with 1% formaldehyde. Chromatin was amplified by PCR with primers surrounding the putative Sox5 RBS in murine *CRMP5* promoter (5'-ACC TCA GGC TTG GGT AGG AT-3' and 5'-GGC CTC TGA TGA ACT TTT GC-3'). Lane 1 ladder; lane 2 input chromatin before immunoprecipitation (IP); lane 3 IP with a non-specific antibody; lane 4 IP with Sox5 antibody. Specific binding of Sox5 transcription factor to *CRMP5* promoter is seen by the amplification of chromatin in the lane 4. Experiment was conducted twice

expression levels with or without L-Sox5 expression. We used transfected N1E115 cells overexpressing L-Sox5 (Cag-L-Sox5 plasmid) or not (empty plasmid, pCAGEN) and *CRMP5* mRNA expression level was assessed using qPCR. We found an approximate tenfold increase in *CRMP5* transcripts expression under L-Sox5 overexpression (Fig. 4a) compared with control condition. Thus, these findings support our previous data by showing that L-Sox5 expression lead to augmented endogenous *CRMP5* mRNA levels.

To further dissect this molecular pathway, we studied the impact of L-Sox5 on *CRMP5* transcription and the resulting *CRMP5* protein levels. L-Sox5 overexpressing (CBig-L-Sox5) or control cells (CBig empty) tagged with GFP were used to quantify endogenous *CRMP5* protein level by fluorescent antibody staining (Fig. 4b). We observed a significant increase in *CRMP5* protein expression in L-Sox5 overexpressing cells compared with control cells that are transfected with empty vector (Fig. 4c). Thus, L-Sox5 promoted *CRMP5* transcriptional activation, leading to a concomitant increase in *CRMP5* mRNA and protein expressions.

L-Sox5 inhibits neurite outgrowth in N1-E155 cells

CRMP5 has previously been reported to inhibit neurite outgrowth [10]. Therefore, we hypothesized that if L-Sox5 transcriptionally increases *CRMP5* expression, it would result

in inhibition of neurite outgrowth. To determine L-Sox5 impact in this process, we used our previously described cell model with L-Sox5 overexpression that showed an increase in *CRMP5* promoter activation (Fig. 2), mRNA (Fig. 3a) and protein expression (Fig. 3b, c). In addition, to evaluate whether the biological effect of L-Sox5 on neurite extension is mediated by *CRMP5*, we generated constructs to silence *CRMP5* (ATTO-TEC *CRMP5* siRNA) and used scrambled siRNA as controls (ATTO-TEC scrambled siRNA). We analyzed only the co-transfected cells identified with GFP (CBig and CBig-L-Sox5) and ATTO 550 fluorescence signals (siRNA) (Fig. 5). Differentiating, N1E115 cells exhibited visible neurites in control conditions (Fig. 5, scrambled siRNA + CBig empty). However, L-Sox5 overexpression resulted in a negative effect of L-Sox5 on neurite outgrowth confirmed by the reduced neuritic length (L-Sox5 $26.6 \pm 17.8 \mu\text{m}$ vs control $71.8 \pm 54.8 \mu\text{m}$, $p < 0.01$, ANOVA) (Fig. 6a). Classifying the neurites according to their length revealed an increase in the percentage of short neurites ($< 50 \mu\text{m}$) with almost no neurites longer than $100 \mu\text{m}$ in L-Sox5 overexpressing cells compared with control (Fig. 6b). These results showed a novel role for L-Sox5 in the regulation of neurite outgrowth, potentially through *CRMP5* upregulation. To confirm *CRMP5* requirement in L-Sox5 inhibitory effect, we used a siRNA approach to knock down *CRMP5* expression while overexpressing Sox5. Silencing *CRMP5* prevented the concomitant decrease in

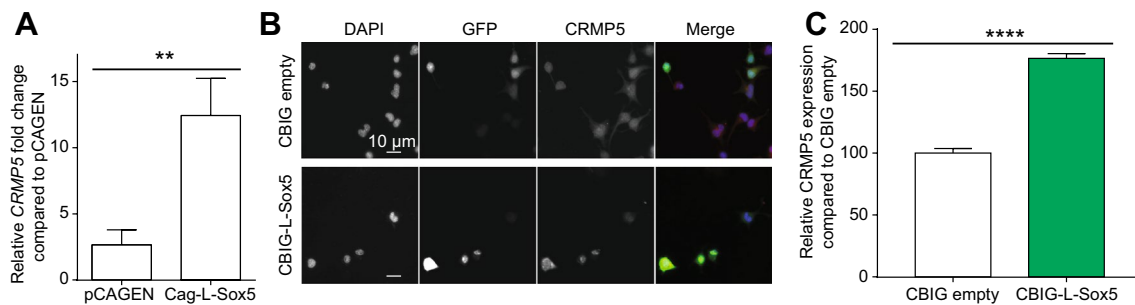


Fig. 4 L-Sox5 augments *CRMP5* mRNA and protein expression. **a** Bar graph representation of the relative *CRMP5* mRNA expression. A semi-quantitative polymerase chain reaction (PCR) analysis of *CRMP5* expression was conducted after N1E115 cells were submitted to L-Sox5 overexpression (Cag-L-Sox5) compared to cells transfected with the empty plasmid (pCAGEN). TRCF was used as a housekeeping gene. Bar graph shows fold change over a calibrator sample ($\Delta\Delta\text{Ct}$ method). Error bars indicate the standard deviation. L-Sox5 overexpression increased the levels of *CRMP5* mRNA in N1E115 cells. $**p < 0.01$ (Mann–Whitney test). Data are the summary of three independent experiments conducted in duplicate. **b** Representative micrographs of N1E115 cells, 48 h after transfection (identified by GFP fluorescence) with either CBig empty plasmid

or CBig-L-Sox5 plasmids which allows for L-Sox5 overexpression. *CRMP5* immunolabeling was performed to assess the protein expression levels in transfected cells. DAPI was used to stain the nucleus of each cell. Scale bars are $10 \mu\text{m}$. **c** Bar graph showing relative *CRMP5* expression in transfected (identified by GFP fluorescence) N1E115 cells 48 h after transfection with either CBig empty or CBig-L-Sox5 plasmid. *CRMP5* fluorescence intensity in CBig-L-Sox5 transfected N1E115 cells was quantified and normalized to *CRMP5* fluorescence levels in CBig empty transfected N1E115 cells. Overexpression of L-Sox5 increased *CRMP5* protein expression ($n = 183\text{--}94$ cells quantified in three independent experiments). Values represent the average \pm SEM, $****p < 0.0001$ compared to CBig-transfected cells, (Student's t test). All analyses were blinded

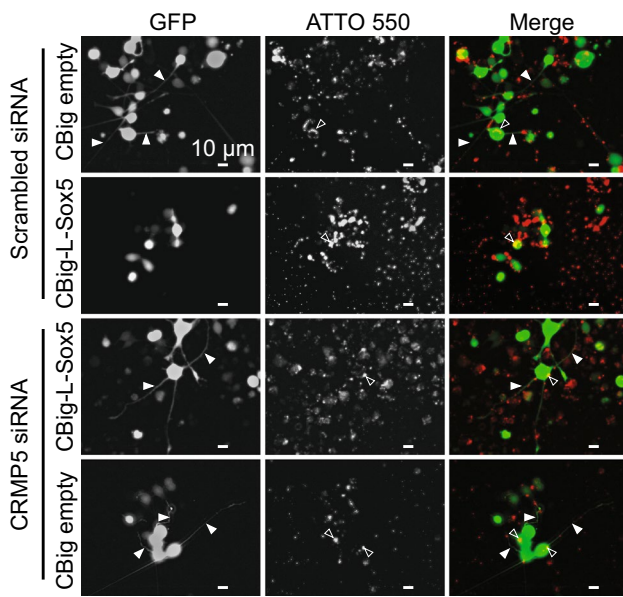


Fig. 5 L-Sox5 controls neurite outgrowth in N1E115 cells with CRMP5. N1E115 cells were cultured in differentiating conditions before transfection with either CBig empty or CBig-L-Sox5 plasmids together with CRMP5 siRNA of scrambled siRNA as indicated. Plasmids transfection was checked by GFP expression (*left column panels*); siRNA co-transfection was checked by ATTO-550 fluorescence (*middle column panels*). The merge (*right column panels*) shows the co-transfection of CBig plasmids and indicated siRNA. *Plain arrowheads* show neurites; *open arrowheads* show intracellular siRNA. Scale bar is 10 μm

neuritic length (Fig. 6a). In addition, CRMP5 knockdown normalized the distribution of neuritic length compared with control (Fig. 6b). Taken together, these findings demonstrate that L-Sox5 acts as a critical player in different aspects of neurite outgrowth, mediated by CRMP5.

L-Sox5 inhibits neurite outgrowth in primary hippocampal pyramidal neurons

To confirm that this L-Sox5–CRMP5 molecular mechanism is also operational in neurons, we used primary hippocampal cultures transfected at DIV2, with either the empty plasmid (CBig, control) or L-Sox5 (CBig-Sox5) associated with or not *CRMP5* silencing. Neurite outgrowth was measured at DIV4 (Fig. 7a). As explained before, we analyzed only the co-transfected cells identified with GFP (CBig and CBig-L-Sox5) and ATTO 550 fluorescence signals (siRNA). Whereas CRMP5 silencing had no effect by itself on neurite length ($113.8 \pm 113.1 \mu\text{m}$) compared to control experiment ($132.2 \pm 140.3 \mu\text{m}$), SOX5 overexpression was able to produce a significant shortening of neurites ($44.9 \pm 47.9 \mu\text{m}$; $p < 0.001$, Kruskal–Wallis) compared to the previous conditions cited. Interestingly, Sox5 overexpression accompanied by *CRMP5* silencing significantly antagonized this shortening ($87.3 \pm 81.8 \mu\text{m}$; $p < 0.001$, Kruskal–Wallis test with Dun’s post hoc test) (Fig. 7b). Classification of the neurites according to their length showed a clear increase of short neurites ($<20 \mu\text{m}$) with very rare longer neurites ($>50 \mu\text{m}$) while overexpressing Sox5. In addition, *CRMP5* knockdown

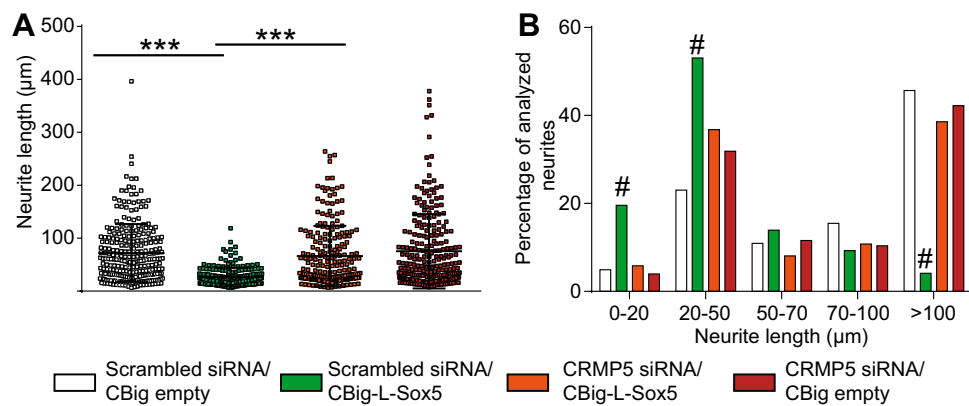


Fig. 6 L-Sox5 inhibits neurite outgrowth through CRMP5 in N1E115 cells. **a** Scatter plot showing the distribution of analyzed neurite length in N1E115 cells, 48 h after transfection with the indicated plasmid/siRNA combinations. L-Sox5 expression decreased the average neurite length in N1E115 cells, $***p < 0.001$ compared to scrambled siRNA/CBig empty, and is reversed by CRMP5 siRNA ($***p < 0.001$). Values represent the average \pm SD, $n = 150\text{--}235$ cells per condition, one-way ANOVA with Dunnett’s post hoc analysis. **b** Bar graph showing the size distribution of analyzed neurites

in N1E115 cells, 48 h after transfection with the indicated plasmid/siRNA combinations. L-Sox5 expression in N1E115 cells, decreased the amount of neurites $>100 \mu\text{m}$ and increased the number of neurites $<50 \mu\text{m}$, $\#p < 0.01$ compared to scrambled siRNA/CBig empty, z test. Results are from three independent experiments. CBig-transfected cells were identified by GFP expression and siRNA transfected cells by ATTO-550 fluorescence as indicated in Fig. 5. All analyses were blinded

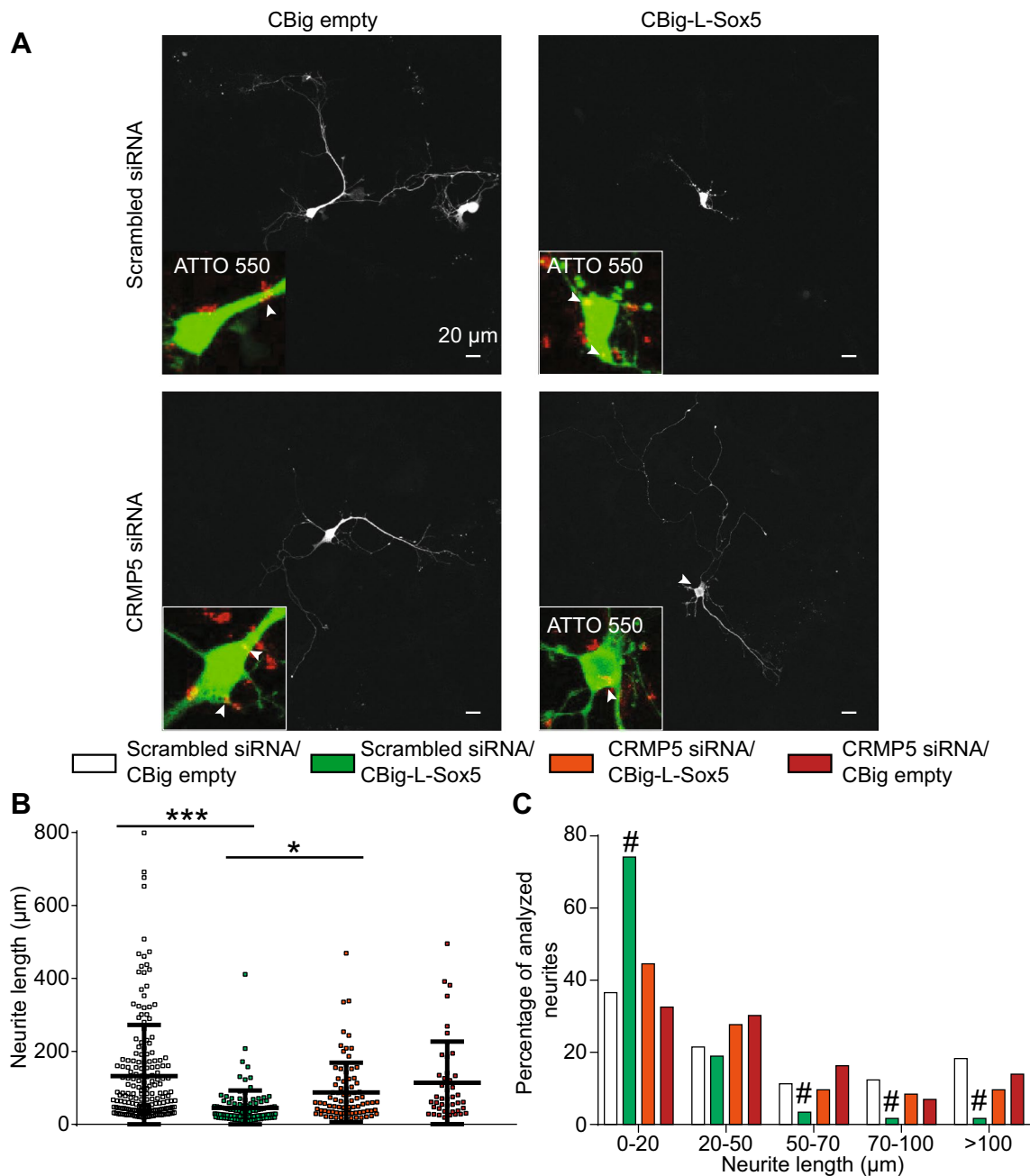


Fig. 7 L-Sox5 inhibits neurite outgrowth in primary hippocampal neurons. **a** Representative micrographs of primary hippocampal neurons, 48 h after transfection (identified by GFP fluorescence) with either CBIG empty or CBIG-L-Sox5 plasmids together with CRMP5 siRNA or scrambled siRNA as indicated. Scale bars are 20 μm. Inset shows the co-transfection of the indicated by neuron by both plasmid and siRNA (indicated by arrows). **b** Scatter plot showing the distribution of analyzed neurite length in primary hippocampal neurons cultures, 48 h after transfection with the indicated plasmid/siRNA combinations,

48 h after transfection with the indicated plasmid/siRNA combinations, *** $p < 0.0001$; * $p < 0.05$ compared to scrambled siRNA/ CBIG empty, mean \pm SD. **c** Bar graph showing the size distribution of analyzed neurites in primary hippocampal neurons, 48 h after transfection with the indicated plasmid/siRNA combinations. L-Sox5 expression in hippocampal neurons decreased the amount of neurites >50 μm and increased the number of neurites <20 μm, # $p < 0.01$ compared to CBIG empty, z test. Results are from two independent experiments. CBIG-transfected cells were identified by GFP fluorescence. $n = 13$ –52 cells per condition. All analyses were blinded

normalized the distribution of neuritic length compared with control (Fig. 7c). Overall these data underline the functional relevance of the L-Sox5-mediated transcriptional control of CRMP5 in hippocampal neurite outgrowth.

Discussion

This study demonstrates that the transcription factor L-Sox5 is responsible for the positive regulation of the cytoskeleton

regulator CRMP5 and uncovers its contribution to neurite development. Based on molecular biology assays, we have identified a regulatory-binding site for SOX transcription factors, in the murine *CRMP5* gene promoter, that promotes transcriptional activity. Our analyses revealed that the expression patterns of both Sox5 and CRMP5 displayed striking similarities in the telencephalic wall from mouse embryo, suggesting their common role in the development of neural circuits. We focused our interest on the long form of Sox5, L-Sox5, which is expressed in neurons [18, 31], whereas the short form is found in testis [32]. We showed that forced expression of L-Sox5 in GL15 cells significantly activates CRMP5 promoter. The mutation of the putative Sox5 RBS core domain allowed a significant but partial decrease in Sox5 mediated activity of the promoter, suggesting that the mutated sequence is not complete enough to totally disrupt L-Sox5 binding, or the presence of additional Sox5 RBS sequence. We showed also that L-Sox5 promotes in situ transcription as well as translation of endogenous CRMP5 by using N1E115 cell line as a neuronal differentiation model. In addition, using a siRNA knockdown strategy, we confirmed that CRMP5 is required for the Sox5-dependent inhibition of neurite outgrowth. However, when Sox5 activation is lacking, the neurite length is not significantly increased with CRMP5 silencing, as previously reported [8], due to different N1E115 cell culture condition with serum deprivation. Similarly, CRMP5 silencing failed to increase neurite length in hippocampal neurons when endogenous CRMP5 level is already low at DIV4 [8]. Altogether our findings demonstrated that CRMP5 effect lies downstream of Sox5 activation. Finally, we provided strong evidence for the functional relevance of this Sox5–CRMP5 molecular pathway to neurite growth in hippocampal neurons.

Our laboratory has shown that CRMP5 antagonizes CRMP2 growth-promoting effect on neurites in hippocampal neurons, which suppresses neurite elongation [8]. This dominant inhibitory action of CRMP5 clearly depends on its tubulin-binding domain since a truncated CRMP5, lacking this domain failed to curb neurite outgrowth [8]. Furthermore, another work documented that phosphorylation of the 516 threonine residue in this domain plays a key role in preventing microtubule polymerization and neurite growth [33]. Thus, CRMP5 appears to be instrumental in determining cytoskeletal dynamics, which raises question about its upstream transcriptional regulation. Transcriptional regulatory elements have been identified for other members of the CRMP family, such as CRMP1 [34, 35], and their interplay results in the regulation of its invasion suppressor activity [35]. In addition, locus-specific changes in CRMP4 promoter methylation have been shown to influence prostate cancer cell dissemination [36]. However, CRMP transcriptional pathways involved in the establishment of neuronal differentiation remain to be elucidated. Insights into the impact of

CRMP transcriptional regulation in neuronal differentiation come from loss-of-function studies in CRMP-deficient mice. Several reports indicate that lack of CRMP proteins results in an alteration of dendritic organization and function in the adult hippocampus or cerebellum [11, 37–40]. Here, we provide new evidence that upstream regulation of CRMP5 gene expression by the L-Sox5 transcription factor, which acts as an activator in the neuronal lineage [13], controls neurite elongation. We now show that L-Sox5 is able to orchestrate neurite outgrowth program in hippocampal neurons through the regulation of CRMP5 levels.

Although Sox transcription factors have been involved in neurogenesis [41], the function of Sox5 remains largely unexplored in the developing brain. Knockout mice models for Sox5 display multiple disorders that reflect brain abnormalities in cerebral structures originating from the telencephalic wall. Sox5 loss of function in mice produces a temporal mismatch in the differentiation program of descending cortical projection neurons in the neocortex that leads to aberrant connectivity to subcortical targets [14, 16, 18, 42]. Thus, a lack of Sox5 triggers a premature differentiation of subsets of cortical neurons [16], suggesting that Sox5 expression levels control neuronal identity and differentiation. Consistently, our new results indicate that a gain in L-Sox5 inhibits neurite outgrowth in neuron-like differentiated neuroblastoma cells as well as in hippocampal neurons. Furthermore, our online analysis revealed that Sox5 is highly expressed in the cortical and hippocampal lineage together with CRMP5, suggesting that the Sox5–CRMP5 transcriptional pathway could have an instructive role in controlling neurite outgrowth that features early steps of neuronal differentiation. Nevertheless, such a correlation of gene expression is not observed in other brain structures or at later developmental stages, supporting the hypothesis that Sox5–CRMP5 transcriptional pathway specifically orchestrates the neuritogenesis of timely and spatially defined populations of neurons.

In a recent study, we demonstrated the implication of CRMP5 in glioblastoma aggressiveness through the control of tumor cell proliferation [14]. Notably, tumor tissues derived from glioblastoma patients have already been shown to display high Sox5 expression [42]. In addition, data mining using the ©2015 Ivy Glioblastoma Atlas Project available from glioblastoma.alleninstitute.org underlined a positive and statistically significant (Spearman $r = 0.44$, $p = 0.03$) correlation between Sox5 and CRMP5 protein expressions. Therefore, it might be hypothesized that Sox5–CRMP5 transcriptional pathway is required in glioblastoma tumor growth. Furthermore, it has been shown that correct gliogenesis partly depends on Sox5 because premature oligodendrocyte specification occurs in Sox5-deficient embryos [43]. Thus, Sox5 appears to be a potential candidate for the maintenance of glial precursors in an undifferentiated

state. Given that migration of oligodendroglial progenitors is directly facilitated by Sox5 [44], it would be interesting to examine whether proliferative and migratory capacities of oligodendroglial progenitors and glioblastoma cells rely on a common molecular mechanism, the Sox5–CRMP5 pathway.

In conclusion, the transcriptional regulation of one of the key cytoskeleton regulators in neuronal development has been deciphered in this study. We demonstrate that this novel Sox5–CRMP5 pathway is a major molecular mechanism underlying neurite outgrowth, but further work must be done to dissect how it might influence neuronal differentiation.

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