Sox2 controls neural stem cell self-renewal through a Fos-centered gene regulatory network

Running head:

A Sox2-Fos axis controlling neural stem cells

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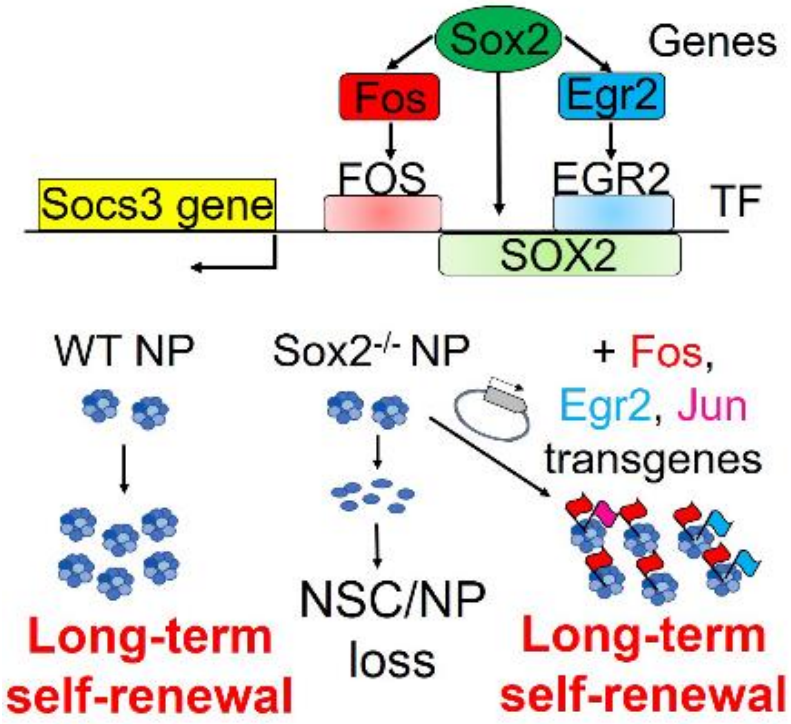
Neural stem cells (NSCs), Sox2, Fos, transcription factors, self-renewal, gene expression, lentiviral vector, CRISPR, conditional knockout, cell biology

Abstract
The Sox2 transcription factor is necessary for the long-term self-renewal of neural stem cells (NSC). Its mechanism of action is still poorly defined. To identify molecules regulated by Sox2, and acting in mouse NSC maintenance, we transduced, individually or in combination, into Sox2-deleted NSC, genes whose expression is strongly downregulated following Sox2 loss (Fos, Jun, Egr2). Fos alone rescued long-term proliferation, as shown by in vitro cell growth and clonal analysis. Further, Fos requirement for efficient long-term proliferation was demonstrated by the strong reduction of NSC clones capable of long-term expansion following CRISPR/Cas9-mediated Fos inactivation. Previous work showed that the Suppressor of cytokine signaling 3 (Socs3) gene is strongly downregulated following Sox2 deletion, and its reexpression by lentiviral transduction rescues long-term NSC proliferation. Fos appears to be an upstream regulator of Socs3, possibly together with Jun and Egr2; indeed, Sox2 reexpression in Sox2-deleted NSC progressively activates both Fos and Socs3 expression; in turn, Fos transduction activates Socs3 expression. Based on available SOX2 ChIPseq and ChIA-PET data, as well as results from the literature, we propose a model whereby Sox2 is a direct activator of both Socs3 and Fos, as well as possibly Jun and Egr2; in turn, Fos, Jun and Egr2 may activate Socs3. These results provide the basis for developing a model of a network of interactions, regulating critical effectors of NSC proliferation and long-term maintenance.

Significance statement
Proliferation and maintenance of NSC are essential during normal brain development, and, postnatally, for the maintenance of hippocampal function and memory until advanced age. Little is known about the molecular mechanisms that maintain the critical aspects of NSC biology (quiescence and proliferation) in postnatal age. Our work provides a methodology, transduction of genes deregulated following Sox2 deletion, that allows to test many candidate genes for their ability to sustain NSC proliferation. In principle, this may have interesting implications for identifying targets for pharmacological manipulations.


Sox2
Genes
Fos
Egr2
FOS
EGR2
TF
Socs3 gene
SOX2
WT NP
Sox2-/- NP
+ Fos,
Egr2, Jun
transgenes
NSC/NP
loss
Long-term
self-renewal
Long-term
self-renewal

Introduction

The transcription factor Sox2 is critically important in the development of the brain. In humans, Sox2 mutations cause brain abnormalities (hippocampal dysplasia, learning disabilities, epilepsy, motor control problems, eye and vision defects)[1-4]. Sox2 is expressed in neural stem and progenitor cells; its deletion during mouse embryogenesis causes early postnatal loss of NSC in the hippocampus, and its postnatal loss causes decreased hippocampal neural stem/progenitor cell proliferation [5]. In vitro experiments on NSC showed that Sox2 loss causes progressive exhaustion of NSC, in contrast to the long-term proliferation of control wild type cells (Favaro et al 2009); differentiation defects of progenitor cells have also been reported in Sox2-mutant cells by [6] and [7]. The loss of self-renewal in Sox2-mutant cells grown in vitro is reversible; in fact, re-expression, in Sox2-deleted cells, of Socs3, the gene most downregulated following Sox2 loss, rescued long-term self-renewal of mutant cells [8]. These findings point to the possibility of identifying the network of regulatory processes acting downstream to Sox2 in allowing NSC proliferation and preserving their functional integrity. To this end, we expressed, in Sox2-deleted NSC, some of the most downregulated genes identified by RNAseq comparison of Sox2-deleted and wild type NSC. We show that Fos, a transcription factor, efficiently rescues long-term NSC proliferation of Sox2-deleted cells, and increases Socs3 levels; also, its mutation in wild type NSC greatly reduces their ability to generate long-term proliferating clones. Mining of our previous NSC genomic analyses of Sox2 ChIPseq and RNApolII ChIA-PET, which detect long-range promoter-enhancer chromatin interactions [8], shows that Socs3, Fos and its partner Jun, and Egr2 (a regulator of Socs3), are all bound by Sox2, defining a network of regulatory interactions sustaining Sox2-dependent long-term NSC self-renewal.

Materials and Methods

Primary ex-vivo neural stem/progenitor cell cultures

Brain-derived NSC cultures were obtained from dissected telencephalon of wild-type and Sox2-deleted P0 mice, and grown in fresh medium (FM): DMEM-F12 with Glutamax (GIBCO), supplemented with 1/50 vol/vol B27 (Life Technologies), 1% of Penicillin-Streptomycin (Euroclone) supplemented with EGF (10 ng/ml, Tebu-bio) as mitogen, as described in [5,8,9].

Lentiviral constructs

Fos, Jun and Egr2 cDNAs were derived from Addgene plasmids: Fos: "pcDNA3FLAGFos WT", from John Blenis (#8966); Jun: "FlagJunWTMyc", from Axel Behrens (#47443); Egr2:"mEgr2/LZRS", from David Wiest (#27784). cDNAs were amplified by PCR and cut with BamHI, and cloned into a unique BamHI site upstream to the IRES-dNGFR cassette of the pHR SIN BX IR/EMW [10](a gift from A. Ronchi, Milano).

The lentiviral vector for CRISPR-Cas9 experiments was lentiCRISPRV2puro (Addgene #98290, RRID: Addgene_104990). The guide RNA (sgRNA) for Fos mutagenesis was designed and cloned according to:

https://media.addgene.org/data/plasmids/52/52961/52961attachment_B3xTwla0bkYD.pdf and is:

5'-CACCGTGATGTTCTCGGGTTTCAAC-3'

As a control, we designed also a scramble non-targeting guide RNA (Mock):

5'- CACCGATGTTGCAGTTCGGCTCGAT-3'

Lentiviral vectors were produced by calcium phosphate transfection into the packaging human embryonic kidney cell line 293T, of the VSV-G plasmid (encoding ENV), CMV R8.74 (packaging) and pRSV-REV (encoding reverse transcriptase)[8,10]. Briefly, after transfection, following replacement with DMEM high glucose (Euroclone), containing 10% Fetal Bovine Serum (Sigma), 1% penicillin-streptomycin (Euroclone), 1% of L-Glutamine (Euroclone), the cell supernatants were collected 24-48 hours after transfection. Lentiviral vectors were titrated on HEK-293T cells by measuring the percentage of eGFP (for the Sox2-transducing vector) or of dNGFR-positive cells (for the Fos, Jun, Egr2-transducing vectors) by Flow Cytometry [10].

NSC transduction with lentiviral constructs encoding Fos, Jun, Egr2, or Empty Vector.

NSC transduction [8]: wild-type and Sox2-deleted neurospheres were grown for 2-3 passages (3-4 days each) in FM supplemented with bFGF (10ng/ml, Tebu-bio) and EGF (10 ng/ml, Tebu-bio), and for one more passage in FM supplemented with EGF only. For passaging, neurospheres were first incubated in 0,25% trypsin (GIBCO) for 5 minutes at 37°C and, subsequently, ovomucoid (Leibovitz's L15 medium (GIBCO) containing trypsin inhibitor (Sigma), BSA (Sigma) and 40 µg/ml DNaseI (Sigma) for 5 minutes at 37°C). Spheres were then carefully dissociated mechanically by gently pipetting up and down, centrifuged at 1200 rpm for 4 minutes and cells were resuspended in 1ml of FM, counted and replated at a density of 25,000 cells/1ml/well (9 wells for each cell type) in 24-well plates, in FM. After 4 hours cells (Sox2-deleted or wild-type) were transduced with appropriate lentiviral vectors (single or in combination), at a multiplicity of

infection (MOI) 5.5 for each vector. Cells were incubated overnight at 37°C, then 1ml per well of FM with EGF was added both to transduced and non-transduced (control) cells. After 4 days, cells were dissociated to single cells as above, counted, and seeded at a density of 20,000 cells/well in FM with EGF. In addition, 500,000 cells for each sample (from pooled wells) were fixed using PFA 4% and stained as in [10] with an anti-human CD271 (dNGFR) antibody, conjugated with Phycoerythrin (PE) (BioLegend, Cat. No. 345106, RRID: AB_2152647) (dilution 1:200) and analyzed by CytoFLEX (Beckman-Coulter) to determine the percentage of infected cells: 10,000 events were analyzed for each sample. Cells were dissociated, counted and seeded at the same density every 3-4 days, to obtain a cumulative growth curve (Fig 2A, 2B).

Identification of Fos, Jun, Egr2 lentivirus integrated in clones of lentivirally transduced NSC

Cell populations of NSC transduced with lentiviruses encoding Fos, Jun, Egr2, obtained after 6 or more passages from the transduction, were plated at limiting dilution into 96-well plates (down to a dilution of 7.5 cells/well, which gave either one or no clone in every well). Individual clones (neurospheres) were analyzed by PCR following a short expansion (2-3 passages), DNA was extracted and analyzed by PCR using primers designed to detect the virally transduced, but not the endogenous, Fos, Jun and Egr2 (forward primer is in the viral SFFV promoter driving the cDNA expression; reverse primer is in the cDNA).

Forward primer SFFV: 5'-CTCACAACCCCTCACTCG-3'

Fos Reverse primer: 5'- AGGTCATTGGGGATCTTGCA-3' (and 5'-GGCTGGGGAATGGTAGTAGG-3')

Jun Reverse primer: 5'- GGTTCAAGGTCATGCTCTGT-3'

Egr2 Reverse primer: 5'- TGCCCGCACTCACAATATTG-3'

Transduction of Sox2-deleted NSC with Sox2-encoding lentiviral vector

Sox2-deleted NSCs were dissociated to single cells and seeded at a density of $5x10^5$ cells/T25cm^2 flask/5ml, in FM. After 4 hours NSCs were transduced with a GFP-Sox2- expressing lentivirus [5], at a multiplicity of infection (MOI) 8 and 12 (Suppl. Fig. 3). Cells were incubated overnight at 37°C. Virus was removed by medium change at 24 h: cells were centrifuged at 1000 rpm for 4 minutes and resuspended in FM with EGF. Non-transduced controls were treated equivalently (without virus). At every passage, every 3-4 days, cells were dissociated to single cells as above, counted, and replated in FM with EGF at a density of 20,000 cells/well/1ml, to generate a cumulative growth curve (Suppl. Fig 3). At successive passages, aliquots of Sox2-transduced cells (50,000-500,000 cells, from pooled wells) were fixed using 4% paraformaldehyde (PFA) and

analyzed for GFP fluorescence by CytoFLEX (Beckman-Coulter) to determine the percentage of infected cells: 10,000 events were analyzed for each sample.

Measure of Fos and Socs3 expression levels in Sox2-deleted cells transduced with Sox2- or Fos-encoding lentiviral vectors

Sox2-deleted NSCs were dissociated to single cells and seeded at a density of $5x10^5$ cells/T25cm^2 flask/5ml, in FM. After 4 hours NSCs were transduced with lentiviruses expressing Sox2 or Fos at a multiplicity of infection (MOI) 10. Cells were incubated overnight at 37°C. Virus was removed by medium change at 24 h: cells were centrifuged at 1000 rpm for 4 minutes and resuspended in FM. Non-transduced controls were treated equivalently (without virus).

At every passage, every 3-4 days, cells were dissociated to single cells as above, counted, and replated in FM with EGF at a density of 800,000 cells/T75cm^2/12ml.; 50,000-500,000 cells were analyzed by FACS for GFP or dNGFR expression, as described above, to determine the percentage of infected cells. Additionally, aliquots of cells (100,000-500,000 cells) were lysed in TRIzol (Life Technologies) for total RNA extraction. RNA was purified using Direct-zol RNA Miniprep (Zymo Research), treated with DNase (Zymo Research) for 30 minutes at 37° C and 400ng of RNA were retrotranscribed (High Capacity cDNA Reverse Transcription Kit, Applied Biosystem). About 5µl of a 1:25 dilution (adjusted following normalization by *Hprt* expression) were used for the real time PCR. Negative control reactions (without Reverse Transcriptase) gave no PCR amplification. Real time analysis was performed using ABI Prism 7500 (Applied Biosystems). Samples from each experiment were analyzed in duplicate. Specific PCR product accumulation was monitored by using SsoAdvanced™ Universal SYBR® Green Supermix (Bio-Rad) fluorescent dye in a 12-µl reaction volume. Dissociation curves confirmed the homogeneity of PCR products.

Primers for the quantification of Fos and Socs3 mRNAs are:

Fos Forward primer: 5'-CTGTCCGTCTCTAGTGCCAA-3'

Fos Reverse primer: 5'-TGCTCTACTTTGCCCCTTCT-3'

Socs3 Forward primer: 5'-ACCTTTGACAAGCGGACTCT-3'

Socs3 Reverse primer: 5'-AGGTGCCTGCTCTTGATCAA-3'

HPRT Forward primer: 5'-TCCTCCTCAGACCGGTTT-3'

HPRT Reverse primer: 5'-CCTGGTTCATTCATCGCTAATC-3'

All qRT-PCRs for Fos and Socs3 were performed in parallel with HPRT qRT-PCR, for normalization.

CRISPR-Cas9 assays

Wild-type neurospheres were dissociated into single cells, as described above, and seeded at a density of $5x10^5$ cells/T25cm^2 flask/5ml, in FM with EGF. Cells were transduced, after 4 hours from plating, with a previously defined amount of lentiCRISPRV2puro lentivirus expressing the anti-Fos guide RNA or the scramble non-targeting guide RNA. Medium was changed after 3 days and cells were selected in FM with 5µg/ml Puromycin (Sigma-Aldrich, cod. P8833) for 3 days. To remove puromycin, cells were then centrifuged at 1000 rpm for 4 minutes and resuspended in FM without puromycin and grown for 15 days to allow recovery from the stress of transduction and selection. Neurospheres were then dissociated to single cells; similar numbers of cells treated with scramble guide RNA (Mock) or with anti-Fos guide RNA (Mut) were plated at clonal dilution in individual wells of a 96-well plate in FM with EGF. With 15 cells/well, essentially all wells showed, after 10 days, at least one or more neurospheres; at a nominal limiting dilution of 7.5 cells/well, single clones (neurospheres) appeared only in a proportion of the wells (the "primary clones"). These clones were then further used to determine the proportion of long-term growing clones (see below). Two experiments were performed: 258 and 207 primary clones were obtained from cells treated with scramble guide RNA; 73 and 198 primary clones were detected from cells treated with anti-Fos guide RNA.

Individual primary clones from wells showing a single neurosphere were picked up, disaggregated and replated ("replated clones", Fig. 4C, 4D) in a single well of a 48-well plate, for further expansion in a 24-well plate and, finally, in a 6-well plate and in flasks. At every passage, the number of clones which did not proliferate was annotated. Clones that continued to grow in flasks or 6-well plates were considered to be "long-term growing clones".

To verify the efficiency of Fos mutagenesis, at the beginning of the experiment (see above and Fig. 4B) an aliquot of the puromycin-resistant cells was collected, DNA was extracted and amplified by PCR with primers surrounding the site of hybridization of the sgRNA. The amplified DNA was cloned in pGEM®-T Easy (Promega, A1360) by transformation of TOP10 E.coli, and inserts from individual colonies were sequenced. The sequences from CRISPR-Cas9-treated cells were compared to wild-type sequences, by using BLAST NCBI.

PCR primers:

Fos Forward: 5'-TCACAGCGCTTCTATAAAGGC-3' and 5'-CTACTACTCCAACCGCGACT-3'

Fos Reverse: 5'-CTGCGAGTCACACCCCAG-3' and 5'-CGCCAGTCTCCCTCCAGA-3'

Immunocytochemistry

Transduced Sox2-deleted, or wild type control NSCs, were dissociated to single cells and seeded on Matrigel™-coated glass coverslips. After 4h cells were fixed (20 minutes) with 4% PFA in phosphate-buffered saline (PBS; pH 7.4) and rinsed 3 times with PBS. Coverslips were then incubated for 90 minutes at 37°C in PBS containing 10% normal goat serum (NGS), 0.2% Triton X-100. Coverslips were incubated with the primary anti-SOX2 antibody (mouse monoclonal IgG2a, 1:100, R&D Systems), overnight at 4°C. Following thorough washing with PBS, cells were incubated for 45 minutes (room temperature) with secondary rhodamine (TRITC)-conjugated goat anti-mouse IgG2a antibodies (1:1000, AlexaFluor Life Technologies). Coverslips were rinsed 3 times in PBS and mounted on glass slides with Fluoromount (Sigma) with DAPI (4′,6-diamidino-2-phenylindole).

Results

Reactivation of Fos expression in Sox2-deleted neural stem cells restores long-term self-renewal

We identified, by RNAseq, about 900 genes downregulated in NSC grown in vitro from neonatal brain, after Cre-mediated Sox2 deletion during embryogenesis [8]. To identify the network of genes mediating Sox2 function in NSC self-renewal, we reasoned that genes downregulated following Sox2 deletion likely include those required for self-renewal; indeed, we previously showed that the most downregulated gene, Socs3, encoding a signaling protein acting at the cell membrane, rescued long-term self-renewal when expressed via a lentivirus in Sox2-deleted cells [8]. We therefore overexpressed, in Sox2-mutant cells, three among the most downregulated genes (Fig. 1A), Fos, Jun, and Egr2, encoding transcription factors which might possibly be involved in the regulation of Socs3 (or other critical factors).

To test for the functional role of the downregulated genes, we individually cloned their cDNAs into a lentiviral expression vector, and transduced them into Sox2-mutant cells, cultured from P0 mouse brains from which Sox2 had been deleted in vivo, at embryonic day (E) 11.5, via a nestin-Cre transgene [5,8](Fig. 1B). We first tested a combination of vectors expressing Fos and Jun, since FOS and JUN are known to act together, forming the AP1 complex, in transcriptional regulation [11]. The vector contains a delta-NGF receptor (dNGFR) marker gene that allows to identify transduced cells by anti-dNGFR immunofluorescence (Fig. 1B). In preliminary experiments, we transduced NSC using single vectors, including each of the genes to be tested, to evaluate the proportion of the cells that were transduced by each type of vector: the transduction efficiency was 23,3% for Fos; for Jun and for Egr2, it was 18,5% and 17,1%, respectively.

We then transduced mutant cells at early stages of their culture, using combinations of different vectors at the same multiplicity of infection (MOI) as that used for single-vector transduction (Fig. 2). After initial passages, non-transduced mutant cells progressively slowed down their proliferation and stopped to grow, the culture eventually becoming exhausted, as expected [5,8]; by contrast, cells transduced with Fos and Jun continued to grow exponentially, with a kinetics matching that of control wild-type cells (Fig. 2A). Taking advantage of the delta-NGF receptor (dNGFR) marker, that is coexpressed with the cDNAs from the lentiviral vectors, we followed the percentage of dNGFR-positive (transduced) cells though cell passaging. While, at the beginning (passage 2 after transduction), dNGFR-positive cells represented about 50% of total cells, their percentage progressively rose to 100% (passage 15) through passaging (Fig. 2C; Suppl. Fig. 1), indicating a selective advantage of transduced cells.
We also verified that cells that had recovered self-renewal were all SOX2-negative, by immunofluorescence (IF) for SOX2 (Suppl. Fig. 2A); this ruled out the possibility that a positive selection of rare, SOX2-positive cells persisting in Sox2-deleted brains could contribute to the recovery of mutant cells.

We also tested a second combination of vectors, expressing Fos and Egr2 (Fig. 2B). In parallel with this vector combination, we also transduced mutant cells with the Fos-expressing vector only (Fig. 2B). The viral upregulation of Fos and Egr2 in mutant cells prevented cell exhaustion, and restored long-term self-renewal (Fig. 2B), similarly to what had been found with Fos+Jun (Fig. 2A). A similar result was obtained also with the Fos vector alone; self-renewal was restored, and the growth kinetics of the cells was similar to that of wild type cells (Fig. 2B). We then measured the percentage of transduced cells by FACS analysis with an anti-dNGFR antibody, as previously done for the Fos+Jun-transduced cells; this was about 20% at the beginning (passage 2), and progressively rose to about 100% through passaging (for both Fos+Egr2 and Fos only), again pointing to a selective advantage of the transduced cells (Fig. 2C; Suppl. Fig. 1).
As a control experiment, also wild type cells were transduced with the Fos-expressing vector; this did not lead to any change in the growth kinetics of the cells, that remained the same as that of non-transduced cells (Suppl. Fig. 2B). As a further control, we also verified that mutant cells, transduced with an “empty” dNGFR lentiviral vector, did not change their growth kinetics in comparison to that of non-transduced cells (Suppl. Fig. 2B).
Overall, these results point to Fos as the major player in the rescue of NSC self-renewal in transduced cells.

Do any of the other co-transduced cDNAs play any additional role in the effect shown? To this end, we plated the transduced cells (Fos + Jun transduction) at limiting dilution, in 96 well plates, to obtain clones. These were then individually expanded, and tested for the presence of the two lentiviral inserts (Fos, Jun). Out of 14 clones tested by PCR analysis, only two contained Jun, but all contained the Fos-expressing vector (Fig. 3A). This indicated that Fos is required for the observed recovery of self-renewal, as suggested. Importantly, no clones exclusively transduced with Jun (but not with Fos) were detected, implying that Jun, by itself, is not sufficient to rescue Sox2-deleted stem cells. The proportion of doubly transduced clones (Fos+, Jun+) is roughly that expected on the basis of the probability of chance transduction of a Fos-transduced cell also with a Jun vector, based on the previous assessment of the transduction efficiency of individual vectors (see above).

Similarly, in the Fos+Egr2-transduction, out of 14 clones expanded and analyzed, 8 contained the Egr2 vector, but all contained the Fos vector; none contained the Egr2 vector alone (Fig. 3B). Again, this indicates that Egr2 alone cannot rescue NSC long-term proliferation.

Fos mutation antagonizes NSC growth

Having found that Fos, a putative target of Sox2, is able to replace Sox2 to maintain NSC self-renewal, when expressed in Sox2-deleted cells, we asked if Fos itself is required to maintain NSC, in the presence of wild type Sox2. We thus mutated the endogenous Fos gene, using the CRISPR/Cas9 system. A short guide RNAs (sgRNA) was identified, which efficiently mutagenized Fos (sgRNA-fos) (Fig. 4A; Methods). Wild type NSC were transduced with a Cas9+sgRNA-expressing lentivirus, carrying a puromycin-resistance gene, or a control virus expressing "scramble" sgRNA that is not expected to mutate Fos; transduced cells were then selected by puromycin for 3 days (Fig. 4B). After puromycin withdrawal, the cells were grown for 15 days to allow recovery from the stress of transduction and selection; at this initial stage, control and mutated cells proliferated in bulk culture with comparable kinetics (not shown). In parallel, at the time of puromycin withdrawal, an aliquot of the puromycin-resistant cells was collected, DNA was extracted and amplified by PCR with primers surrounding the site of hybridization of the sgRNA, and the PCR product cloned in a plasmid, and sequenced (Fig. 4B). 9/10 individual clones were mutated with the anti-Fos sgRNA, whereas no mutated Fos copy was found in scramble-treated cells (Suppl. Table 1).

We then tested the ability of NSC to self-renew generating stem cells capable of giving rise to clonal progeny [12,13](Fig. 4C). In this type of experiment, cells were plated at clonal dilution in individual wells, and the appearance of single neurospheres in a well was evaluated at the

microscope. When the growth of the neurosphere had generated a relatively large number of cells (a "primary clone"), this was picked up, disaggregated and replated ("replated clones", Fig. 4C,D) in a single well, for further expansion. Primary clones could be obtained from both scramble-treated controls (mock) and sgRNA-fos treated cells. We then asked whether each of these clones represents the bona fide progeny of a long-term self-renewing stem cell, by testing for its ability to expand through serial passaging, to give long-term growth (Fig. 4C,D, Suppl. Table 2, "long-term proliferating clones"). We performed two experiments (Fig. 4D, Suppl. Table 2): in both experiments, the number of primary clones (from sgRNA-fos treated cells), capable to reform secondary clones demonstrating long-term growth, was greatly, and significantly, diminished in comparison to controls from mock-treated cells (Fig. 4D, Suppl. Table 2, line 2). These data indicate that Fos-mutant NSC progressively deteriorate during culture, failing to self-renew, a phenomenon reminiscent of the previous observations obtained with Sox2-deleted NSC [8].

Sox2 upregulation in Sox2-mutant cells restores expression of endogenous Fos and Socs3

These observations made us wonder if re-expression of Sox2 into Sox2-deleted cells, leading to recovery of self-renewal [5](Suppl. Fig. 3), would also re-increase levels of Fos and Socs3 expression. We thus analyzed by qRT-PCR the expression of Fos, and of Socs3, in mutant cells (three different mutants) transduced with Sox2, which re-acquire the ability to self-renew long term. Transduction of Sox2 initially (until passages 5-6) had little or no effect on the expression of Fos and Socs3 (Fig. 5 A,B); however, at later stages in culture, after 5-8 passages, there was a substantial increase of both Fos and Socs3 mRNAs that lasted at least until late passages (passages 20-22, when the experiment was interrupted)(Fig. 5A,B). In some experiments, we also checked the expression of Socs3 in Sox2-mutant cells transduced with Fos (Fig. 5C)(alone or together with Jun or Egr2); again, an increase of Socs3 expression was observed. A selective advantage of Sox2-transduced cells was again observed by FACS analysis through passaging, until almost 100% of the cells were represented by transduced cells (Suppl. Fig. 3).

Discussion

Socs3 overexpression is able to rescue the self-renewal defect of Sox2-mutant NSC [8]. Do any additional genes, upstream to Socs3, or acting in parallel with it, contribute to mediate Sox2 effects on self-renewal? In the present work, we tested some candidate genes which are strongly downregulated in Sox2-mutant cells. We found that Fos alone is able to fully rescue Sox2-mutant cells (Fig. 2). The analysis of the rescued clones, obtained by Fos+Jun or Fos+Egr2 transduction,

shows that neither Jun, nor Egr2 play critical roles in the rescue. In fact, we do not find any rescued clones that have been transduced exclusively with one of these genes, and, when these transduced genes are present in clones, they are always accompanied by Fos. Further, the proportion of Fos-transduced clones, that are also transduced by Jun (Fos + Jun), is not higher than that expected on the basis of chance cotransduction of a Fos-transduced cells also by a Jun vector, indicating that Jun does not significantly cooperate with Fos in the rescue (Fig. 3). However, the proportion of Fos/Egr2 cotransduced rescued clones (8 out of 14 total rescued clones) is higher than expected based on the assumption that Fos transduced cells should be transduced with Egr2, by chance, with a probability of 0.17, the frequency of Egr2 transduction; the result obtained might suggest that Fos-transduced cells, also transduced with Egr2, have some proliferative advantage over Fos-only-transduced NSC, indicating that Egr2 could cooperate with Fos. Finally, the progressive decrease of clonogenicity that occurs in Sox2-positive NSC mutated in Fos by CRISPR/Cas9 (Fig. 4; Suppl. Tables 1, 2) confirms that Fos is a critical component of the genetic program controlling in vitro self-renewal of NSC.

Fos, a component, with Jun or related factors, of the AP-1 protein complex, is well known to control cell life and death by regulating the expression and function of cell cycle regulators such as Cyclin D1, p53, p21cip1/waf1, p19ARF and p16 [14]. In NSC grown in vitro, factors such as FGF2 and bEGF control cell proliferation through a cascade involving ERK signaling, Fos, and cyclins [15]. The strong decrease of Fos in Sox2-deleted cells (Fig. 1A), although less profound than that observed with Socs3, is in line with the known role of Fos in cell proliferation control. Thus, three lines of evidence support the conclusion that Fos is an important mediator of Sox2 function in the maintenance of NSC self-renewal: i) the rescue of self-renewal of Sox2-deleted cells by Fos overexpression (which, by itself, has no effect in wild type NSC); ii) the decreased long-term self-renewal in Fos-mutated NSC; iii) the known proliferation-related activity of Fos in several different cell types.

The effects on Fos and Socs3 levels of Sox2 deletion, and of Sox2 re-expression in Sox2-deleted cells, suggest that Sox2 might act directly on these two genes. In favor of this hypothesis is the observation that Fos is connected by long-range chromatin interactions to two different functionally validated enhancers [8,16], shown by SOX2 ChIPseq to be bound by SOX2 (see Table S7 in [8]), and that Socs3 is bound by SOX2 on its promoter (Fig. 6B) as well as on a connected functional enhancer (Fig. 6A) [8]. Additionally, also Jun and Egr2 regions show SOX2 binding (Fig. 7). Sox2 loss disrupts a large proportion of promoter-enhancer interactions in NSC, including those in the Fos, Socs3, Jun and Egr2 regions (Fig. 7), providing a plausible explanation for the decreased expression of all these genes.

Finally, additional studies in non-neural cells showed that both the AP1 (FOS+JUN) complex and Egr1/2 interact with the Socs3 promoter to activate it in transfection experiments (Fig. 7) [17,18]. Preliminary experiments (not shown) do in fact suggest that Egr2 transduction in Sox2-deleted cells transiently increases Socs3 levels during the initial cell culture passages.

Conclusions and Summary

Overall, these data indicate that a Sox2-dependent Fos/Jun/Egr-Socs3 network is involved in the regulation of in vitro NSC self-renewal (Fig. 6, 7). Within this network, Fos and its putative target Socs3 appear to be essential for NSC renewal. While our transduction results do not document a critical role for the Jun and Egr genes in NSC maintenance, their proposed involvement in Socs3 regulation (Fig. 6), dependence on Sox2 for expression, and binding by Sox2 (Fig. 7) point to some role of these genes in Sox2-dependent proliferation. Indeed, it remains possible that the overall residual levels of Jun and Egr1/2 in Sox2-deleted cells are sufficient (and required) to sustain their regulatory functions in NSC, once Fos is provided to the cells by transduction, as in Fig. 2. Under these conditions, further adding Jun and Egr expression, as in Fig. 2, is not expected to reveal their necessity.

The present data open a new window on factors necessary for NSC maintenance.

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Disclosure of potential conflicts of interest

The authors declare no potential conflicts of interest.

Data availability

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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A

Gene	wt	Sox2-mut	Fold ratio wt/Sox2-mut
Socs3	254.43	29.32	8.7
Fos	2868.27	818.55	3.5
Jun	621.48	248.82	2.5
Egr2	164.05	82.58	2.0

B

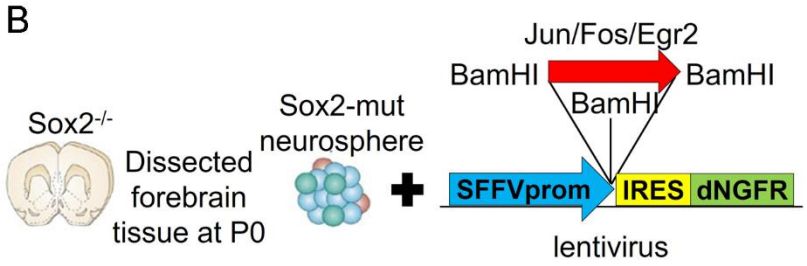
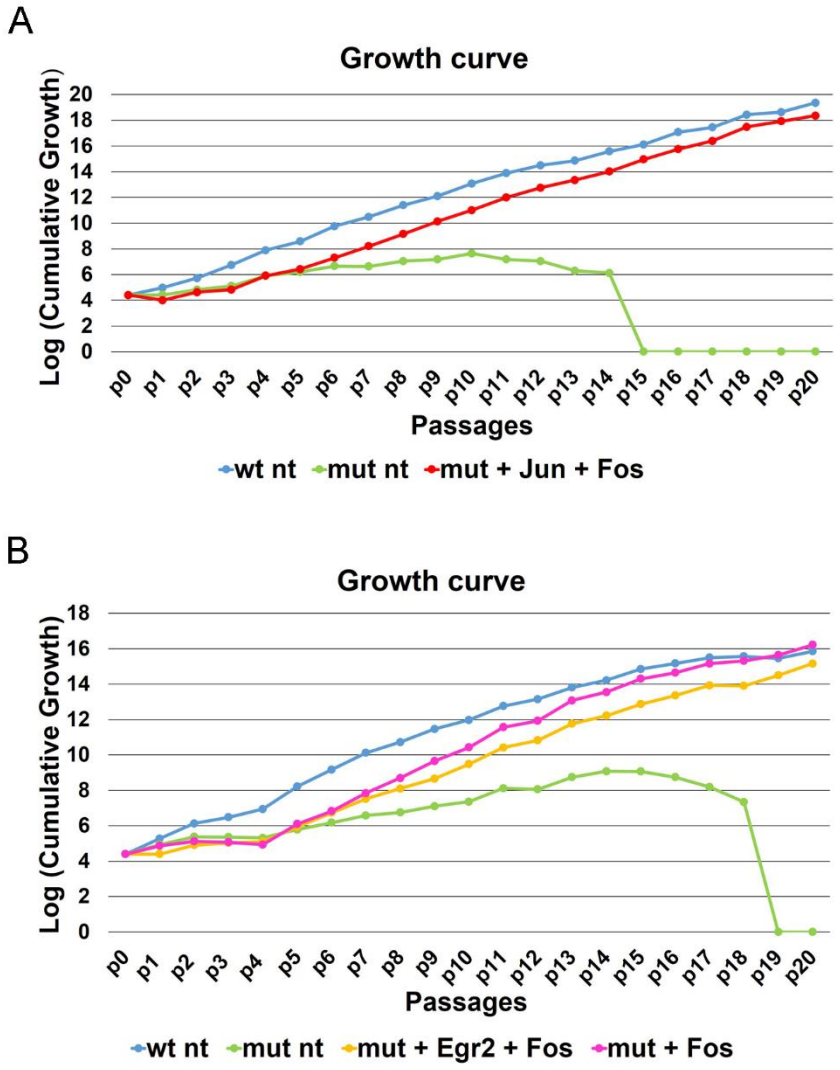


Figure 1

A, Expression levels of selected transcription factor genes among the most downregulated genes in Sox2-deleted NSC (transcripts per million; data from Bertolini et al, 2019).

B, Scheme of the Sox2 target transduction experiment and of the lentiviral vector



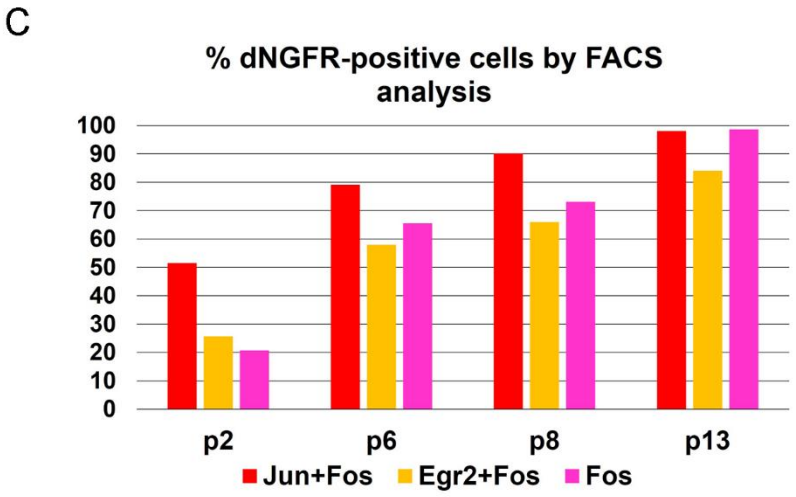


Figure 2

Sox2-mutant cells transduced with Fos+Jun, Fos+Egr2, or Fos alone recover the ability to self-renew long term

A,B, growth curves of Sox2-deleted cells (green), and of the same cells transduced with the indicated vectors

C, Percentage of dNGFR-positive cells by FACS analysis

A

Fos+Jun transduction	
Fos+, Jun-	12
Fos+, Jun+	2
Fos-, Jun+	0
Fos-, Jun-	0

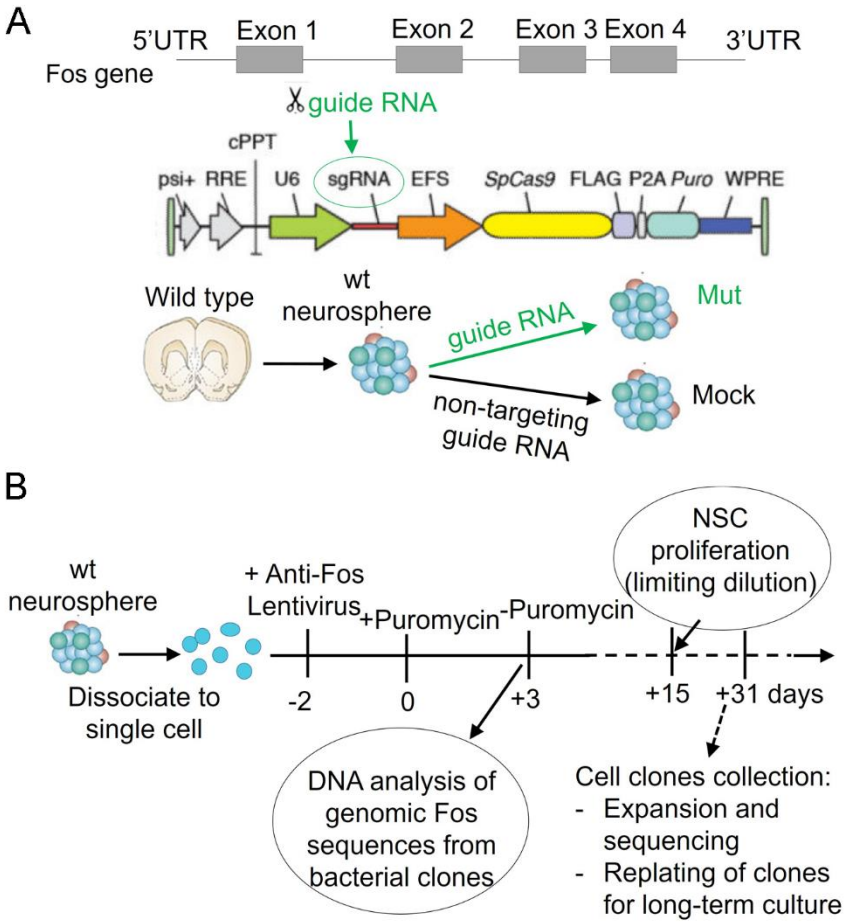
B

Fos+Egr2 transduction	
Fos+, Egr2-	6
Fos+, Egr2+	8
Fos-, Egr2+	0
Fos-, Egr2-	0

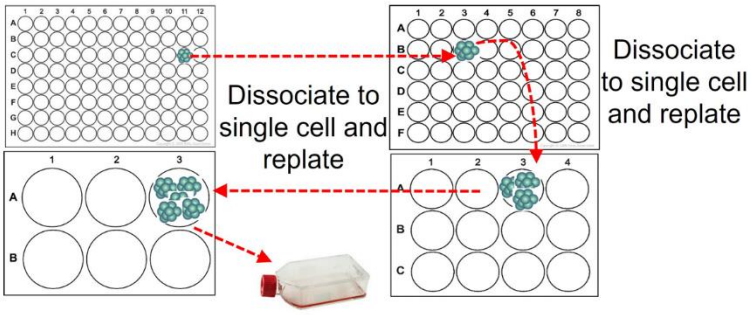
Figure 3

Clones cultured from Sox2-mutant cells rescued with Fos+Jun (A), or Fos+Egr (B), always contain the Fos-expressing lentivirus

Numbers of clones are indicated. The transduction efficiency of the cells by Jun alone, in preliminary experiments, was 18.5%; hence, we would expect that 18.5% of the 14 Fos-positive clones, i.e 2-3 clones, should also be Jun-positive, by chance. For Egr2, the expected frequency of Fos/Jun positive clones is 17.1% of the 14 Fos-positive clones, i.e. 2-3 clones.



C



D

Long-term proliferating clones				
	Experiment 1		Experiment 2	
	Mut	Mock	Mut	Mock
Total replated clones	23	11	25	20
Long-term proliferating clones	7 (30,4%)	8 (72,7%)	8 (32%)	16 (80%)

Figure 4

Fos mutagenesis impairs the clonogenic ability of NSC

A, Position of the guide RNA-recognized sequence in the Fos gene; lentiviral vector for mutagenesis expressing the guide RNA and Cas9

B, Fos mutagenesis experiment and obtainment of transduced, puromycin-resistant NSC as single clones at limiting dilution (primary clones)

C, Replating of primary single clones for progressive expansion into long-term growing NSC populations (long-term proliferating clones).

D, Fos mutagenesis in wild type NSC reduces the percentage of long-term proliferating clones. The significance of the difference between number of clones obtained with anti-Fos (Mut) and scramble (Mock) sgRNA was validated by paired one-tailed t-test ($p=0.03127$)

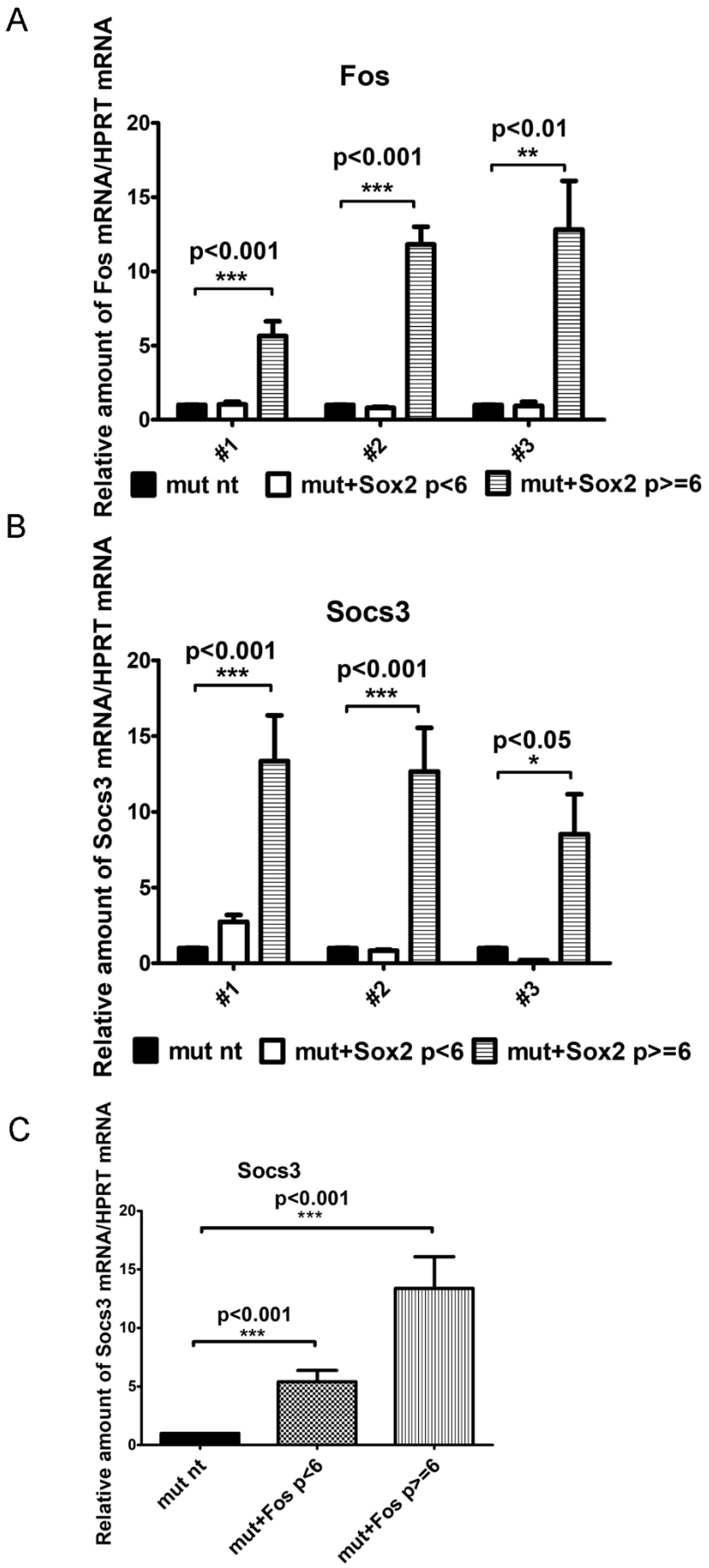

A
Fos
Relative amount of Fos mRNA/HPRT mRNA
20
15
10
5
0
p<0.001
***
p<0.001
***
p<0.01
**
#1
#2
#3
mut nt
mut+Sox2 p<6
mut+Sox2 p>=6
B
Socs3
Relative amount of Socs3 mRNA/HPRT mRNA
20
15
10
5
0
p<0.001
***
p<0.001
***
p<0.05
*
#1
#2
#3
mut nt
mut+Sox2 p<6
mut+Sox2 p>=6
C
Socs3
Relative amount of Socs3 mRNA/HPRT mRNA
20
15
10
5
0
p<0.001
***
p<0.001
***
mut nt
mut+Fos p<6
mut+Fos p>=6

Figure 5

Sox2-rescued Sox2-deleted cells recover expression of endogenous Fos and Socs3

A,B, Expression of Fos (A) and Socs3 (B) in Sox2-mutant cells (three different mutants, #1, #2, #3) transduced with Sox2-expressing lentivirus. P<6 and p>6 are the average expression values for passages 2-5 and 6-22, respectively.

C, Expression of Socs3 in Sox2-deleted cells transduced with Fos. mut: Sox2-deleted. nt: non transduced.

In A,B,C, expression values are calculated as ratios of Fos or Socs3 mRNA/HPRT mRNA, as determined by qRT-PCR. The values obtained for untransduced mutant (mut nt) are set = 1, and the values obtained for Sox2- or Fos-transduced mutants (mut + Sox2; mut + Fos) are normalized to the corresponding mut nt.

Histograms in A,B,C represent the mean of results obtained with the following numbers of qRT-PCRs at different passages: mut 1 and 2: 20 qRT-PCRs performed in duplicate for mut nt and mut+Sox2 p>6, and 13 qRT-PCRs performed in duplicate for mut nt and mut+Sox2 p<6; mut 3: 10 qRT-PCRs for nt and mut+Sox2 p>6; 3 qRT-PCRs for mut nt and mut+Sox2 p<6. ***p<0.001; *p<0.05, paired t-test. ns: not significant.

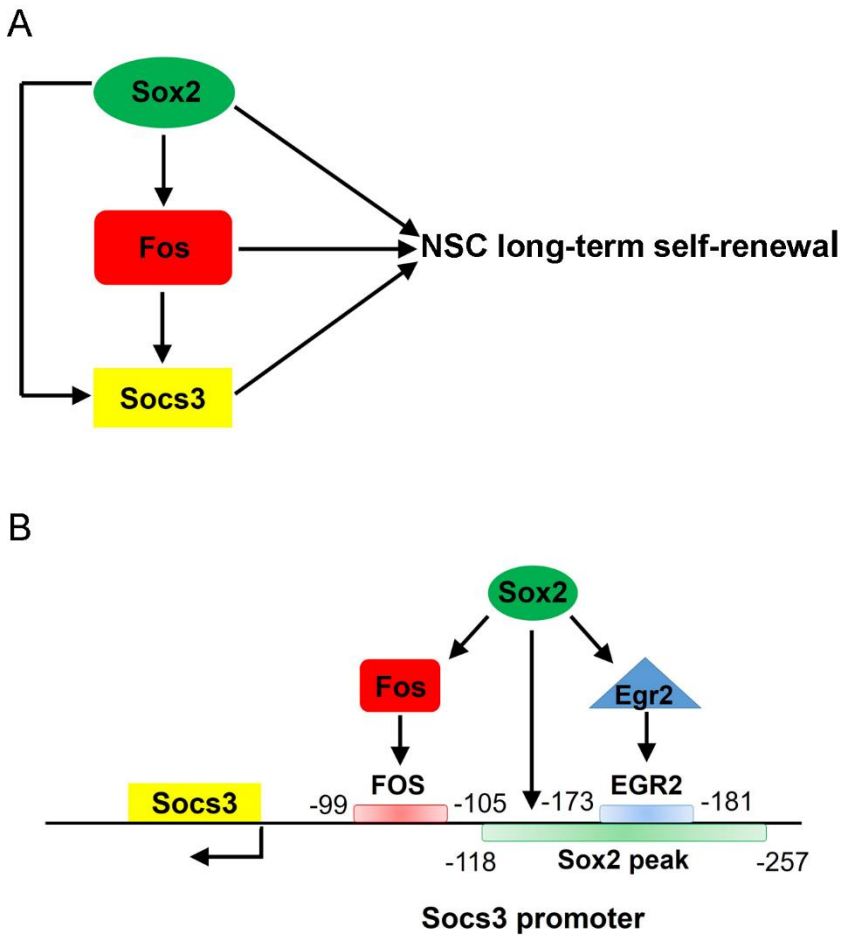


Figure 6

A model for a Sox2-controlled gene regulatory network involving Fos, Egr2 and Socs3

A, Regulatory relations between Sox2, Fos, and Socs3, in NSC long-term self-renewal control

B, Sox2, Fos and Egr2 functional interactions on the Socs3 promoter. For Fos and Egr2 binding data, see [17,18]. For Sox2 binding, see [8].

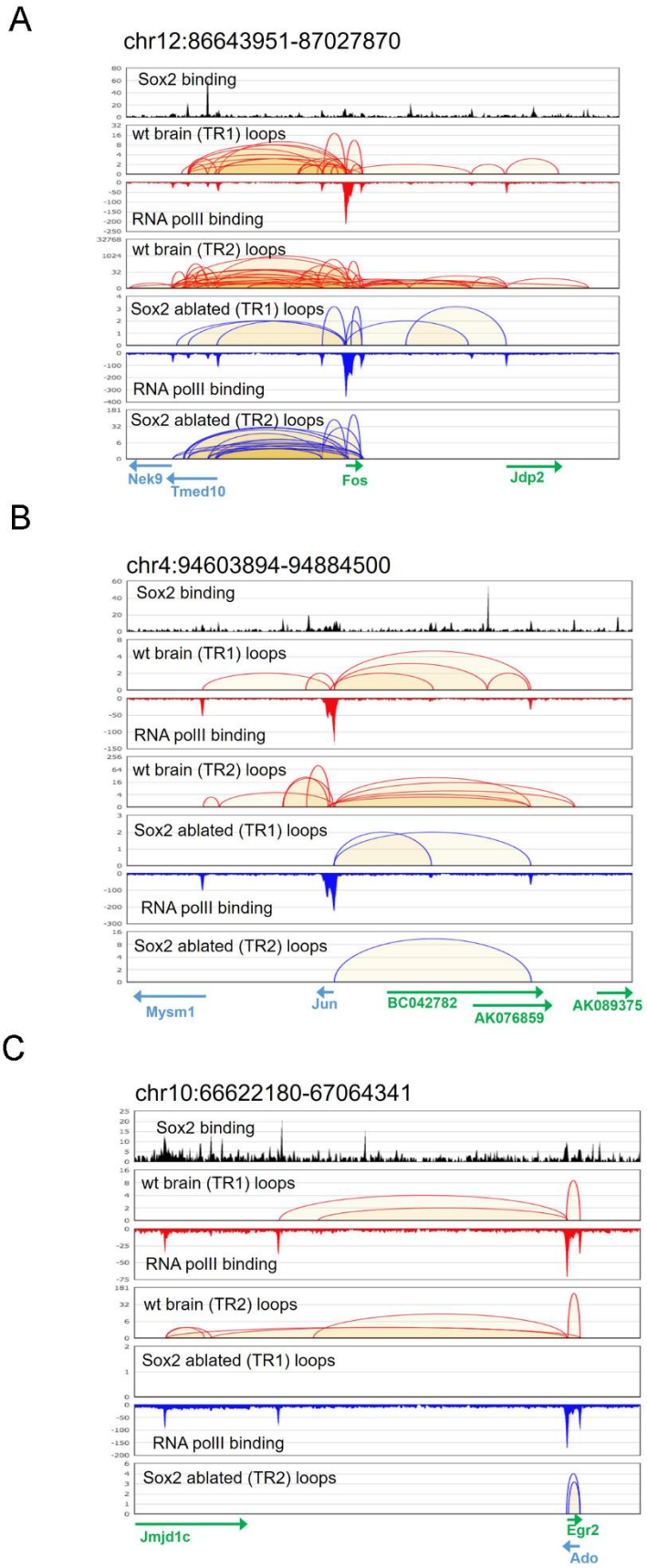

A
chr12:86643951-87027870
Sox2 binding
wt brain (TR1) loops
RNA polII binding
wt brain (TR2) loops
Sox2 ablated (TR1) loops
RNA polII binding
Sox2 ablated (TR2) loops
Nek9
Tmed10
Fos
Jdp2
B
chr4:94603894-94884500
Sox2 binding
wt brain (TR1) loops
RNA polII binding
wt brain (TR2) loops
Sox2 ablated (TR1) loops
RNA polII binding
Sox2 ablated (TR2) loops
Mysm1
Jun
BC042782
AK076859
AK089375
C
chr10:66622180-67064341
Sox2 binding
wt brain (TR1) loops
RNA polII binding
wt brain (TR2) loops
Sox2 ablated (TR1) loops
RNA polII binding
Sox2 ablated (TR2) loops
Jmjd1c
Egr2
Ado

Figure 7

Long-range chromatin interactions detected by ChIA-PET on the Fos (A), Jun (B), and Egr2 (C) regions in wild type and Sox2-deleted NSC

The Fos (A), Jun (B) and Egr2 (C) genes are indicated below each panel. Long-range interactions between the genes and distal regions are indicated by loops (wild type cells, red; Sox2-deleted cells, blue). Tracks with SOX2 and RNApolII binding peaks are shown. TR1 and TR2 refer to different ChIA-PET experiments. Note the occurrence of SOX2 binding peaks in correspondence of distant regions connected with each of the three genes. Data from (Bertolini et al, 2019).