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Sox2 is required for embryonic development of the ventral telencephalon through the activation of the ventral determinants Nkx2.1 and Shh

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SUMMARY

The Sox2 transcription factor is active in stem/progenitor cells throughout the developing vertebrate central nervous system. However, its conditional deletion at E12.5 in mouse causes few brain developmental problems, with the exception of the postnatal loss of the hippocampal radial glia stem cells and the dentate gyrus. We deleted Sox2 at E9.5 in the telencephalon, using a Bf1-Cre transgene. We observed embryonic brain defects that were particularly severe in the ventral, as opposed to the dorsal, telencephalon. Important tissue loss, including the medial ganglionic eminence (MGE), was detected at E12.5, causing the subsequent impairment of MGE-derived neurons. The defect was preceded by loss of expression of the essential ventral determinants Nkx2.1 and Shh, and accompanied by ventral spread of dorsal markers. This phenotype is reminiscent of that of mice mutant for the transcription factor Nkx2.1 or for the Shh receptor Smo. Nkx2.1 is known to mediate the initial activation of ventral telencephalic Shh expression. A partial rescue of the normal phenotype at E14.5 was obtained by administration of a Shh agonist. Experiments in Medaka fish indicate that expression of Nkx2.1 is regulated by Sox2 in this species also. We propose that Sox2 contributes to Nkx2.1 expression in early mouse development, thus participating in the region-specific activation of Shh, thereby mediating ventral telencephalic patterning induction.

KEY WORDS: Brain development, Sox2, Ventral telencephalon, Mouse, Neurogenesis, Sonic hedgehog, Nkx2.1

INTRODUCTION

The transcription factor Sox2 is necessary for the maintenance of pluripotency in epiblast and embryonic stem cells; its knockout is early embryonic lethal (Avilion et al., 2003; Masui et al., 2007). Later in development, Sox2 is required in various tissue stem cells and early progenitors, in particular in the nervous system (Que et al., 2009; Basu-Roy et al., 2010; Pevny and Nicolis, 2010). Throughout vertebrate evolution, Sox2 is expressed in the developing neuroectoderm from its earliest stages (Wegner and Stolt, 2005). In the embryonic nervous system, Sox2 marks undifferentiated neural precursor cells, including neural stem cells (NSCs). Postnatally, Sox2 is expressed in NSCs within the neurogenic niches of the subventricular zone (SVZ) and hippocampus dentate gyrus (DG) (Zappone et al., 2000; Ellis et al., 2004; Ferri et al., 2004; Suh et al., 2007). Sox2 is also expressed in some differentiating neural cells and neurons (Ferri et al., 2004; Taranova et al., 2006; Cavallaro et al., 2008).

Interestingly, heterozygous Sox2 mutations in humans cause a characteristic spectrum of CNS abnormalities, including eye, hippocampus, hypothalamus and basal ganglia defects, with neurological pathology including epilepsy and motor control problems (Fantes et al., 2003; Kelberman et al., 2008; Sisodiya et al., 2006).

Sox2 gain-of-function and dominant-negative experiments established roles for Sox2 in the maintenance of NSC/progenitor cells in chicken and frog (Kishi et al., 2000; Bylund et al., 2003; Graham et al., 2003). Moreover, neonatal and embryonic NSCs grown *in vitro* from mice with a nestin-Cre-driven conditional ablation of Sox2 in the neural tube at embryonic day of development (E) 12.5 became prematurely exhausted in long-term culture experiments (Favaro et al., 2009).

Despite the severe *in vitro* defects of NSC maintenance, *in vivo* embryonic brain abnormalities in Sox2-nestin-Cre mutants are rather limited (Miyagi et al., 2008; Favaro et al., 2009); the only prominent defect is early postnatal failure to maintain hippocampal NSCs (radial glia) and neurogenesis, followed by loss of the hippocampal dentate gyrus. These defects were preceded by embryonic-perinatal loss of sonic hedgehog (Shh) expression in the telencephalon (but not in midbrain and in spinal cord), and could be rescued by a chemical Shh agonist (Favaro et al., 2009).

The reasons for the limited effects of Sox2 deletion on brain development remain unclear. Other Sox proteins, such as Sox1 and Sox3, which play roles similar to those of Sox2 (Bylund et al., 2003; Graham et al., 2003), might compensate *in vivo* for Sox2 absence. Alternatively, the timing of embryonic Sox2 deletion in previous experiments (Favaro et al., 2009) might have been too late, thus failing to uncover essential earlier functions of Sox2.

Here, we have used an early-acting Bf1 (Foxg1)-Cre transgene, which completely ablated Sox2 by E9.5 in the developing telencephalon, two days earlier than the deletion with nestin-Cre (Miyagi et al., 2008; Favaro et al., 2009). This caused defects much more severe than those observed with nestin-Cre (Miyagi et al.,

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2008; Favaro et al., 2009). Unexpectedly, these defects were markedly region specific, with much more pronounced ventral than dorsal telencephalic alterations. The medial ganglionic eminence (MGE) was completely lost at E12.5, preceded by an earlier failure to express the ventral determinants Nkx2.1 (Nkx2-1) and Shh. Treatment with a Shh agonist (Shh-ag) *in vivo* was sufficient to rescue the ventral (MGE) phenotype to a significant, but not complete, extent. Furthermore, we show that Sox2 regulates Nkx2.1, a known direct activator of Shh (Jeong et al., 2006).

MATERIALS AND METHODS

Mouse strains

Sox2$^{flox/+}$ mice (Favaro et al., 2009) were bred to *Bf1-Cre* mice (Hébert and McConnell, 2000) to obtain compound *Sox2$^{flox/+}$ Bf1-Cre* heterozygotes, which were bred to *Sox2$^{flox/flox}$* mice to generate Sox2-deleted embryos. *Bf1-Cre* mice were maintained by brother-sister mating, and subsequently on a 129 background (Hébert and McConnell, 2000).

Histology, *in situ* hybridisation (ISH), immunohistochemistry and Shh agonist treatment

Histology, ISH and immunohistochemistry were carried out as previously described (Ferri et al., 2004; Favaro et al., 2009). Antibodies used were: anti-SOX2, anti-SOX1, anti-SOX3, anti-SOX9 mouse monoclonals (R&D Systems); anti-Nkx2.1 rabbit polyclonal (BIOPAT); anti-SHH rabbit polyclonal (Santa Cruz); and anti-SHH mouse monoclonal [Developmental Studies Hybridoma Bank (DSHB)]. BrdU (Sigma B5002, 15 mg/ml in PBS) was administered to pregnant females at 6 μl/g body weight; females were sacrificed after 30 minutes. BrdU immunofluorescence and TUNEL analysis were carried out as described by Favaro et al. (Favaro et al., 2009) and Ferri et al. (Ferri et al., 2004), respectively.

Shh agonist #1.2 (Frank-Kamenetsky et al., 2002) was administered to pregnant females at E8.5 and E10.5, by oral gavage of a 1.5 mg/ml solution in 0.5% methylcellulose/0.2% Tween 80 at 100 μl/g body weight.

Mosaic deletion of *Sox2* by Sox2CreERT2 was by tamoxifen administration at E8.5 by oral gavage of a 20 mg/ml solution in 1:10 ethanol/corn oil, 0.1 mg/g body weight (Favaro et al., 2009).

Nkx2.1 regulation studies

Transgenic constructs

The genomic sequence spanning nucleotides −495 to +1842 relative to the mouse upstream *Nkx2.1* transcription start site was PCR amplified (primers: forward: 5′-GAGTAGAGAGCACTCTTCAAGGAG-3′; reverse: 5′-GGCGTCGGCTGGAGGAGGAAGGAAG-3′) and cloned into the vector IsceI-EGFP (Conte and Bovolenta, 2007) generating mNkx2.1 wt long:EGFP. The Sox2 consensus sites were mutated using the Multisite Quickchange Lightening Kit (Strataclone).

Luciferase constructs

Appropriate fragments were amplified by PCR (with primers: forward: 5′-AT<u>CTCGAG</u>CCGACCAAATTGGACCGCGG-3′, added *Xho*I site underlined; reverse: 5′-GCG<u>AGATCT</u>TGCCAAATATTCTGGTGTT-ACCTTAACG-3′, added *Bgl*II site underlined) and cloned upstream to the luciferase gene into the TK-LUC vector (provided by A. Okuda, Saitama Medical School, Saitama, Japan) previously deleted of the TK minimal promoter.

Chromatin immunoprecipitation (ChIP)

ChIP was performed using stage 16-18 Medaka fish (*Oryzia latipes*) embryos. Chromatin was immunoprecipitated with 2 μg of anti-Sox2 (R&D Systems) or a non-related IgG (Sigma). DNA was analysed by Q-PCR (Roche). Fold-enrichment was expressed as the ratio of Sox2 to IgG signal. Q-PCR of the 18S rRNA region and the 3′ UTR of the *Nkx2.1* gene, lacking *Sox2*-binding consensuses (negative controls), and of the *Nkx2.1* promoter/enhancer, were performed using the following specific primers: 18S Forward: 5′-GGTAACCCGCTGAACCCCAC-3′; 18S Reverse: 5′-CCATCCAATCGGTAGTAGCG-3′; Nkx2.1-3′UTR Forward: 5′GCCC-TACAGGTTCAGTCCAG-3′; Nkx2.1-3′UTR Reverse: 5′ACTGGG-ACTGGGGTTCTTTT-3′; Nkx2.1enhancer Forward: 5′-CAATTAAG-GCGGACTTGAGG-3′; Nkx2.1enhancer Reverse: 5′-AGAAGGCA-AGGCAATCTCTC-3′.

Transfection experiments

P19 cells (2×10^5/well) were plated in 6-well plates and transfected after 24 hours in 1 ml of Opti-MEM (Invitrogen) with Lipofectamine 2000 (Invitrogen) with 1 μg luciferase plasmid (Nkx2.1-luciferase, or ‘empty’-luciferase), and increasing amounts of *Sox2* expression vector (Favaro et al., 2009). In control experiments, equimolar amounts of *Sox2* ‘empty’ vector were used. pBluescript was added to each transfection to equalise total DNA to 2 μg. Luciferase activity was measured after 24 hours. For transgenesis experiments in Medaka, plasmids purified using the Genopure Plasmid Midi Kit (Roche) were injected at the one-cell stage into Medaka oocytes CAB strain, at 15 ng/μl (Conte and Bovolenta, 2007). Embryos were analysed for EGFP expression (by fluorescence and confocal microscopy) in the hypothalamus at stage 19. To determine whether Sox2 regulates reporter expression, Nkx2.1 *wt-long*-EGFP was co-injected with *Sox2* mRNA or a Sox2-specific, already validated morpholino (MO) (Beccari et al., 2012). ISH was as described (Conte and Bovolenta, 2007) using probes against Medaka *Nkx2.1*, *Arx* and *Dmbx* (*Arx* and *Dmbx* representing diencephalic and mesencephalic markers, respectively). Subsequently, three independent stable transgenic lines were selected.

In utero electroporation

E13.5 C57/Bl6 pregnant mice were anesthetised and DNA introduced by electroporation *in utero* as described (Sanchez-Camacho and Bovolenta, 2008) using a solution containing a 1:1 mixture of Nkx2.1 *wt-long*::EGFP and pCAG-Cherry (2μg/μl). Embryos were collected and analysed after 48 hours (E15.5) by sectioning the brains in 50-μm-thick frontal sections. GFP expression was enhanced by immunostaining with rabbit anti-GFP (1:1000, Molecular Probes).

RESULTS

Sox2 early deletion severely impairs embryonic brain development

To ablate Sox2 in the early embryonic brain, we bred mice carrying a *Sox2^{flox}* conditional mutation (Favaro et al., 2009) to mice expressing the Cre-recombinase gene under the control of the *Bf1* regulatory regions, specifically active in the developing telencephalon from embryonic day (E) 9.5 of development (Bf1cre ‘knock-in’) (Hébert and McConnell, 2000). In *Sox2$^{flox/flox}$;Bf1cre* embryos, Sox2 protein was completely ablated by E9.5 in the telencephalon, though not in more posterior neural tube regions, as expected (Fig. 1A). This caused early morphological defects: at E12.5, telencephalic vesicles were reduced and the eyes were abnormal (Fig. 1B,C). Interestingly, although the whole telencephalon was affected, the ventral part was much more severely compromised than the dorsal one (Fig. 1C,F); histological sections (Fig. 1F) showed that the ventral primordia of the medial ganglionic eminence (MGE), involved in the generation of the basal ganglia (Sur and Rubenstein, 2005; Hébert and Fishell, 2008), were severely reduced (Fig. 1F, arrowhead). These initial defects developed into profoundly abnormal development, leading to death just after birth. At E18.5, mutant pups had a smaller head (Fig. 1E) and the telencephalon was smaller than in wild type (Fig. 1D,G: compare with the almost unaffected midbrain); also, the olfactory bulbs and the midline ventral structures were absent (Fig. 1D, black arrowhead pointing to ventral ‘hole’). In tissue sections, the ventral midline and the immediately adjacent territories were missing (Fig. 1G, arrowheads).

In agreement with the early MGE abnormalities, GABAergic cortical interneurons, which originate in the MGE and then migrate to more dorsal locations (Sur and Rubenstein, 2005; Hébert and Fishell, 2008; Elias et al., 2008), were strongly decreased in mutants, as indicated by the almost complete loss of somatostatin (SS)-positive and the strong reduction of the neuropeptide Y (NPY)-

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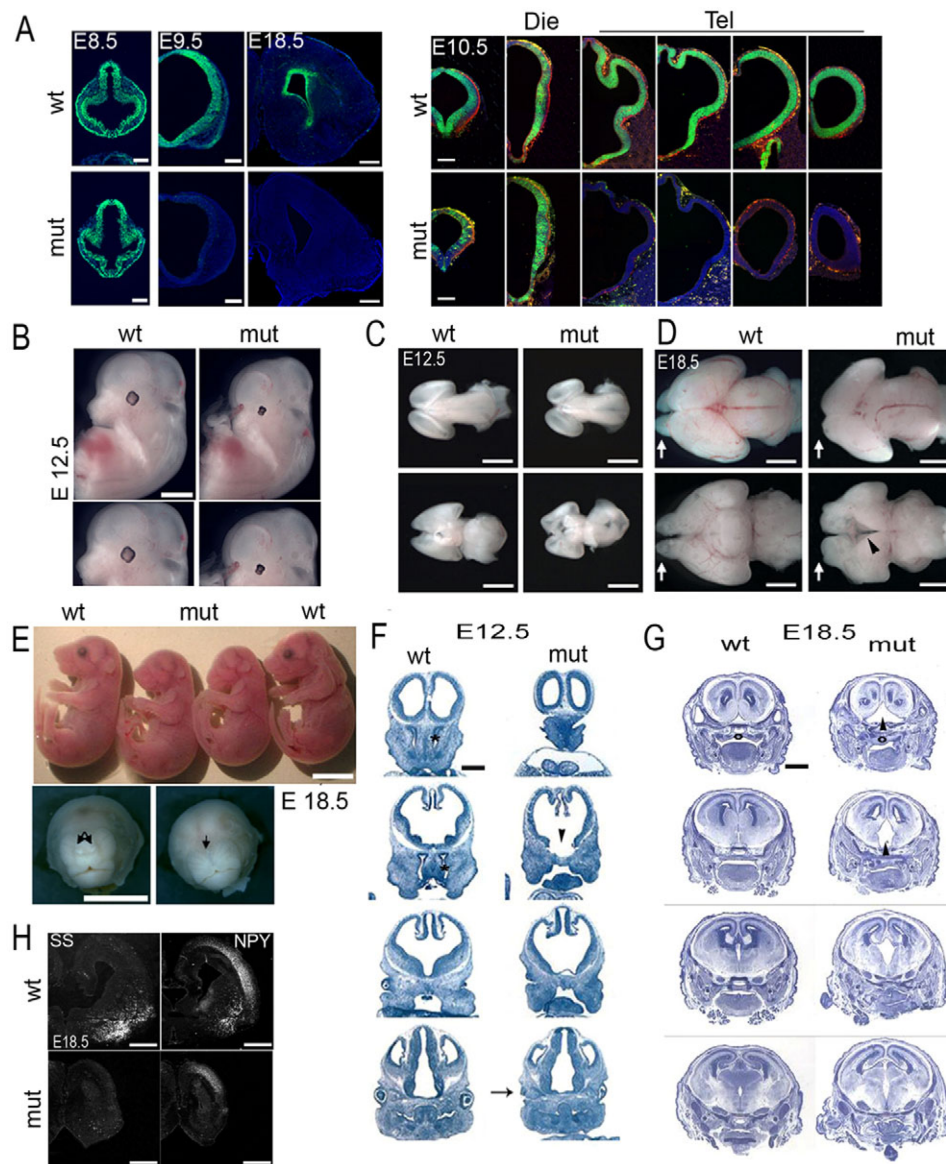

Fig. 1. Early telencephalic ablation of Sox2 with Bf1Cre causes impairment of embryonic brain development. (**A**) Sox2 immunofluorescence (green) on telencephalic sections of normal (*Sox2flox/flox*) and mutant (*Sox2flox/flox;Bf1cre*) mouse embryos. Left: E8.5, E9.5 and E18.5 sections. Sox2 ablation is complete by E9.5. Right: E10.5 sections (posterior left to anterior right). Sox2 ablation is seen in the telencephalon (Tel) but not in the diencephalon (Die). (**B-D**) Brain abnormalities. (B) E12.5 whole embryos. Note the reduced telencephalon, the comparatively unaffected midbrain and the undeveloped eye. (C) Dissected E12.5 brains, viewed dorsally (top) and ventrally (bottom). Note the smaller telencephalic vesicles and the initial ventral tissue loss. (D) Dissected E18.5 brains viewed dorsally (top) show, in mutant, smaller telencephalon (compare to unaffected midbrain) and absence of olfactory bulbs (arrows). Ventral view (bottom) reveals extensive tissue loss (arrowhead) in mutant. (**E**) Mutant E18.5 embryos show smaller head and eyes compared with wild type (wt; top), and facial abnormalities including fusion of the anterior nasal plate (bottom; double arrow in wt, single arrow in mutant) and slightly increased eye proximity. (**F**) E12.5 coronal sections, thyonine stained, anterior (top) to posterior. Arrowhead indicates ventral tissue loss (MGE) in mutant; arrow indicates defective mutant eye. Note olfactory epithelium (asterisk in wt) is missing in the mutant. Note the comparatively unaffected diencephalon in the last section. (**G**) E18.5 coronal sections (thyonine stain) reveal major loss of ventral territories, including striatum region (arrowheads). Circle indicates defective maxillary region (palate). (**H**) ISH for somatostatin (SS) and neuropeptide Y (NPY) shows strong downregulation in the mutant, particularly for SS. Scale bars: 150 µm.

positive subsets of neurons (Markram et al., 2004; Toledo-Rodriguez et al., 2005; Elias et al., 2008; Hébert and Fishell, 2008) (Fig. 1H). SS-positive interneurons originate from the (dorsal) MGE progenitors and require the Nkx2.1 transcription factor for their development (see below) (Hébert and Fishell, 2008; Butt et al., 2008; Flandin et al., 2011). NPY-positive neurons originate from the progenitor domain of the adjacent preoptic area (Gelman et al., 2009), which may be somewhat less severely affected.

Additional abnormalities included absence of the olfactory epithelium [Fig. 1F, asterisk in wild type (wt)] and face abnormalities: the nasal plate, normally developing a characteristic bilateral symmetry, was consistently centrally fused (Fig. 1E, arrows) and underdeveloped. Furthermore, the eyes were abnormal and extremely reduced in size (Fig. 1B,E,F) (see also Taranova et al., 2006); maxillary structures, e.g. the palate, were also abnormal (Fig. 1G); the cortex (Fig. 1B,D,G) was reduced; and the

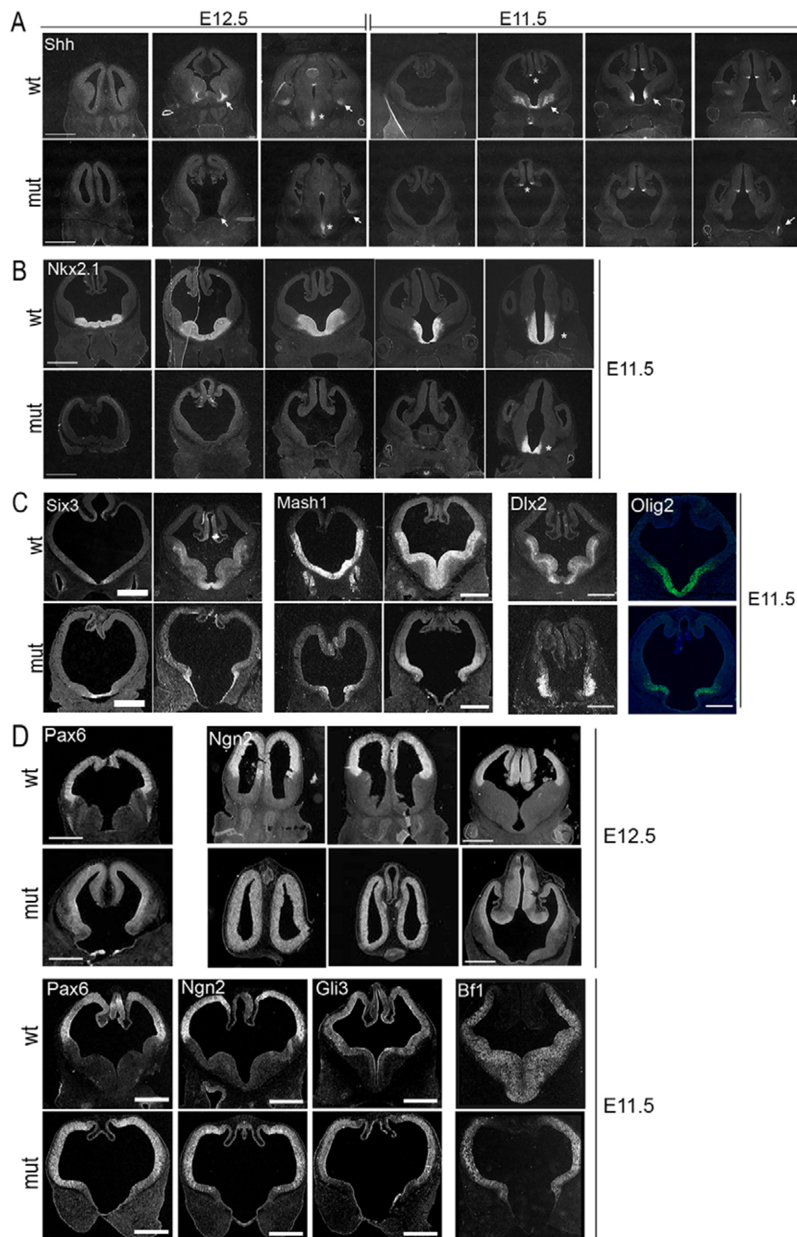

Fig. 2. Expression of ventral determinants is impaired in *Sox2* mutants. (**A**) ISH with *Shh* probe on E12.5 (left) and E11.5 (right) normal (top) and mutant (bottom) mouse embryos (left anterior to right posterior). Arrows indicate the *Shh* signal in wild type, and its absence (midline region) or important reduction (amygdala region) in mutants. Asterisks indicate the signal in diencephalon, a non-*Sox2*-deleted region, as an internal control, showing similar intensity. Arrows in the bottom far-right panel indicate the impaired mutant eyes. (**B**) ISH with *Nkx2.1* probe on E11.5 embryos (left anterior to right posterior). The signal is detected in all telencephalic sections in wild type, but not in mutant. Asterisks indicate the signal in non-*Sox2*-deleted diencephalon, as internal control. (**C**) ISH with probes for ventrally expressed genes at E11.5. Probes are indicated on each panel. Ventral gene expression shows loss or strong downregulation in mutants. Note *Mash1* and *Six3* hybridisation to the olfactory epithelium of wt, but not mutants. (**D**) Expression of some dorsally, or dorsally/ventrally, expressed genes in E12.5 and E11.5 mutants, compared with wild type. Expression of *Pax6* and *Ngn2* is maintained but clearly shifted ventrally in E12.5 mutants. Expression of *Bf1* at E11.5 is retained in the mutant (though lost ventrally where tissue loss is observed). Scale bars: 150 μm.

hippocampus (at E18.5) was severely underdeveloped (not shown). None of the defects described above was seen in control mice (*Sox2* $^{flox/+}$ *Bf1-Cre*; *Sox2*$^{flox/+}$; *Sox2*$^{flox/flox}$) (not shown).

Early expression of ventral forebrain determinants is impaired in Sox2 mutants

We focused on the most severely affected region, the ventral telencephalon, to study genes known to be involved in its specification and development. We first analysed embryos by ISH at E12.5, when the morphological defect becomes overt, and at E11.5, when the defective morphology can first be appreciated. The *Shh* gene is expressed in the developing ventral telencephalon, and is crucial at early stages for the development of this region (Fuccillo et al., 2004; Sousa and Fishell, 2010). Furthermore, we had previously found that *Shh* is a Sox2 target, acting as its functional effector in postnatal hippocampal development (Favaro et al., 2009). By E12.5, *Shh* mRNA is completely absent in the midline region following the loss of the tissue expressing it, and is strongly downregulated in the amygdala region (Fig. 2A); in E11.5 mutant embryos, *Shh* is already severely downregulated in the medial ventral telencephalon (Fig. 2A). Indeed, deletion of the *Shh* gene, or that of its receptor Smo, from the early ventral telencephalon using the same *Bf1-Cre* transgene (Fuccillo et al., 2004) produces abnormalities very similar to those of our mutants. Importantly, these abnormalities are less severe than those seen in the complete *Shh* knockout, in which *Shh* expression in the prechordal plate mesoderm is also lost (Chiang et al., 1996).

The transcription factor Nkx2.1, a direct regulator of Shh (Sussel et al., 1999; Jeong et al., 2006), is specifically expressed in the MGE within the developing brain, and is absolutely required for its development (Sussel et al., 1999; Butt et al., 2008; Nòbrega-Pereira et al., 2008). In *Sox2* mutants, *Nkx2.1* expression was already undetectable at E11.5 in the telencephalon (Fig. 2B), but still observed in the non-*Sox2*-deleted diencephalon (Fig. 2B).

Six3, another transcription factor essential for ventral telencephalic development (Lagutin et al., 2003; Geng et al., 2008),

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is also a direct activator of Shh (Jeong et al., 2008); expression of *Six3* was only slightly reduced at E11.5, in coincidence with the initial tissue loss (Fig. 2C). Expression of the gene encoding Mash1 (Ascl1 – Mouse Genome Informatics), a transcription factor expressed in the MGE and lateral ganglionic eminence (LGE) and important for GABAergic interneuron development (Guillemot 2007), was essentially lost in regions close to the midline, and reduced more laterally (Fig. 2C). The genes encoding Dlx2 and Olig2, two transcription factors expressed in the MGE and LGE, downstream of Shh activity (Fuccillo et al., 2004), and required for ventral telencephalic development (Sur and Rubenstein, 2005; Hébert and Fishell, 2008), were similarly reduced (Fig. 2C). The Ebf1 transcription factor is expressed within the developing LGE, but not the MGE (Fuccillo et al., 2006; Geng et al., 2008); expression of *Ebf1* was maintained, to some extent, in mutants (supplementary material Fig. S2). These data are consistent with a severe loss of MGE, but some degree of maintenance of LGE primordia.

In contrast to the strong reduction of the 'ventral' effectors described above, expression of transcription factor genes marking the dorsal brain and required for its development, such as *Pax6*, *Ngn2* (*Neurog2* – Mouse Genome Informatics) and *Gli3*, was maintained at E11.5-12.5 in mutants, with a clear tendency for dorsal-specific expression to spread ventrally (Fig. 2D), particularly at E12.5.

Expression of the gene encoding Bf1, a transcription factor expressed both dorsally and ventrally, but required mainly in ventral regions (Gutin et al., 2006; Hébert and Fishell, 2008), was maintained in lateral and dorsal regions, though it was severely reduced in the area affected by initial tissue loss (Fig. 2D, lower-right panel).

Early downregulation of Nkx2.1 precedes ventral tissue loss

As morphological abnormalities are already evident at E11.5, we investigated whether any gene expression defects precede their development. At E10.5 and E9.5, *Nkx2.1* expression was clearly detectable in the ventral telencephalon of the wild type, but was strongly downregulated or absent in the mutant (Fig. 3A). Consistent with a relationship between *Sox2* and *Nkx2.1* expression, the latter was clearly present in diencephalon (Fig. 3A), where *Sox2* was normally expressed (Fig. 1A). Similarly, *Shh* expression, which largely overlaps with that of *Nkx2.1*, was absent or weak in a few of the mutant embryos at E10.5 (not shown). *Six3* expression was only slightly decreased in mutants at E10.5 and E9.5 (Fig. 3B,C). By contrast, the gene encoding Bf1, which acts in parallel with Shh (Hébert and Fishell, 2008), was normally expressed in *Sox2* mutants, compared with controls (Fig. 3C). *Sox1* and *Sox3*, members of the same Sox transcription subfamily as *Sox2*, are widely co-expressed with *Sox2* in the telencephalon; they do not show major variations in mutant embryos at these stages (Fig. 3C). Sox9, which stimulates NSC growth after E10.5-11.5 (Scott et al., 2010), was normally expressed at these early stages (supplementary material Fig. S1).

We conclude that *Sox2* deletion affects the expression of early, important determinants of brain development, in a region-specific manner: several ventral fate genes are severely affected, whereas activity of dorsal genes is maintained. Notably, one essential effector of ventral telencephalon and MGE development, and activator of Shh, Nkx2.1, is downregulated at early stages.

Increased apoptotic cell death in early *Sox2*-mutant ventral telencephalon

We investigated whether ventral tissue loss in *Sox2* mutants was due to impaired cell proliferation and/or increased cell death. Cell

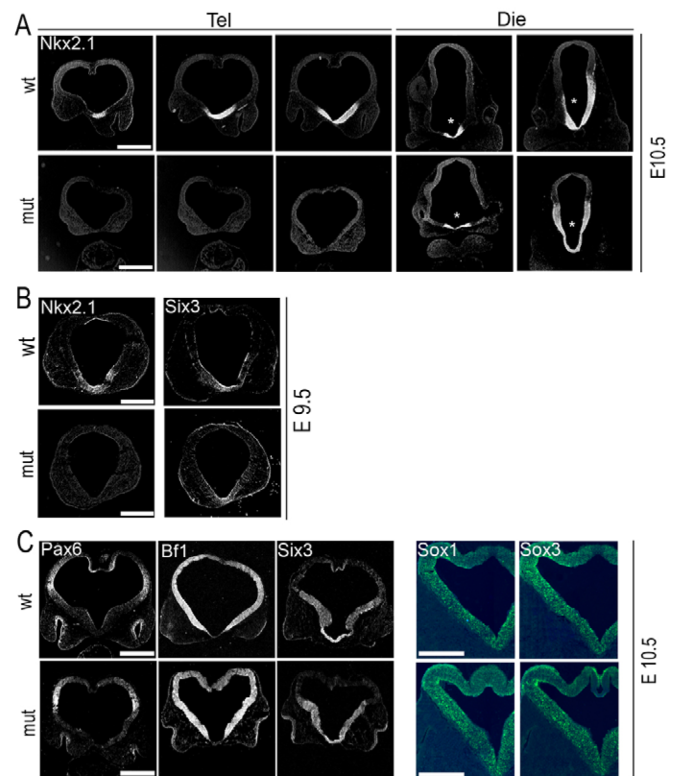

Fig. 3. Gene expression abnormalities are detected by *in situ* hybridisation at early stages of development, preceding morphologic impairment in mutants. (**A**,**B**) *Nkx2.1* expression is not established in the telencephalon (Tel) of mouse mutants at E10.5 (A) or E9.5 (B), but is preserved in the adjacent non-*Sox2*-deleted diencephalon (Die). *Six3* expression is only slightly reduced at E9.5. Asterisks indicate the Nkx2.1 signal in non-Sox2-deleted diencephalon. (**C**) *Pax6*, *Bf1*, *Six3* and (by immunofluorescence) Sox1 and Sox3 do not show major changes in mutants at E10.5. Scale bars: 200 μm.

proliferation, assessed by BrdU labelling at E9.5 and E10.5 just prior to the appearance of morphological defects, was not decreased overall in mutant telencephalon or specifically in the ventral region (Fig. 4A). Apoptotic cell death, assayed by TUNEL, was comparable between normal and mutant embryos at E9.5, but a threefold increase in TUNEL-positive cells was observed in the ventral telencephalon of E10.5 mutants (Fig. 4B).

Thus, increased cell death could directly cause ventral tissue loss in the mutants. Apoptotic death is a possible consequence of impaired ventral gene expression (e.g. loss of Shh, which has anti-apoptotic activities) (Cayuso et al., 2006), which precedes by at least one day the increase in cell death.

Defective expression of ventral genes and morphological abnormalities of *Sox2* mutants are rescued by a Shh agonist

The ventral defects observed in Bf1-cre-deleted *Sox2* mutants are very similar to those observed in mutants of the sonic hedgehog pathway [in which the Shh receptor smoothened (Smo) is conditionally ablated with the same deleter, Bf1cre] (Fuccillo et al., 2004), as well as to that of *Nkx2.1* mutants (Sussel et al., 1999). Indeed, *Sox2* mutants show (Figs 2, 3) severely impaired expression of both Shh and Nkx2.1, a direct activator of Shh (Jeong et al., 2006).

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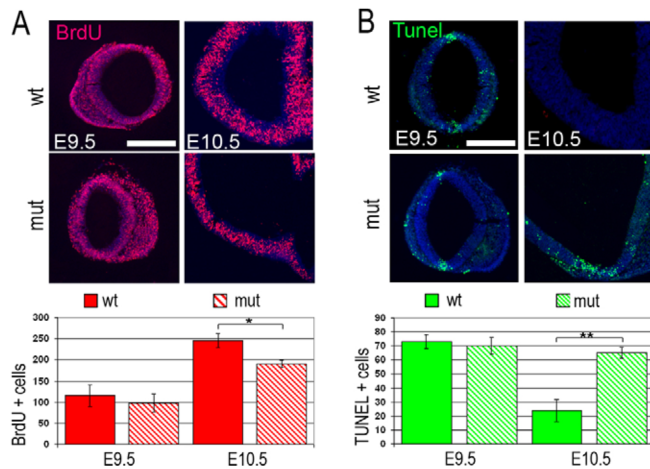

Fig. 4. Cell death is ventrally increased in *Sox2* mutant telencephalon. (**A**) Immunofluorescence for BrdU in normal (wt) and mutant (mut) mouse telencephalon; histogram shows quantification of BrdU-positive cells in the ventral half of the telencephalon. (**B**) TUNEL assay of normal and mutant telencephali. Sections (top) show increased TUNEL signal in mutant, concentrated ventrally. Histogram shows quantification; significantly higher numbers of TUNEL-positive cells are found in mutants compared with wild type at E10.5 (n=5 wild-type and mutant embryos analysed, for both assays). Values on the y-axis represent the mean±s.d. of the total number of cells counted, on every fifth 20-μm section throughout the telencephalon (four or five total sections counted for E9.5 or 10.5 brains, respectively). *P<0.01; **P<0.001 (Student's t-test). Scale bars: 150 μm.

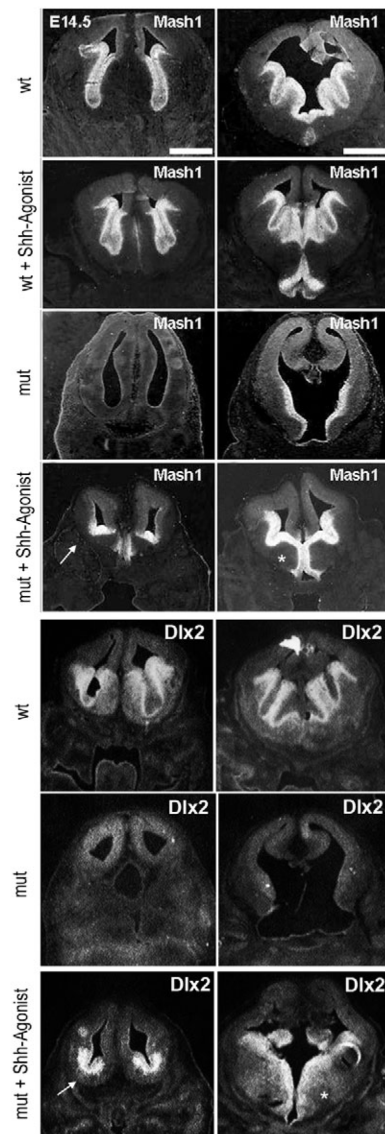

Fig. 5. A pharmacological Shh agonist significantly rescues ventral gene expression and morphology in *Sox2* mutants. ISH for ventral markers *Mash1* (top) and *Dlx2* (bottom) on normal (wt) and *Sox2*-deleted mouse embryos (mut), treated with Shh agonist or untreated. Telencephalic sections at two levels, anterior (left) and posterior (right), are shown. Expression of *Dlx2* and *Mash1*, strongly impaired (particularly anteriorly) in mutants by E14.5, is significantly rescued in treated mutants, together with an improved ventral morphology. No major effect is seen in the same region on normal embryos from the same litter. A representative experiment is shown out of n=4 mutant embryos analysed. Scale bars: 200 μm.

Hence, we tested whether Shh signalling was involved in the *Sox2* mutant phenotype, by administering mice an agonist (Shh-ag) that activates the Shh co-receptor smoothened (Frank-Kamenetsky et al., 2002). Shh-ag was administered at E8.5 (just prior to *Sox2* ablation) and E10.5. Already at E14.5, expression of ventral determinants *Mash1* and *Dlx2*, which is impaired in the untreated mutants, recovered to a significant, albeit not complete, extent (Fig. 5); morphologically, the ventral brain also recovered a somewhat more normal shape, with ventral bulges reminiscent of wild-type ganglionic eminences (Fig. 5). By contrast, no major effect was observed on brain morphology or gene expression of treated wild-type littermates (Fig. 5).

We conclude that failure to activate Shh signalling is an important cause of the defects observed in *Sox2*-mutant embryonic telencephalon.

Sox2 activates Nkx2.1 cell-autonomously

Nkx2.1 is a direct activator of the *Shh* gene and is required for its expression *in vivo* (Sussel et al., 1999; Jeong et al., 2006; Sousa and Fishell, 2010); however, *Nkx2.1* expression is also stimulated in response to Shh signalling (Fuccillo et al., 2004; Xu et al., 2005; Gulacsi and Anderson, 2006; Sousa and Fishell, 2010). *Nkx2.1* expression failed to be established early in *Sox2* mutants, and remained absent at later stages (Figs 2, 3); we thus investigated whether loss of *Nkx2.1* expression depends on Sox2 cell-autonomously or is secondary to the loss of Shh expression. To this end, we used a *Sox2CreERT2* transgene, encoding the tamoxifen-activatable Cre guided by the *Sox2* telencephalic enhancer/promoter (Favaro et al., 2009). Tamoxifen treatment at E8.5 and E10.5 caused a 'salt-and-pepper' deletion of Sox2, as seen by immunofluorescence at E14.5 (Fig. 6); some Shh expression, presumably arising from non-deleted cells, was maintained (Fig. 6), and no major abnormality was noticed in these mosaic-deleted embryos. We analysed Nkx2.1 and Sox2 expression by immunofluorescence in the ventral telencephalic ventricular zone. In control embryos, most cells co-expressed Sox2 and Nkx2.1 (Fig. 6). In tamoxifen-treated embryos, Sox2-expressing cells were strongly reduced; Nkx2.1 expression was retained in cells in which Sox2 was still expressed, but was rarely, if ever, seen in cells that did not express Sox2 (Fig. 6). We conclude that expression of Nkx2.1 requires Sox2 cell-autonomously.

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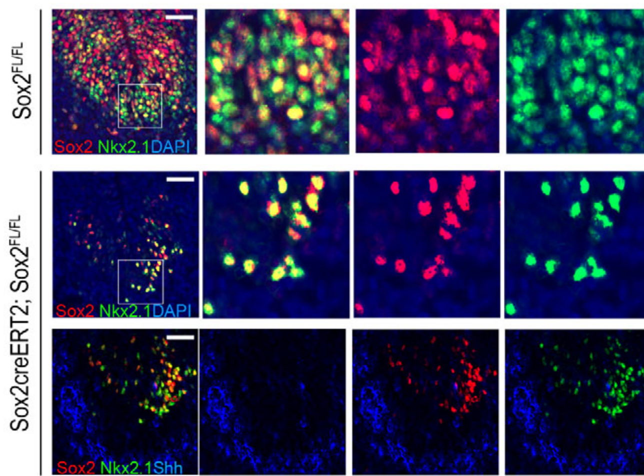

Fig. 6. Mosaic Sox2 ablation via a *Sox2CreERT2* transgene leads to cell-autonomous loss of Nkx2.1. Immunofluorescence for Nkx2.1 (green) and Sox2 (red) in normal mouse embryos (*Sox2flox/flox*; top row) at E14.5 in the ventral telencephalon. Left-hand panel: general view at low magnification; right-hand panels (top and intermediate rows) show a magnification of the boxed area, with merged and separated colour channels. Sox2 and Nkx2.1 are co-expressed in most cells in the wild type. In *Sox2CreERT2; Sox2flox/flox* embryos treated with tamoxifen at E8.5 and E10.5, only a fraction of ventral telencephalic cells retains Sox2 expression (second row, compare stained cells with total DAPI-labelled nuclei); Nkx2.1 is detected in those cells that show Sox2 expression. Shh is detectable by immunofluorescence (blue in lower row) in this region. One representative experiment is shown out of *n*=3 embryos analysed. Scale bars: 500 µm.

Regulation of *Nkx2.1* by Sox2

The early loss of *Nkx2.1* following Sox2 telencephalic ablation (Figs 2, 3) raised the possibility that *Nkx2.1* expression is directly controlled by Sox2, within a specific subregion of the Sox2 pan-neural expression domain.

In a survey for evolutionarily conserved regions in the *Nkx2.1* genomic locus, we detected a small conservation peak just upstream to the second *Nkx2.1* exon (Fig. 7A,B). Evolutionary conservation within this region was present across vertebrate evolution (Fig. 7A). This region included a single and a twin potential Sox2-binding sites; both sites are conserved in mammals, and at least one site is conserved in vertebrates, including teleostean fishes (Fig. 7A). The *Nkx2.1* gene has two promoters, one upstream to the first exon ('distal' promoter), the other in the intron between exon 1 and 2 ('proximal' promoter), both of which are functional *in vitro* and *in vivo* (including E10.5 and E14.5 telencephalon; supplementary material Fig. S3), though the latter might be the stronger (Pan et al., 2004; Hamdan et al., 1998). The Sox2-binding sites (Fig. 7B) lie in the region between the 'distal' and 'proximal' transcriptional start sites. ChIP from E14.5 embryos gave a moderate (2.5-fold) enrichment for this region (not shown). To develop a functional reporter assay for promoter sequences, we cloned a fragment including the conserved *Sox2* sequences from the *Nkx2.1* region upstream to a green fluorescent protein (*GFP*) gene (Fig. 7B), and tested it in Medaka embryos. These sequences drove GFP activity in forebrain regions superimposable with those showing endogenous *Nkx2.1* expression (Fig. 8A,B). In line with these observations, ChIP from stage 16-18 Medaka embryos with anti-Sox2 antibody revealed a 30-fold enrichment (relative to ChIP with a non-related IgG) of the *Nkx2.1* intronic conserved element, which was not observed in negative control regions (a different region of the *Nkx2.1* gene devoid of Sox2 consensus sites and the 18S RNA-encoding gene) (Fig. 8C), indicating that Sox2 binds to the *Nkx2.1* promoter/enhancer *in vivo*.

Co-injection of *Sox2* mRNA enhanced expression of the *Nkx2.1-GFP* transgene (Fig. 8F,J, compare with 8D,H), as well as of the endogenous *Nkx2.1* gene, which is both increased and expanded anteriorly, as detected by ISH (Fig. 8N, compare with 8L). Conversely, co-injection of anti-*Sox2* morpholino (Sox2 MO) (Beccari et al., 2012) (Fig. 8H,I) antagonised the activity of the co-injected *Nkx2.1-GFP* transgene (Fig. 8E,I, compare with 8D,H), as well as endogenous *Nkx2.1* expression, the domain of which was also reduced (Fig. 8M, compare with 8L).

To evaluate the importance of a direct action of Sox2 on transgene regulation, we mutated the conserved Sox2 sites within the *Nkx2.1-GFP* transgene. GFP expression required the integrity of the Sox2-consensus sites, as their mutation caused a substantial loss of transgene activity (Fig. 8G,K, compare with 8D,H). This result is consistent with experiments showing that mutation of the same Sox2 sites in a luciferase-reporter gene driven by the 'proximal' promoter abolishes the response to co-transfected Sox2 in P19 teratocarcinoma cells (Fig. 7C).

These experiments show that Sox2 is an important regulator of *Nkx2.1* expression in Medaka fish. In Medaka fish, the telencephalon is substantially reduced in size and lacks detectable endogenous *Nkx2.1* expression (Fig. 8A,L). This raises the question of whether the *Nkx2.1* regulatory elements studied here are sufficient to drive expression in the ventral telencephalon of the mouse. We thus tested the GFP construct described above in E13.5 mouse telencephalon by transient electroporation. Supplementary material Fig. S4 shows that two days after electroporation the transgene is expressed in the ventral telencephalon. At E13.5-15.5, mutation of the Sox2 sites had little effect on telencephalic expression, indicating that, at this developmental stage, other transcription factor-binding sites play a role in the regulation of this construct in the telencephalon (see Discussion).

DISCUSSION

The Sox2 transcription factor is crucial for the maintenance of several types of stem cells, including pluripotent, neural and osteogenic stem cells (Masui et al., 2007; Favaro et al., 2009; Basu Roy et al., 2010). Despite the importance of Sox2 in NSCs *in vitro*, major abnormalities in brain development were not detected by conditional ablation of Sox2 at midgestation (E12.5) in mouse, with the exception of defects in postnatal development of the hippocampus dentate gyrus and of the retina (Taranova et al., 2006; Miyagi et al., 2008; Favaro et al., 2009). Here, we examined the hypothesis that Sox2 is required in the developing telencephalon at early developmental stages. By conditionally deleting *Sox2* by E9.5 in all the developing telencephalon, we discovered that *Sox2* deletion strongly affects embryonic development of the ventral telencephalon. Patterning of the ventral telencephalon is crucially dependent on the induction of the diffusible factor Shh, which is mediated by the transcription factor Nkx2.1 (Sousa and Fishell, 2010). The crucial role of Shh is highlighted by the severe abnormalities in patients affected with holoprosencephaly, a developmental defect of the brain ventral midline, caused by *SHH* mutations (Dubourg et al., 2004; Roessler and Muenke, 2010). Here, we show that Sox2 is required for the early expression of *Nkx2.1*, thus controlling downstream ventral patterning genes such as *Shh*.

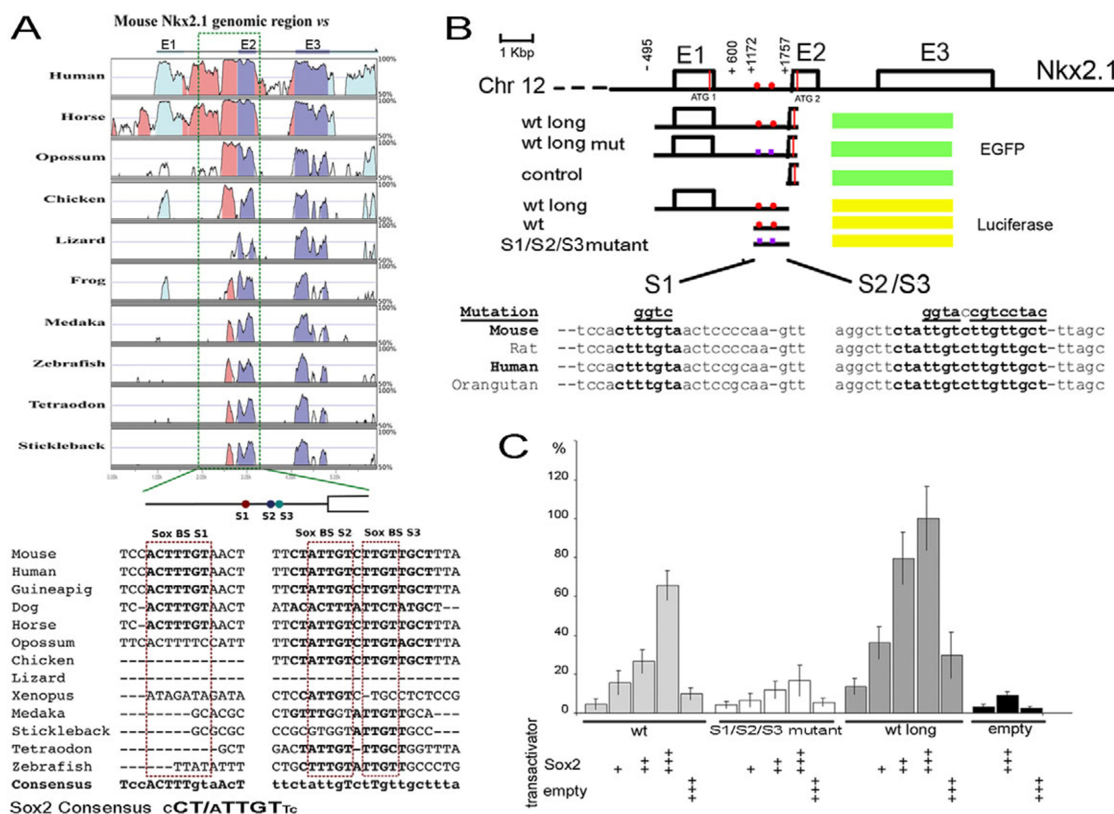

Fig. 7. Nkx2.1 regulation by Sox2. (**A**) The *Nkx2.1* intronic promoter/enhancer is evolutionary conserved in vertebrates. The genomic *Nkx2.1* sequences from the different vertebrate species were retrieved from the UCSC genome browser and aligned using the Shuffle-LAGAN of Vista. Pink, pale-blue and violet peaks represent conserved non-coding elements (75% conservation over 100 bp), mRNA untranslated sequence and coding sequence, respectively. Putative Sox2-binding sites, indicated as S1, S2 and S3, localise to a conserved element in the first intron. The S1 binding site is conserved among mammals but not in other vertebrates. S2 and S3 binding sites were conserved among most vertebrates. The indicated Consensus within the alignment was derived with Simple Consensus Maker (http://www.hiv.lanl.gov/content/sequence/CONSENSUS/SimpCon.html). The general Sox2 consensus is from Engelen et al. (Engelen et al., 2011). (**B**) Schematic of the *Nkx2.1* gene (Sox2 sites indicated by red dots) with constructs used for transgenic (EGFP) and transfection experiments (luciferase); sequence shows the Sox sites and the mutations introduced. EGFP constructs: *wt-long*: a region from nucleotide –495 (5′ to exon 1) to nucleotide +1942 in the second exon was cloned in frame with the EGFP reporter (green box); this fragment comprises the conserved elements in the first intron. *wt-long-mut*: carries mutations in the Sox2 sites, shown below (underlined). *control*: promoter-less EGFP construct. Luciferase constructs: *wt-long*: same region as in the EGFP construct, here linked to a luciferase reporter. *wt*: a shorter region encompassing the two Sox2 sites, from +1172 to +1757. *S1/S2/S3 mutant*: same as wt, with the same Sox2 mutations as in the *wt-long-mut*-EGFP reporter. (**C**) Co-transfection in P19 cells of *Nkx2.1* promoter with luciferase vectors (1 μg) and their mutant versions (shown in B), with increasing amounts (+, 0.125 μg; ++, 0.25 μg; +++, 0.5 μg) of *Sox2*-expressing vector (Sox2), or with the corresponding 'empty' vector. Results are the mean of at least three independent transfections, in triplicate.

Early Sox2 loss affects *Nkx2.1* and *Shh* expression

In *Bf1-cre Sox2*-deleted embryos, extensive ventral tissue loss occurs starting at ~E11.5, developing into major abnormalities of the ganglionic eminences (particularly the MGE) and of MGE-derived GABAergic neurons at later stages (Fig. 1); the expression of the dorsal markers *Pax6* and *Ngn2* (Fig. 2) also tends to spread ventrally, pointing to abnormalities of the ventral versus dorsal specification of the telencephalon. These defects strongly resemble those observed in *Nkx2.1* germ-line deletion and in the conditional ablation (via Bf1-cre) of the Shh receptor smoothened (Sussel et al., 1999; Fuccillo et al., 2004; Sousa and Fishell, 2010). We confirmed the connection to Shh by showing that *Shh* expression is strongly diminished in the ventral region of the mutant telencephalon concomitantly and prior to the onset of tissue loss (Figs 2, 3). Moreover, treatment of the embryos with a Shh agonist substantially rescued ventral development in the mutant brain (Fig. 5), though prenatal lethality still occurred. This rescue is reminiscent of that of the hippocampal dentate gyrus stem cells and postnatal growth by the same drug, in *nestin-cre Sox2*-deleted mice (Favaro et al., 2009). Local cell death in the ventral telencephalon is detected just prior to the onset of tissue loss (Fig. 4); this might also relate to loss of Shh signalling, which activates the anti-apoptotic gene *Bcl2* (Cayuso et al., 2006). These data, together with those of Favaro et al. (Favaro et al., 2009), highlight an unexpected role of Sox2 in mediating the development of specific brain regions at defined stages via Shh-dependent regulation.

How does Sox2 regulate *Shh* expression in the ventral telencephalon? Sox2 might regulate genes involved in local specification of ventral regions. A prime candidate target is *Nkx2.1*, essential for ventral brain development and correct dorsoventral patterning (Sussel et al., 1999; Sousa and Fishell, 2010). Nkx2.1 is thought to mediate the early 'homogenetic' induction of Shh in the ventral telencephalon, in response to the gradient of mesendoderm-derived Shh (Sousa and Fishell, 2010). Indeed, *Nkx2.1* mutant mice fail to express Shh in the ventral region (Sussel et al., 1999), and their phenotype resembles both that of *Shh* or smoothened mutants

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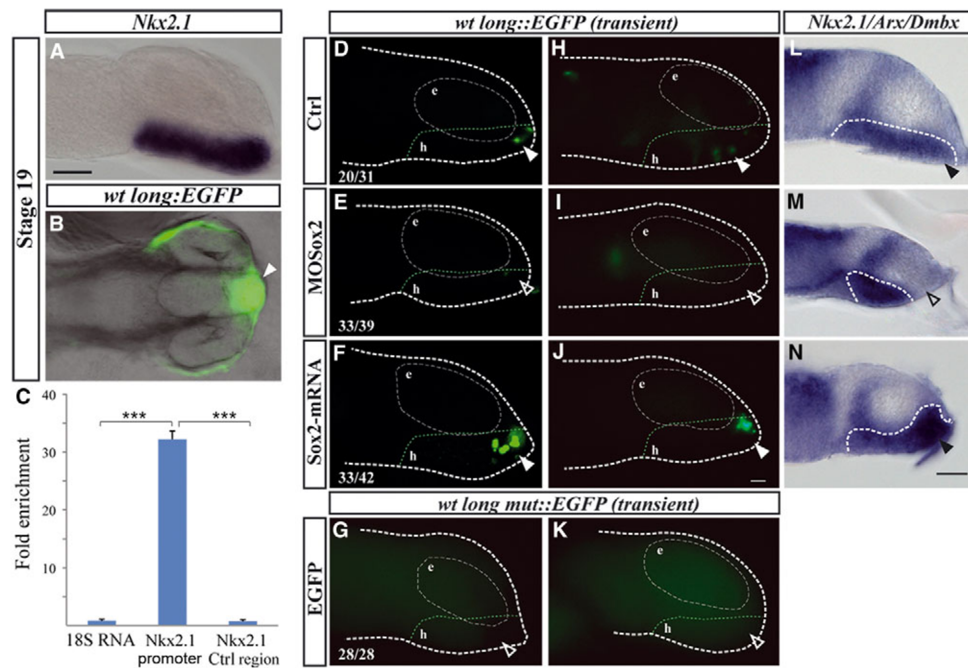

Fig. 8. *Nkx2.1* transgene and endogenous gene regulation by Sox2 in Medaka. (**A**) Lateral view of stage 19 Medaka embryo hybridised with an *Nkx2.1*-specific probe. (**B**) Dorsal view of a stable transgenic Medaka fish embryo carrying the EGFP reporter driven by the mouse *Nkx2.1* promoter/enhancer (*wt long*). Note the expression of the EGFP reporter corresponding to anterior hypothalamus (white arrowhead). (**C**) ChIP performed with anti-Sox2 on chromatin from stage 19 Medaka embryos. The histograms show the mean value+s.e.m. of a representative experiment performed in triplicate. Fold enrichment for the tested regions (*Nkx2.1* promoter, and a control region (Nkx2.1-Ctrl region) located 2783 bp downstream of the predicted *Nkx2.1* start codon) was normalised to control IgG and compared with 18s RNA. ***$P<0.0001$. (**D-N**) Medaka fish embryos were co-injected with control (D,H,L,G,K), Sox2 MO (E,I,M) or *Sox2* mRNA (F,J,N). Lateral views of transient transgenic Medaka fish embryos carrying the EGFP reporter driven by the *wt long* (D-F,H-J) or mutated (*wt long mut*) (G,K) mouse promoter/enhancer (confocal microscopy, G-N) and of embryos hybridised *in toto* with probe against diencephalic (*Arx*), mesencephalic (*Dmbx1*) and hypothalamic (*Nkx2.1*) markers (L-N). As expected for transient transgenic embryos, injections of the *wt long::EGFP* construct activates reporter expression with a variable (compare D with H, white arrowheads) mosaic pattern in discrete cells always in the anterior domain of the hypothalamus. Reporter expression is no longer observed when embryos are injected with the mutated version (unfilled arrowheads in G,K) or co-injected with Sox2 MO (empty arrowheads in E,I), whereas expression is increased in intensity upon *Sox2* mRNA injection (white arrowheads in F,J). Note also the parallel reduction (unfilled arrowhead in M) or expansion (black arrowhead in N) of the anterior hypothalamic mRNA distribution of *Nkx2.1* (black arrowhead in L), whereas the distribution of diencephalic and mesencephalic markers did not change. The shape of the embryos and of the eye (e) are outlined by dashed white lines. The *Nkx2.1*-positive hypothalamic domain (h) is outlined by green dotted lines. Frequency of the observed phenotype for each one of the experimental conditions is indicated in the bottom-left corner of panels (D-G). Two prototypic embryos are shown for each experimental condition. Scale bars: 50 μm in A-K; 40 μm in L-N.

(Fuccillo et al., 2004), and that of the present *Sox2* mutant. Furthermore, mutations destroying a consensus *Nkx2.1*-binding site in a distant *Shh* enhancer, active in telencephalon, impair the transcription of reporter constructs in transgenic mice (Jeong et al., 2006). Finally, *Nkx2.1* is required for expression of transcription factors Lhx6 and Lhx8 (also known as Lhx7) (Sussel et al., 1999), which coordinately activate Shh in neurons in the developing MGE (Flandin et al., 2011).

The early severe impairment of *Nkx2.1* expression in *Sox2* mutants already by E10.5 (Fig. 3), and the absence of *Shh* at least from E10.5/11.5 onwards (Fig. 2), are consistent with the hypothesis that a large part of the phenotypic effects of *Sox2* ablation is initially mediated by *Nkx2.1* deficiency.

Do other transcription factors mediate the effects of *Sox2* deficiency?

Presently, we can neither rule out nor implicate other genes besides *Nkx2.1* (and *Shh*) in the early effects of *Sox2* ablation. *SIX3* mutations are found in some human patients affected with holoprosencephaly (Jeong et al., 2008), and *Six3* haploinsufficiency caused by the 'knock-in' of a human mutant *SIX3* gene impairs Shh expression and MGE development in mouse, recapitulating features of the human phenotype (Geng et al., 2008). Moreover, a mutation in a *SIX3*-binding site, within a *SHH* long-range acting enhancer, has been detected in a human holoprosencephalic patient (Jeong et al., 2008). In our mutants, *Six3* expression was only slightly diminished in the ventral region at early stages, when *Nkx2.1* expression was already substantially affected (Figs 2, 3), making it unlikely that the effects of *Sox2* ablation are mediated by *Six3* deficiency. Interestingly, in the Medaka telencephalon, *Sox2* activates *Six3*, but the two genes seem to have antagonistic function in the hypothalamus (Beccari et al., 2012). The expression of *Bf1*, another candidate gene (Gutin et al., 2006; Hébert and Fishell, 2008), was also not significantly affected at these early stages, despite the *Bf1* hemizygosity due to cre 'knock-in' (Fig. 3).

After *Nkx2.1* (and thus *Shh*) expression is initially activated by mesendoderm-derived Shh, its activity is normally maintained, at later stages, by Shh itself (Xu et al., 2005; Xu et al., 2010; Sousa and Fishell, 2010). Following mosaic *Sox2* deletion at E8.5 (Fig. 6), Nkx2.1 is poorly expressed later on in *Sox2*-deleted cells, even in the presence of Shh. These data do not contradict the notion that Shh maintains later expression of Nkx2.1 (Xu et al., 2005; Xu et

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al., 2010), but simply highlight an early requirement for Sox2 in establishing this process.

The ventral telencephalic defects due to *Sox2* early ablation point to a marked regional specificity of Sox2 requirement in development (Fig. 1). As an example, Sox2 is required for *Nkx2.1* (and *Shh*) (see also Favaro et al., 2009) expression only within a specific subregion of the Sox2 pan-neural expression domain (Figs 2, 3). This might depend on local Sox2 concentrations, and/or on the presence of additional co-regulators. The transcription factors Sox1 and Sox3 are closely related to Sox2 (Wegner and Stolt, 2005), and recognise similar DNA sequences *in vitro* (Kondoh and Kamachi, 2010; Wegner, 2010). Thus, in regions in which Sox2 ablation causes few or no defects, Sox1 and/or Sox3 might compensate for Sox2 deficiency. Indeed, embryos doubly mutant for *Sox2* and *Sox3* ($Sox2^{+/-}$; $Sox3^{-/Y}$) develop diencephalic defects, mirroring those observed following early diencephalic deletion of the *Shh* gene (Zhao et al., 2012); by contrast, no such defects were observed in single mutants. Thus, Sox3 does compensate for some Sox2 functions in the diencephalon. By contrast, in the ventral telencephalon (present paper), Sox2 has some region-specific functions that cannot be complemented by Sox3 and Sox1, in spite of their normal expression levels (as also observed in Medaka) (Beccari et al., 2012). Most sequence diversity between Sox1, Sox2 and Sox3 occurs outside the DNA-binding domain; unique partnerships between Sox2 and co-factors (Kondoh and Kamachi, 2010; Bernard and Harley, 2010; Wegner, 2010) might mediate its specific functions in ventral (and hippocampal) (Favaro et al., 2009) brain development.

Among Sox2-specific interactors/DNA-binding proteins, CHD7 is known to activate a set of common targets relevant for anophtalmia (caused by *SOX2* mutations in humans and mice) and CHARGE syndrome (caused by *CHD7* mutations) (Engelen et al., 2011). The specific expression of different Sox2 interacting/ cooperating factors in various tissues might impart regional specificity to the defects caused by the absence of Sox2; indeed, an important (antagonistic) relationship of Sox2 with Pax6 was reported in a study of the development of neural competence in the optic cup (Matsushima et al., 2011).

How does Sox2 regulate *Nkx2.1*?

In Medaka, modulation of Sox2 levels correlates with changes in endogenous *Nkx2.1* expression intensity and spatial distribution (Fig. 8L-N). Moreover, the *Nkx2.1-GFP* construct faithfully recapitulates endogenous *Nkx2.1* expression, and requires intact Sox2-binding sites for activity (Fig. 8B,D-K), consistent with transfection results in P19 cells (Fig. 7C). These data identify *Nkx2.1* as a Sox2 target in Medaka. In Medaka, unlike in mouse, Nkx2.1 is not active in the telencephalon, and we thus cannot directly extrapolate from the Medaka results to infer direct regulation of Nkx2.1 by Sox2 in the telencephalon. We tested the same *Nkx2.1* construct in mouse by electroporation in the telencephalon at E13.5-15.5, showing that it is active in the telencephalon, preferentially in the MGE and other ventral regions (supplementary material Fig. S4); however, the mutation of the Sox2 sites did not substantially affect the activity of the construct (not shown). These results formally rule out the possibility that, at the E13.5-15.5 stage, the Sox2-binding sites, per se, are required for activity of the *Nkx2.1* promoters in the telencephalon. As a consequence, it remains unclear whether, in *Sox2* mutant mouse, the observed loss of *Nkx2.1* expression depends on the loss of a direct activity of Sox2 on the *Nkx2.1* promoter (so far unproven), on additional effects on other regulatory elements, or on indirect effects mediated by other Sox2-dependent factors.

How do we reconcile the data obtained by electroporation in mouse telencephalon with the low activity of the Sox2-mutated reporter in Medaka (and in *in vitro* transfected P19 cells)? We speculate that the requirement for Sox2 binding to the *Nkx2.1* promoter (if any) might be limited to the early stages of development. We know that late *Sox2* ablation (E12.5) has little effect on ventral telencephalic development and gene expression (Favaro et al., 2009; unpublished data), whereas early ablation (E9.5) causes important defects. We therefore hypothesise that Sox2-binding sites in the *Nkx2.1* promoter might be required for *Nkx2.1* regulation in mouse telencephalon at early (~E9.5), but not late, stages of development. Once established, *Nkx2.1* expression might be maintained, at E14.5, by transcription factors other than Sox2, and additional regulatory regions might be involved in controlling *Nkx2.1* expression. Unfortunately, the present constructs show very low activity following electroporation at E9.5, and similar transgenic constructs were not expressed in embryonic ventral telencephalon (Pan et al., 2004), making it difficult to test this hypothesis. Several regions adjacent to *Nkx2.1* bind Sox2 in ChIP experiments, and distal intergenic regions exhibit long-range interactions with the *Nkx2.1* gene (not shown), and might potentiate the promoter. These sequences will be investigated in the future in order to determine to their regulatory potential.

Conclusions

Sox2, despite its ubiquitous expression in neural stem/progenitor cells at all levels of the developing central nervous system, is absolutely required, in a stage- and region-specific way, in a limited set of locations, here exemplified by the early ventral telencephalon and by the hippocampus (Favaro et al., 2009). In the ventral telencephalon, Nkx2.1 is likely to be the main (although not necessarily the exclusive) mediator of Sox2 effects; other factors might mediate Sox2 activities in different regions. Expression defects of Shh (a target of Nkx2.1 in the ventral telencephalon), are common to both territories affected by Sox2 loss (ventral telencephalon and hippocampus); it will be interesting to examine other embryonic brain sites expressing Shh for defects caused by Sox2 ablation at different developmental stages.

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Competing interests statement

The authors declare no competing financial interests.

Supplementary material

Supplementary material available online at
http://dev.biologists.org/lookup/suppl/doi:10.1242/dev.073411/-/DC1

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