Sp8 exhibits reciprocal induction with Fgf8 but has an opposing effect on anterior-posterior cortical area patterning

Setsuko Sahara¹, Yasuhiko Kawakami², Juan Carlos Izpisua Belmonte² and Dennis DM O’Leary*¹

Address: ¹Molecular Neurobiology Laboratory, The Salk Institute, N. Torrey Pines Road, La Jolla, CA 92037, USA and ²Gene Expression Laboratory, The Salk Institute, N. Torrey Pines Road, La Jolla, CA 92037, USA

Email: Setsuko Sahara - sahara@salk.edu; Yasuhiko Kawakami - kawakami@salk.edu; Juan Carlos Izpisua Belmonte - belmonte@salk.edu; Dennis DM O’Leary* - doleary@salk.edu

* Corresponding author

Published: 17 May 2007

Neural Development 2007, 2:10 doi:10.1186/1749-8104-2-10

Received: 16 March 2007
Accepted: 17 May 2007

This article is available from: http://www.neuraldevelopment.com/content/2/1/10

© 2007 Sahara et al.; licensee BioMed Central Ltd.

This is an open access article distributed under the terms of the Creative Commons Attribution License (http://creativecommons.org/licenses/by/2.0), which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

Abstract

Telencephalic patterning centers, defined by the discrete expression domains of distinct morphogens, Fgf5 in the commissural plate (CoP), Wnts and Bmps in the cortical hem, and a ventral domain of Sonic hedgehog (Shh), are postulated to establish during development the initial patterning of the telencephalon, including the neocortex. We show that the expression patterns of Sp5, Sp8, and Sp9, members of the Sp8-like family that are homologues of Drosophila buttonhead, correlate during early embryonic development with these three telencephalic patterning centers. To study potential functional relationships, we focused on Sp8, because it is transiently expressed in the CoP coincident with the expression of Fgf8, a morphogen implicated in area patterning of the neocortex. We also show that Sp8 is expressed in cortical progenitors in a high to low anterior-medial to posterior-lateral gradient across the ventricular zone. We used in utero electroporation of full-length and chimeric expression constructs to perform gain-of-function and loss-of-function studies of interactions between Sp8 and Fgf8 and their roles in cortical area patterning. We show that Fgf8 and Sp8 exhibit reciprocal induction in vivo in the embryonic telencephalon. Sp8 also induces downstream targets of Fgf8, including ETS transcription factors. In vitro assays show that Sp8 binds Fgf8 regulatory elements and is a direct transcriptional activator of Fgf8. We also show that Sp8 induction of Fgf8 is repressed by Emx2 in vitro, suggesting a mechanism to limit Fgf8 expression to the CoP. In vivo expression of a dominant negative Sp8 in the CoP indicates that Sp8 maintains expression of Fgf8 and also its effect on area patterning. Ectopic expression of Sp8 in anterior or posterior cortical poles induces significant anterior or posterior shifts in area patterning, respectively, paralleled by changes in expression of gene markers of positional identity. These effects of Sp8 on area patterning oppose those induced by ectopic expression of Fgf8, suggesting that in parallel to regulating Fgf8 expression, Sp8 also activates a distinct signaling pathway for cortical area patterning. In summary, Sp8 and Fgf8 robustly induce one another, and may act to balance the anterior-posterior area patterning of the cortex.
Background

Many homeotic genes originally identified in *Drosophila* have important roles in vertebrate development. *Button-head* (*btd*), *empty spiracles* (*ems*), and *orthodenticles* (*otd*) were identified through mutagenesis screens as genes required for anterior head development in *Drosophila* [1-3]. Vertebrate homologues of *ems* and *otd*, which encode the *Emx* and *Otx* families of transcription factors (TFs), respectively, have been studied extensively, particularly their roles in mammalian forebrain development [4,5]. Vertebrate homologues of *btd*, a member of the *Sp1-Zn* finger family of TFs, have also been identified [6-8], but in contrast to homologues of *ems* and *otd*, they have been studied relatively little, particularly their roles in brain development.

Nine genes have been identified in mammals as *btd* homologues and the proteins they encode share *btd* and *Zn* finger domains. Based on the domain structures of their protein products, these nine genes are divided into a *Sp1*-like family (Sp1-4) and a *Sp8*-like family (Sp5-9). However, because Sp8 can rescue the defects in head development in *Drosophila btd* mutants, whereas Sp1 cannot [9], Sp8 and its family are viewed as the functional homologues of *Drosophila btd*. Mice deficient for either Sp5 or Sp8 have been reported. Sp5 knockout mice reportedly have no overt phenotypes [10]. In contrast, analyses of Sp8 knockout mice [9,11], complemented by retrovirus-mediated overexpression studies in embryonic chicks using dominant negative and active forms of Sp8 [8], indicate that Sp8 is required for limb and head formation.

A prominent role for Sp8 in limb development is to maintain the expression of several morphogens, including fibroblast growth factor (*Fgf8*), bone morphogenetic protein (*Bmp*4), and Sonic hedgehog (*Shh*) [9,11]. Because these morphogens have prominent roles in forebrain development, we examined the expression of the Sp8-like family members in the developing mouse forebrain to determine their potential for roles in forebrain patterning, particularly the patterning of the neocortex into ‘areas’, which are anatomically and functionally distinct divisions with specialized functions in sensory perception, motor performance, learning and memory.

The genetic mechanisms that control cortical area patterning, that is, arealization, have come under intense study in the past few years, but remain sketchy. Current evidence indicates that morphogens secreted by signaling centers located at the perimeter of the developing dorsal telencephalon (dTel) establish gradients of TFs across the dTel ventricular zone (VZ), which in turn determine the area fate of cortical progenitors and their progeny [12-17]. These morphogens include *Fgf8* expressed by the anterior neural ridge (ANR), which later becomes the commissural plate (CoP), located at the anterior midline of the dTel, and also members of the *wingless-int* (*Wnts*) and *Bmp* families expressed by the cortical hem and choroid plexus epithelium (CPE), located at the dorsal midline of more posterior dTel. In addition, the morphogen Sonic hedgehog (*Shh*) is expressed in the forebrain, but in ventral structures, including the prechordal plate and the medial ganglionic eminence (MGE).

Here we describe that each of these three major signaling centers in mouse forebrain is uniquely associated with the discrete expression patterns of a member of the Sp8-like *btd* family, Sp5, Sp8, and Sp9. These associations are suggestive of functional interactions between the Sp family members and the respective morphogens. To address this issue, we focused on functions of Sp8, which at early stages of cortical development is expressed in association with the expression domain of *Fgf8* in the ANR/CoP. We employed both gain and loss of function strategies using *in utero* electroporation to manipulate gene function in a localized manner, a technique similar to that recently used by others to show roles for *Fgf8* in cortical area patterning [18,19]. We complemented these *in vivo* studies with *in vitro* assays of inductive pathways and interactions. These approaches allowed us to overcome the early embryonic lethality and forebrain defects exhibited by Sp8 knockout mice, including the failure of the anterior neural tube to close and the cerebral cortex to develop, that preclude an analysis of Sp8 in cortical patterning [9,11].

We show that Sp8 and Fgf8 can robustly induce one another, and that the induction of Fgf8 by Sp8 is repressed by the homeodomain TF, Emx2, which itself has been implicated in controlling area patterning [19-22]. The reciprocal induction between Sp8 and Fgf8 exhibits significant specificity among family members. In addition, we show that Sp8 affects cortical area patterning, but in a manner that opposes that of Fgf8, raising the possibility that Sp8 influences area patterning not only by regulating Fgf8 and its signaling pathway, but also through a distinct mechanism. Our findings show that Sp8 interacts with Fgf8 to balance the anterior-posterior (A-P) area patterning of the cortex.

Results

Forebrain expression of the Sp8-like family, mouse btd homologues, and correlation with signaling centers in the forebrain

We used *in situ* hybridization to analyze the expression patterns of the Sp8-like family members (Sp5–Sp9) on brain whole mounts at E9.5 and sections through the forebrain from E9.5 through E13.5 (Figure 1, 2, 3; data not shown). We did not detect expression of Sp6 and Sp7 (data not shown) [23,24]. At early stages of cortical development, E9.5 to E13.5, we detect expression of Sp5 and
Sp8 in dTel within the cortical ventricular zone (VZ), whereas Sp9 expression is not detected within dTel but is strong in ventral telencephalon. As detailed in the following sections, each of these Sp expression domains correlates with a forebrain signaling center defined by the expression of unique sets of morphogens in the telencephalon.

**Sp5 expression**

Between E9.5 and E11.5, expression of Sp5 is highest in the medial and posterior dTel, including the cortical hem and CPe, and exhibits a very steep gradient with the cortical VZ, quickly declining from high to low levels within more posterior and medial dTel, resulting in very low levels in more anterior and lateral dTel (Figure 1a, b, d, e, g).

**Figure 1**

Expression patterns of Sp5 in embryonic mouse forebrain and relationship with cortical hem and choroid plexus. (a-i) Sp5 expression relates to the cortical hem, a source of Wnts and Bmps. Shown are in situ hybridizations using DIG-labeled riboprobes for Sp5 on sagittal (a, d, g) or coronal (b, e, h) sections and Wnt2b (c, f, i) of mouse forebrain at E10.5 to E13.5. Sp5 expression is observed around the cortical hem and Cpe as being highest in medial and posterior parts of dorsal telencephalon (dTel; arrowhead), and quickly downregulated at E13.5. Unlike the expression of Sp8, Sp5 expression domains overlap those of Wnts throughout E10.5 to 13.5. Ctx, cortex; GE, ganglionic eminence; Hp, hippocampus. Scale bars: 0.5 mm.
h). The pattern of Sp5 expression is considerably more extensive than the cortical hem/CPe, but most of the caudomedial part of the Sp5 expression domain contains the Wnt2b expression domain (Figure 1c, f, i), a marker for the cortical hem [16]. By E13.5, Sp5 expression declines to non-detectable levels in the cortex, but persists in the hem and CPe (Figure 1). Sp5 is also expressed at lower levels in the mantle of the MGE.

**Sp9 expression**

At E10.5, Sp9 is modestly expressed in the mantle zone of the ganglionic eminence (GE) (Figure 2a, b) and, therefore, likely in postmitotic neurons. At E11.5 (Figure 2d, e), Sp9 is robustly expressed in the MGE, throughout the mantle zone and likely also in the subventricular zone (SVZ) of the MGE. At this age, the MGE mantle includes postmitotic GABAergic interneurons that migrate into and
Expression patterns of Sp8 in embryonic mouse forebrain and relationships with expression domains of Fgf8 in the CoP. (a-i) Shown are in situ hybridizations of sections of mouse forebrain at E10.5 to E13.5 using DIG-labeled riboprobes for Sp8 on sagittal (a, d, g) or coronal (b, e, h) sections, and for Fgf8 (c, f, i). Sp8 is expressed in the dTel VZ, with highest levels in the medial and anterior parts. Sp8 is also expressed robustly in the LGE and at lower levels in the MGE. Sp8 expression overlaps the Fgf8 expression domain in the CoP at E10.5 (arrowhead in (b, c)), but gradually become excluded at E11.5 (arrowhead in (e, f)). (j, k) Whole mount in situ hybridizations done on E9.5 embryos with DIG-labeled riboprobes for Sp8 (j), and Fgf8 (k), respectively. The Sp8 A/P gradient is evident at this stage (j). Arrowheads indicate expression domains. Ctx, cortex; GE, ganglionic eminence; Hp, hippocampus. Scale bars: 0.5 mm (a-f); 0.5 mm (g-i); 0.5 mm (j, k).
populate the cortex [25], and the SVZ contains proliferating cells. Sp9 is also expressed robustly in the SVZ of the lateral GE (LGE). Sp9 expression overlaps with Shh expression in the MGE mantle. However, elsewhere in ventral telencephalon, Shh is predominantly expressed in largely non-overlapping, complementary patterns with Sp9 (Figure 2).

**Sp8 expression**

From E9.5 through E13.5, Sp8 is expressed in a high to low medial to lateral gradient across the dTel VZ, and is expressed across the A-P axis of dTel, with a shallow high to low A-P gradient; by E10.5, Sp8 is expressed across the entire A-P axis. The Sp8 domain is associated with the Fgf8 expression domain in the CoP (Figure 3b, c). The correlation of the Sp8 domain with the Fgf8 expression domain in the CoP is dynamic over the brief time window of Fgf8 expression (Figure 3b, c, e, f, h, i). At E9.5 and E10.5, the Sp8 and Fgf8 domains overlap, although the Sp8 domain is more expansive and extends laterally and posteriorly from the CoP through the dTel VZ (Figure 3b, c, j, k). At E11.5, Sp8 is robustly expressed, but it is beginning to segregate from the Fgf8 domain at the posterior domain, with Fgf8 largely limited to the midline CoP, and Sp8 largely excluded from it and expressed in a high to low gradient extending laterally from the midline (Figure 3e, f). At E13.5, Sp8 expression in the dTel VZ is still present but substantially diminished, and Sp8 has become excluded from the increasingly restricted Fgf8 domain in the CoP (Figure 3h, i).

Sp8 is also expressed in the SVZ of the LGE, a source of interneurons that migrate into the olfactory bulbs, and at low levels in the mantle of the LGE and MGE. Sp8 has been shown to regulate the specification, survival and migration of olfactory interneurons [26].

**Sp8 and Fgf8 exhibit reciprocal induction**

For functional analyses, we have focused on Sp8 and its relationship with the Fgf8 expression domain in the CoP, the potential for an interaction between Sp8 and Fgf8, and its influence on cortical area patterning. The expression pattern of Sp8 suggests that Sp8 might be a target of Fgf8, which is consistent with the finding that Sp8 expression is diminished in conditional or hypomorphic Fgf8 knockout mice [27]. To address whether Fgf8 induces Sp8, we ectopically expressed Fgf8 in vivo in the telencephalon by in utero electroporation of a CAG-Fgf8 expression vector; in this, and all other similar experiments, a enhanced green fluorescent protein (EGFP) expression vector was co-electroporated as a reporter to mark the transfection domain. Electroporations were done on E11.5 and the brains analyzed at E13.5. Adjacent sections were processed by in situ hybridization for ectopic expression of Sp8, Sp9 and Sp5. Sp8 is consistently induced by ectopic Fgf8, (n = 8 of 9 cases, Figure 4b), whereas we do not detect induction of ectopic expression of either Sp9 (n = 4 of 4, Figure 4c) or Sp5 (n = 4 of 4, Figure 4d). The control vector has no influence on Sp expression (n = 4 of 4, Figure 4f–h). These findings show that Fgf8 induces Sp8, indicating that Sp8 is a downstream target of Fgf8 but Sp5 and Sp9 are not.

To address whether Sp8 reciprocally induces Fgf8, we ectopically transfected expression vectors for Sp8, or to assess specificity, Sp5 and Sp9. We found that ectopic expression of Sp8 robustly induces Fgf8 expression (n = 9 of 10, Figure 4j). Ectopic expression of Sp9 only modestly induces Fgf8 (n = 8 of 9, Figure 4l), whereas Sp5 does not induce detectable levels of Fgf8 (n = 2 of 2, Figure 4n). These findings show that Sp8 can induce Fgf8, and, therefore, that Sp8 and Fgf8 form a reciprocal inductive loop.

To address whether Sp8 is a transcriptional activator of Fgf8, we used a strategy of fusing the btd and Zn finger functional binding domains of Sp8 to a VP16 domain, which is a potent transcriptional activator [8]. This chimeric construct acts as a dominant active form of Sp8 that can induce Fgf8 in the developing chick limb [8]. We found that ectopic expression of the Sp8-VP16 construct also induces Fgf8 (n = 12 of 14, Figure 4p ), indicating that Sp8 is a transcriptional activator of Fgf8. The VP16 construct alone (n = 6 of 6, data not shown) does not induce Fgf8. In summary, we demonstrate that Fgf8 and Sp8 exhibit reciprocal induction in vivo in the embryonic telencephalon. Furthermore, our findings show that Sp8 regulates transcription of Fgf8.

Fgf17 is expressed within the CoP, albeit in a broader domain than Fgf8, and has a similar effect as Fgf8 on A-P cortical patterning [18]. Therefore, we also performed in utero electroporations to test whether Sp8 and Fgf17 exhibit reciprocal induction. However, we did not detect reciprocal induction (n = 3 of 3 cases, data not shown), suggesting that Sp8 is not directly relevant to Fgf17 signaling. Thus, Sp8 and Fgf17 do not induce one another.

In conclusion, the reciprocal induction between Sp8 and Fgf8 exhibits a specificity that does not extend significantly to related Fgf and Sp8-like family members.

**Sp8 activates the Fgf8 signaling pathway**

Our findings show that Sp8 is a transcriptional activator of Fgf8, suggesting that ectopic Sp8 induction of Fgf8 should also induce the Fgf8 downstream targets, including Fgf8-target genes such as the ETS (E-twenty-six) family TFs, Pea3, Erm, and Er81 [19]. To address this issue, we carried out additional in utero electroporations of Sp8 expression vectors as described above on adjacent sections through the transfection site identified by the GFP signal, and per-
formed in situ hybridization for Fgf8 as well as ETS family members (Figure 5). Ectopic expression of Sp8 does indeed induce the expression of Pea3 (n = 4 of 4, Figure 5c), Ern (n = 3 of 3, Figure 5d), and Er81 (n = 3 of 3, Figure 5e), but coincident to the Sp8-transfection domains. Control vectors had no effect (n = 3 of 3, Figure 5g–j). These findings indicate that Sp8 not only induces Fgf8, but also components of its signaling cascade, suggestive of the activation of a functional Fgf8 signaling pathway.

**Sp8 binds the Fgf8 promoter and activates Fgf8 expression**

To gain more insight into the regulation of Fgf8 expression, we searched the genomic sequence of Fgf8 for putative Sp8 regulatory elements. Because Sp8 is a member of the Sp1-Zn finger family, we expected that Sp8 would bind DNA sequences similar to those described for Sp1 [28]. Within the 585 base-pairs (bp) upstream of the Fgf8 transcription start site, we identified six putative Sp8 bind-
Figure 5
Sp8 induces Fgf8 downstream targets. (a-j) Induction of ectopic ETS TFs, Pea3, Erm and Er81 by the ectopic expression of Sp8. Coronal sections of E13.5 forebrains that were electroporated at E11.5 with Sp8 (a-e) or a control vector (f-j), mixed with EGFP, and processed for *in situ* hybridization using 3S riboprobes for Sp8 (b, g), Pea3 (c, h), Erm (d, i) and Er81 (e, j), respectively. EGFP (a, f) marks the electroporation domains (arrowheads). Pea3 (c), Er81 (d) and Erm (e) were induced by ectopic expression of Sp8. Scale bars: 0.5 mm.
ing sites, and a position at which three of six putative sites overlap with one another (Figure 6a).

To show Sp8 binding to Fgf8 promoter sequences, we first carried out a gel retardation assay. This 585 bp fragment of the Fgf8 promoter region was labeled with P32 and incubated with lysate from reticulocytes expressing the Sp8 protein under the control of a T7 promoter, or control lysate. We detected two different bands in the Sp8 lysate but not in the control lysate (Figure 6b). To corroborate the specificity of these results, we performed a competition experiment by repeating the gel retardation assay as above, but in the presence of an excess of the 585 bp DNA fragment not labeled with P32. In this competition assay, the bands were absent, indicating that Sp8 protein directly binds to these fragments of the Fgf8 promoter region.

As a complementary approach, we tested Sp8 binding to the Fgf8 promoter sequences by a pull-down assay. Lysates from cells transfected with hemagglutinin (HA)-tagged Sp8 or control vector were incubated with a biotinylated oligonucleotide corresponding to the putative Sp8 binding site, or with one mutated at the core region of the Sp1-recognition sequences. We found co-precipitation of Sp8 with the wild-type oligonucleotide but not with the mutated version (Figure 6c). Thus, these data indicate that Sp8 recognizes and binds to this region of the Fgf8 promoter defined by sequence similarity to Sp1 binding sites.

To further investigate if Sp8 regulates Fgf8 expression through binding to this promoter region, we used a luciferase reporter assay (Figure 6d). Control or test vectors containing Sp8, Sp9, or Emx2 were transfected independently or in combinations into C3H10T1/2 cells also transfected with an Fgf8 reporter construct consisting of the same 585 bp promoter element for Fgf8 used in the gel retardation assay, and a firefly luciferase reporter vector [29]. Transfection of an Sp8 expression vector robustly induces expression of the Fgf8 reporter construct, whereas Sp9 has only a modest inductive effect.

Noggin, a Bmp inhibitor, has been recently reported to induce Fgf8 expression when overexpressed in forebrain [30]. Therefore, we tested whether a suppression of Bmp signaling could contribute to Sp8-mediated Fgf8 upregulation. However, we found that manipulation of Bmp activity has no effect on Sp8 transcriptional activation of Fgf8 in the in vitro reporter assay (Additional file 1).

Taken together these findings show that Sp8 binds to a proximal promoter region of the Fgf8 gene of Fgf8, and that this binding induces Fgf8 expression.

**Sp8 induction of Fgf8 is repressed by Emx2**

As described above, Sp8 is expressed in the CoP coincident with Fgf8. However, Sp8 is also expressed outside of the CoP, within the VZ of dTel (neocortex), locations where Fgf8 is not expressed, suggesting that factors expressed in the cortical VZ repress the ability of Sp8 to induce Fgf8 and restrict its expression to the CoP. Emx2 is a candidate repressor because the domain of Fgf8 expression in the CoP is broader in Emx2 null mice, and ectopic Emx2 expression in the CoP in slice culture suppresses Fgf8 expression [19].

Therefore, we tested whether Emx2 can repress Sp8 induction of Fgf8 in a luciferase reporter assay. An Emx2 construct transfected alone has no effect on expression of the Fgf8 reporter, and is indistinguishable from a control empty vector alone. However, when Emx2 is co-transfected with Sp8, Emx2 represses Sp8 induction of the Fgf8 reporter construct. Emx2 also represses the modest induction of Fgf8 exhibited by Sp9.

In summary, these findings are consistent with our in vivo analyses, and provide a molecular mechanism for them, demonstrating that Sp8 binds Fgf8 regulatory elements and is likely a direct transcriptional activator of Fgf8. Furthermore, they show that Emx2 can act as a repressor of Sp8 induction of Fgf8.

**Sp8 is required to maintain Fgf8 expression in vivo**

To obtain further evidence that Sp8 regulates Fgf8 expression in vivo, we used a dominant negative strategy by fusing the btd and Zn finger DNA binding domains of Sp8 to an engrailed repressor domain, which has been shown in developing chick limb to act as a dominant negative form of Sp8 [8]. We electroporated the Sp8-engrailed construct in the anterior domain of dTel at E11.5, and analyzed expression at E14.5. An engrailed control vector, consisting of the engrailed repressor sequences but lacking the Sp8 DNA binding domains has no effect on Fgf8 expression when transfected into the CoP (n = 9 of 9, Figure 7a). In dramatic contrast, transfection of the dominant negative Sp8-engrailed construct into the CoP markedly suppresses Fgf8 expression in this anterior domain, although intense GFP expression is evident (n = 10 of 13, Figure 7b).

These results were also confirmed by the expression of Pea3, a member of the Pea3 ETS TF subfamily that is rigidly regulated by Fgf8 [31,32]. We found that transfection of the dominant negative Sp8-engrailed construct into the CoP results in a substantially diminished expression of Pea3 (n = 7 of 8, Figure 7f), whereas the control engrailed construct has no effect (n = 9 of 9, Figure 7e). These results demonstrate that Sp8 is required to maintain Fgf8 expres-
Sp8 directly binds the Fgf8 promoter and Emx2 represses Sp8 induction of Fgf8. (a) Sequence of a 585 bp fragment, containing the 555 bp immediate upstream region of the mouse Fgf8 transcription start site and the 30 bp 5’ UTR of Fgf8 having six putative Sp8 binding sites. Putative Sp8 binding sites predicted from SpI-binding motifs (GGGGCGG or CCCCGGC) are underlined. (b) Gel retardation assay of Sp8 and Fgf8 promoter fragments. P32-labeled Fgf8 promoter fragments show slow mobility after incubation with the Sp8-expressed lysate (black arrow) compared to the unbound DNA (gray arrow). These bands are not detected in the control lysate sample and are diminished in the presence of an equal (×1) or five-fold (×5) amount of unlabeled DNA compared to labeled probes. (c) Sp8 binding to the oligonucleotide of the Fgf8 promoter region. HA-Sp8 was co-precipitated with biotinylated non-mutated oligonucleotide corresponding to a putative Sp8 binding site, but not with oligonucleotides with a mutated core recognition sequence. (d) Luciferase reporter assay for induction of Fgf8. Control or test vectors (Sp8, Sp9, Emx2) were transfected independently or in combinations into C3H10T1/2 cells also transfected with an Fgf8 reporter construct consisting of a promoter element for Fgf8 and a firefly luciferase reporter vector. The relative effectiveness of Fgf8 induction was assessed by measuring luciferase activity. Sp8 robustly induces expression of the Fgf8 reporter construct; this Sp8 induction is suppressed by co-expression of Emx2. Sp9 modestly induces expression of the Fgf8 reporter construct; again, this is suppressed by Emx2. Emx2 alone has no effect on Fgf8 induction, similar to a control empty vector.
Anterior electroporation of a dominant negative form of Sp8 inhibits endogenous Fgf8 expression in the commissural plate and results in an anterior shift in area patterning. (a-h) Anterior electroporation of a dominant negative form of Sp8 inhibits endogenous Fgf8 expression in the CoP. Electroporation of the Sp8 dominant negative chimeric construct, Sp8-engrailed, suppresses endogenous Fgf8 expression in the CoP (b), as well as expression of Pea3, a downstream target of Fgf8 (f) at E14.5. Fgf8 and Pea3 expression in brains with similar electroporation of the control engrailed construct (a, e) is indistinguishable from non-transfected brains (not shown). Arrowheads indicate Fgf8 (a, b) and Pea3 expression domains (e, f). An EGFP expression construct co-electroporated with Sp8-engrailed and engrailed control constructs are shown in parallel and define the transfection domain (c, d, g, h). (i, j) Anterior ectopic expression of Sp8-engrailed results in an anterior shift of cortical areas. Tangential cortical sections through layer 4 of a P7 brain electroporated at E11.5 with control vector (i) or Sp8-engrailed (j) and processed for serotonin immunostaining to reveal the primary sensory areas: somatosensory (S1), auditory (A1) and visual (V1). In cortex of brains with anterior electroporation of Sp8-engrailed, S1 shifts far anteriorly, resulting in a very small amount of cortex remaining for motor (M) areas. The arrow in (j) indicates putative visual areas. Scale bars: 0.5 mm (a-h), 1.0 mm (i, j). (k) Quantification of motor area size in Sp8-engrailed electroporated brains. The length of motor areas is measured as a ratio of the length from the edge of anterior flattened brains to the staining of S1 regions (ML) and the total length (TL) of serotonin staining of flattened brains. Compared to engrailed control vector cases (28.89 ± 1.35% standard error of the mean (SEM, n = 5), brains electroporated with Sp8-engrailed show shrunken motor areas (13.66 ± 1.34% SEM, n = 6), as indicated in the middle panel. The right panel shows a scatter plot of individual cases.
sion within the CoP in vivo, as well as expression of its targets.

**Maintained expression of Fgf8 by Sp8 is required for proper cortical area patterning**

If Sp8 is required to maintain Fgf8 expression and activation of its signaling pathway, as our results above show, then electroporation of the dominant negative form of Sp8 (Sp8-engrailed) into anterior dTel should affect cortical area patterning by diminishing Fgf8 signaling. We predict that the effect on area patterning should resemble the anterior area shifts produced by ectopic expression in anterior dTel of the extracellular domain of Fgf receptor 3c (sFGFR3c), which is interpreted to be due to sFGFR3c binding and suppressing the action of endogenous Fgf8 protein secreted by the CoP [18].

To test this prediction, we performed in utero electroporations of the Sp8-engrailed construct into anterior dTel at E11.5 as above, but rather than analyzing the brains at embryonic ages, we analyzed their area patterning at P7 using serotonin immunostaining on tangential sections, which clearly delineate the primary sensory areas [33]. We observed that Sp8-engrailed electroporation induces a substantial shift of area patterns toward the anterior pole of the cortex and a substantial reduction in the sizes of the primary sensory areas (n = 6 of 6, Figure 7i). Electroporation of the control engrailed domain alone does not affect area patterning (n = 5 of 5, Figure 7i). Thus, these anterior area shifts are consistent with an Sp8-engrailed mediated repression of targets of Sp8, such as Fgf8 and its downstream effectors (for example, Pea3). Thus, Sp8 is crucial for maintaining Fgf8 expression in the CoP and activation of its signaling pathway, as well as the influence of Fgf8 signaling on cortical arealization.

**Changes in area patterning induced by ectopic expression of Fgf8**

As a prelude to addressing the effect of ectopic expression of Sp8 on area patterning, we performed in utero electroporation of a CAG-Fgf8 expression construct into either anterior or posterior dTel at E11.5, and analyzed area patterning at P7 using serotonin immunostaining on tangential sections [33]. These cases allowed us to compare the effect of ectopic expression of Sp8 to that of Fgf8 done in the same manner.

In brains with electroporations done at E11.5 and examined at E13.5/E14.5, about 80% of the transfection domains marked by the EGFP reporter are located at the region of dTel targeted for electroporation (anterior pole or posterior pole) (105 of 137 cases). However, because the EGFP reporter co-electroporated at E11.5 with the Sp8 constructs is no longer detectable at P7, to confirm these results, we repeated these studies using ROSA26 reporter mice, and co-electroporated the Sp8-VP16 dominant active construct or the VP16 control construct with a CAG-Cre Recombinase construct; the CAG-Cre Recombinase activates the β-gal reporter to mark the lineage of transfected cells and hence the location of the transfection domain. The findings confirm that the transfection domains, marked by β-gal labeled cells, are at positioned where the transfection was targeted, either the anterior or posterior cortical pole (Additional file 2), and the area shifts are identical to those described above.

Anterior electroporation of Fgf8 causes a substantial expansion of the anterior cortical field, which includes motor areas (Figure 8a), as previously reported [18]. In contrast, we observed that posterior electroporation of Fgf8 induces an anterior shift of areas accompanied by an expansion of V1 (Figure 8b). Electroporations of Fgf8 that targeted the S1 barrelfield, made from a medial or lateral approach at a mid-posterior level, resulted in aberrant patterning of the barrelfield and shifts in its position (Figure 8c, d). Thus, ectopic expression of Fgf8 in anterior dTel results in a posterior shift in cortical areas, whereas ectopic expression of Fgf8 in posterior dTel results in an anterior shift.

**Sp8 induces area shifts that oppose those induced by Fgf8**

Because both full-length and dominant active VP-16 forms of Sp8 induce Fgf8 and its signaling pathway, we expected that ectopic expression of Sp8 should have an effect on cortical arealization resembling that produced by ectopic expression of Fgf8. Therefore, we predicted that anterior electroporation of these Sp8 constructs would result in a posterior shift of cortical areas, similar to that for anterior overexpression of Fgf8 [18] (Figure 8a). Based on similar logic, we anticipated that posterior ectopic expression of the Sp8 constructs should result in an anterior shift of cortical areas. We performed in utero electroporations of the Sp8 constructs into anterior or posterior dTel at E11.5, and analyzed area patterning at P7 using serotonin immunostaining on tangential sections [33].

Contrary to our prediction, we found that anterior electroporation of Sp8 results in an anterior shift of areas (n = 8 of 10, Figure 9b) judging from serotonin staining of tangential sections at P7, while similar electroporation of the control construct (n = 5 of 5, Figure 9a) results in an area pattern that is indistinguishable from wild type. Anterior electroporation of the Sp8-VP16 also results in an anterior shift of areas (n = 7 of 9, Figure 9d), whereas anterior electroporation of the VP16 control construct (n = 5 of 5, Figure 9c) again results in an area pattern that is indistinguishable from wild type. Because these area shifts oppose those produced by anterior overexpression of Fgf8, they suggest that although Sp8 can induce Fgf8, Sp8
might also activate distinct pathways to overcome Fgf8 signaling for area patterning.

Posterior electroporations of either the full-length Sp8 or Sp8-VP16 constructs result in a posterior shift of areas, accompanied by a substantial reduction in the size of V1, the most posterior primary sensory area (Sp8, n = 5 of 6; Sp8-VP16, n = 16 of 18; Figure 9h, l); again, control electroporations have no effect on area patterning. This finding is consistent with the anterior shift produced by anterior electroporation of Sp8. However, these area shifts induced by Sp8 (Figure 9) are the opposite of those observed when we electroporate an Fgf8 expression construct in posterior dTel, which we find produces an anterior shift in cortical areas (Figure 8).

**Area shifts by Sp8 are paralleled by changes in markers of positional identity**

To address whether area changes induced by Sp8 are accompanied by changes of positional identity of cortical neurons [34,35], Cad8 expression was detected at P1 by whole mount in situ hybridization using digoxigenin-labeled probes, and RORβ and ephrin-A5 were detected using S35 labeled probes on sagittal sections. Expression was analyzed in P1 brains following in utero electroporation with Sp8 expression vectors or control vectors in anterior dTel or posterior dTel at E11.5.

Compared to cases electroporated with a control vector (n = 9 of 9, Figure 10a), we observed that in cases with anterior electroporations of Sp8, the anterior (motor) cad8 expression domain exhibits an anterior contraction (n = 7 of 11, Figure 10d). In contrast, cases with a posterior electroporation of Sp8 results in a posterior expansion of the anterior cad8 domain (n = 5 of 6, Figure 10j) compared to control cases (n = 6 of 6, Figure 10g). In addition, the expression patterns of RORβ (n = 4 of 4, Figure 10e) and ephrin-A5 (n = 4 of 4, Figure 10f) are shifted anteriorly in the anterior Sp8 transfected cases compared to the control transfected cases (n = 4 of 4, Figure 10b, c), or shifted posteriorly in the posterior Sp8 transfected cases (n = 4 of 4, Figure 10k, l) compared to control transfected cases (n = 4 of 4, Figure 10h, i). Thus, these findings indicate that ectopic Fgf8 expression induces shifts in primary sensory areas revealed by serotonin immunostaining at P7, which

---

**Figure 8**

Cortical area shifts by ectopic expression of Fgf8 at anterior or posterior sites. Electroporation of a CAG-Fgf8 vector was done at E11.5 and later, at P7, the brains were processed and tangential sections of their cortical areas were visualized by serotonin staining as described in Methods. (a) Ectopic expression of Fgf8 at an anterior site in E11.5 causes posterior area shifts with motor (M) area expansion (n = 2 of 2 electroporated cases; this finding replicates that of Fukuchi-Shigomori and Grove [18], therefore we did not perform additional cases) compared to control electroporated brains. (b) In contrast, a posterior electroporation of Fgf8 induces anterior area shifts accompanied by an expansion of V1 (n = 4 of 6 electroporated cases). We also performed electroporations using medial and lateral approaches at mid-posterior levels in an attempt to target the presumptive barrelfield of S1 (posteromedial barrel subfield, PMBSF). (c, d) Medial-posterior electroporation of Fgf8 typically causes an 'elongated' S1 (c) (n = 7 of 10 electroporated cases), whereas lateral-posterior electroporation within the cortical field that would develop as S1 can result in a 'split barrel field' (d) (n = 2 of 9 electroporated cases). The middle of the 'PMBSF' in S1 is pointed to by the line. We did not observe 'duplicate' barrels as reported by Fukuchi-Shigomori and Grove [18]. We assume that duplicated barrels are produced only in a unique situation with an appropriate combination of timing, size and position of the ectopic Fgf8 expression domain, and level of Fgf8 expression, required to partially duplicate the barrel pattern. Arrows indicate targeted locations of the electroporation sites. M, motor areas. Scale bar: 1.0 mm.
Ectopic expression of Sp8 and a dominant active form of Sp8 induce area shifts that oppose Fgf8. (a, b) Ectopic expression of Sp8 (b) at an anterior site of E11.5 brains causes anterior area shifts with a substantial reduction of motor (M) areas compared to control electroporated brains (a) that are indistinguishable from wild type (not shown). Areas of tangential section at P7 cortices are visualized by serotonin immunostaining. (c, d) Anterior ectopic expression of a dominant active form of Sp8, Sp8-VP16 (d), but not the VP16 domain alone (c), induces anterior area shifts similar to Sp8. The dashed line is at the E1 barrel position of control brain for the A-P comparison. (e) Quantification of motor areas in Sp8-, Sp8-VP16, or control-transfected brains. Anterior electroporation of either Sp8 or Sp8-VP16 causes reduction of the motor area ratio (Sp8, 18.07 ± 1.52% SEM, n = 10; Sp8-VP16, 19.12 ± 2.18% SEM, n = 9), compared to the control cases (control vector; 29.05 ± 0.691%, SEM, n = 5; VP16, 26.88 ± 0.853%, SEM, n = 5). The right panel shows a scatter plot of individual cases. (f-i) Posterior ectopic expression of Sp8 and Sp8-VP16 induces posterior cortical area shifts. Sp8 (g) and Sp8-VP16 (i) ectopic expression at posterior sites causes a posterior shift in area patterning with a reduction in size of V1 areas, whereas expression of an empty vector (f) or the VP16 domain alone (h) has no effect on area patterning. The dashed line is at the A1 barrel position of control brain. Scale bar: 1.0 mm. (l) Quantification of caudal areas in Sp8-, Sp8-VP16, or control-transfected brains. Posterior electroporation of either Sp8 or Sp8-VP16 causes reduction of caudal area ratio (Sp8, 24.42 ± 2.03%, SEM, n = 6; Sp8-VP16, 24.89 ± 0.700% SEM, n = 18), compared to the control cases (control vector, 33.02 ± 0.581%, SEM, n = 5; VP16, 31.16 ± 0.430%, SEM, n = 5). The right panel shows a scatter plot of individual cases. EP, electroporation; M, motor length; C, caudal length; T, total length.
Figure 10
Sp8 changes region-specific gene expression in the cortex. (a, d, g, j) Dorsal views of P1 brains processed for whole mount in situ analysis with digoxigenin labeled-cad8 riboprobes following electroporation at an anterior (a, d), or posterior (g, j) location of empty control (a, g) or Sp8 (d, j) vectors at E11.5. Arrowheads indicate the posterior border of the cad8 expression domain in anterior cortex. Anterior electroporation of Sp8 results in an anterior contraction of the cad8 domain (d), whereas posterior electroporation of Sp8 results in a posterior expansion of the cad8 domain (j). Electroporation of an empty vector has no effect on the cad8 domain (e, m). (b-f, h-l) Sagittal sections of P1 brains stained with an anterior marker gene, RORb probes (b, e, h, k), or a S1 marker gene, ephrin-A5 probes (c, f, i, l), following electroporation at an anterior (b, c, e, f) or posterior (h, i, k, l) domain of empty vector (b, c, h, i) or Sp8 (e, f, k, l). Arrowheads indicate the posterior border of the RORb expression domain or the putative M1/S1 and S1/V1 borders of ephrin-A5 expression. Coincident to the area shifts detected by cad8 expression, RORb and ephrin-A5 expressions are shifted anteriorly in anterior Sp8-overexpressed brains, and shifted posteriorly in posterior Sp8-electroporated brains. Scale bars: 0.5 mm (e, f, m, l), 0.5 mm (b, c, e, f, h, l, k, i). (m) Quantification of cad8 expression domains in Sp8- or control transfected brains. Anterior electroporation of Sp8 reduces motor area (M1a) size detected as cad8 domain (8.2 ± 1.12%, SEM, n = 13) compared to the control cases (15.2 ± 0.297%, SEM, n = 9). In contrast, posterior electroporation of Sp8 enlarges motor areas (19.97 ± 0.771%, SEM, n = 6) compared to the control cases (14.37 ± 0.241). The bottom panel shows a scatter plot of individual cases. EP, electroporation.
is preceded by changes in the expression patterns of gene markers indicative of the positional identities of cortical neurons.

In summary, although Sp8 can strongly induce Fgf8 and its signaling pathway, the changes in area patterning and parallel shifts in positional identity of cortical neurons induced by ectopic expression of Sp8 oppose those induced by ectopic expression of Fgf8. These unexpected findings suggest the complexity of the role for Sp8 signaling in influencing cortical patterning. Sp8 might interfere with or counteract the influence of Fgf8 on area patterning without disturbing Fgf8 signaling. These opposing effects of Sp8 and Fgf8 may cooperate to balance A-P patterning of the cortex into areas.

Discussion
Expression of the Sp8-like family relates to forebrain patterning centers
We show that three mammalian homologues of the Droso phila btd gene, Sp5, Sp8 and Sp9, which are functionally most closely related to btd, are expressed in the embryonic mouse forebrain in unique patterns that closely relate to three signaling centers defined by the expression domains of distinct classes of morphogens implicated in forebrain patterning. Sp5 is expressed in association with the cortical hem and CPe, which are sources of Wnts and Bmps, Sp8 with the ANR/CoP, a source of Fgfs, including Fgf8, and Sp9 with the Shh domain in ventral telencephalon (Figure 11). In addition, Sp8 and Sp5 are expressed within the cortical VZ in opposing gradients across the A-P axis of the cortex, with Sp8 being expressed in a high to low A-P gradient, whereas both are expressed in a high medial to low lateral gradient. These unique expression patterns of Sp5, Sp8, and Sp9 and their associations with forebrain signaling centers implicate them in forebrain patterning, potentially through their regulation of the relevant morphogens and their signaling.

Based on these expression patterns, we focused on a functional relationship between Sp8 and Fgf8, as well as potential roles for Sp8 in area patterning of the neocortex. It is not possible to study area patterning using constitutive Sp8 knockout mice because they exhibit an early embryonic lethality and have severe neural tube closure defects resulting in a failure of the cortex to develop [9,11]. Indeed, even the use of conditional knockout strategies to study roles for Sp8 in regulating Fgf8 independent from controlling cortical area patterning would be difficult as it would require the use of distinct Cre lines in which Cre recombinase is expressed in very restricted patterns, both spatially and temporally, to dissociate the function of Sp8 in regulating Fgf8 versus a direct role in cortical progenitor cells, and the requirement of Sp8 for neural tube closure and subsequent development of the cerebral cortex. To circumvent these obstacles, we used complementary gain of function and loss of function strategies employed through in utero electroporation of expression constructs, a technique similar to that recently used by others to show roles for Fgf8 in area patterning [18,19].

Sp8 and Fgf8 share a reciprocal induction loop that exhibits significant specificity
We analyzed the relationship between Fgf8 and Sp8 in vivo by electroporating expression constructs into the lateral wall of the telencephalon and analyzing their influence at embryonic stages very early in cortical development, when Fgf8 is normally expressed in the CoP and exerts an influence on area patterning. We show that ectopic expression of Fgf8 in the telencephalon strongly induces Sp8. This finding is consistent with the recent observation that Sp8 expression is diminished in conditional or hypomorphic Fgf8 knockout mice [27].

We also found that ectopic expression of a full length Sp8 construct can strongly induce Fgf8 in dTel. To extend these

Discussion
Expression of the Sp8-like family relates to forebrain patterning centers
We show that three mammalian homologues of the Droso phila btd gene, Sp5, Sp8 and Sp9, which are functionally most closely related to btd, are expressed in the embryonic mouse forebrain in unique patterns that closely relate to three signaling centers defined by the expression domains of distinct classes of morphogens implicated in forebrain patterning. Sp5 is expressed in association with the cortical hem and CPe, which are sources of Wnts and Bmps, Sp8 with the ANR/CoP, a source of Fgfs, including Fgf8, and Sp9 with the Shh domain in ventral telencephalon (Figure 11). In addition, Sp8 and Sp5 are expressed within the cortical VZ in opposing gradients across the A-P axis of the cortex, with Sp8 being expressed in a high to low A-P gradient, whereas both are expressed in a high medial to low lateral gradient. These unique expression patterns of Sp5, Sp8, and Sp9 and their associations with forebrain signaling centers implicate them in forebrain patterning, potentially through their regulation of the relevant morphogens and their signaling.

Based on these expression patterns, we focused on a functional relationship between Sp8 and Fgf8, as well as potential roles for Sp8 in area patterning of the neocortex. It is not possible to study area patterning using constitutive Sp8 knockout mice because they exhibit an early embryonic lethality and have severe neural tube closure defects resulting in a failure of the cortex to develop [9,11]. Indeed, even the use of conditional knockout strategies to study roles for Sp8 in regulating Fgf8 independent from controlling cortical area patterning would be difficult as it would require the use of distinct Cre lines in which Cre recombinase is expressed in very restricted patterns, both spatially and temporally, to dissociate the function of Sp8 in regulating Fgf8 versus a direct role in cortical progenitor cells, and the requirement of Sp8 for neural tube closure and subsequent development of the cerebral cortex. To circumvent these obstacles, we used complementary gain of function and loss of function strategies employed through in utero electroporation of expression constructs, a technique similar to that recently used by others to show roles for Fgf8 in area patterning [18,19].

Sp8 and Fgf8 share a reciprocal induction loop that exhibits significant specificity
We analyzed the relationship between Fgf8 and Sp8 in vivo by electroporating expression constructs into the lateral wall of the telencephalon and analyzing their influence at embryonic stages very early in cortical development, when Fgf8 is normally expressed in the CoP and exerts an influence on area patterning. We show that ectopic expression of Fgf8 in the telencephalon strongly induces Sp8. This finding is consistent with the recent observation that Sp8 expression is diminished in conditional or hypomorphic Fgf8 knockout mice [27].

We also found that ectopic expression of a full length Sp8 construct can strongly induce Fgf8 in dTel. To extend these
findings, we show that ectopic expression in dTel of a ‘dominant-active’ Sp8-VP16 construct, created by the fusion of Sp8 with the transcriptional activator VP16, also robustly induces Fgf8, similar to the full length of Sp8, indicating that Sp8 is a transcriptional activator for Fgf8. We complemented these in vivo studies with an in vitro luciferase assay in transfected cell lines, and show that Sp8 strongly induces expression of an Fgf8 reporter construct in vitro. Thus Sp8 appears to directly induce Fgf8.

The reciprocal inductive loop between Sp8 and Fgf8 appears to be a specific relationship, consistent with the unique relationship in their expression patterns in the developing forebrain. The inductive effect of Fgf8 in the telencephalon is specific for Sp8, because we found that Fgf8 does not induce either Sp5 or Sp9 in vivo. Complementing these findings, we show using in utero electroporation in dTel that in vivo Sp9 only weakly induces Fgf8, and Sp5 has no detectable inductive effect. These findings are paralleled by those from the in vitro luciferase assay in which Sp9 weakly induced expression of an Fgf8 reporter construct. Interestingly, the inductive abilities of these Sps for Fgf8 correlate with sequence homologies: Sp9 has identical bHLH and Zn finger domains to those of Sp8, whereas those of Sp5 are distinct [8].

In addition, the Sp8/Fgf8 inductive loop has specificity within the Fgf family, as indicated by our finding that Sp8 does not induce Fgf17, which is closely related to Fgf8 and is also expressed within the CoP. Thus, the reciprocal induction between Sp8 and Fgf8 exhibits significant specificity for one another. Such unique pairings of reciprocal induction between Sp8-like family members and forebrain patterning centers may also be utilized by Sp9 and Shh in ventral telencephalon and by Sp5 with wnts and Bmps in the cortical hem. This latter possibility is consistent with the report that the zebrafish homologue of Sp5 is induced by Wnt/β-catenin signaling [36].

**Distinct roles for Sp8 in initiation and maintenance of Fgf8 expression in the CoP**

The initiation and maintenance of Fgf8 expression in the CoP are important phenomena for the regulation of cortical area patterning. Our findings show that ectopic Sp8 expression is sufficient to induce Fgf8, but Sp8 does not appear to be required for the normal initiation of Fgf8 expression in the ANR/CoP. Analysis of Sp8 knockout mice show that Fgf8 expression is initiated in the ANR, although its subsequent expression in the ANR, or later in the CoP, is not described [11]. Our findings, however, that Fgf8 expression is diminished when we electroporate a dominant-negative Sp8-engrailed construct into the CoP indicate that Sp8 is required to maintain Fgf8 expression in the CoP. This interpretation is also consistent with our expression data revealing that Fgf8 expression in the CoP diminishes at a stage shortly after Sp8 expression is no longer detected in the CoP but is enhanced outside of it within the cortical VZ. Thus, we conclude that although Sp8 is sufficient to induce Fgf8, it is not normally required for Fgf8 induction in the ANR/CoP. However, Sp8 is required for the maintenance of Fgf8 expression in the CoP. This scenario is similar to conclusions derived from observations of limb development in Sp8 knockout mice that Fgf8 expression is initiated but not maintained [11].

**Sp8 induction of Fgf8 is limited to the CoP by Emx2 repression**

A related important issue is the mechanism by which Fgf8 expression is limited to the CoP even though Sp8, which can robustly induce Fgf8 in the telencephalon outside of the CoP, is more broadly expressed in dTel. One possibility is that other factors uniquely present in the CoP are required to cooperate with Sp8 to induce Fgf8. Another possibility, and not mutually exclusive, is that a repressor of Sp8 induction of Fgf8 is present in the dTel but not the CoP. Multiple lines of evidence indicate that this repressor is Emx2, a homeodomain transcription factor that controls area patterning.

First, we show using the in vitro luciferase assay that Emx2 completely represses the robust induction of Fgf8 by Sp8. In addition, the expression patterns of Sp8 and Emx2 are consistent with this model. At early stages, Sp8 expression overlaps with the CoP, and later becomes excluded from the CoP and spreads across the entire cortical VZ (Figure 12), whereas, Emx2 is never expressed in the CoP but is coincident with the expression of Sp8 in the cortical VZ [20,33]. Further, in Drosophila, homologues of vertebrate Emx2 and Sp8, ems and btd, respectively, interact to influence head development [37]. Taken together, these findings provide a compelling case that Emx2 represses Sp8 induction of Fgf8 in dTel and restricts Fgf8 expression to the CoP.

This interpretation is consistent with the report that the domain of Fgf8 expression expands in dTel beyond the CoP in Emx2 mutants [19]. Furthermore, our findings provide an explanation for the report that ectopic expression of Emx2 constructs electroporated into the CoP of forebrain slices can repress Fgf8 expression when done at E11.5 embryonic stages but not at E13.5 [19]. This temporal change in the ability of Emx2 ectopically expressed in the CoP to repress Fgf8 expression coincides with the early presence then downregulation of Sp8 expression in the CoP (present study). In conclusion, our findings indicate that the report that Emx2 both limits Fgf8 expression to the CoP and represses Fgf8 in the CoP by Emx2 [19] are due to Emx2 repression of Sp8 induction of Fgf8 (Figure 12).
Summary of the Sp8 function related to telencephalic patterning centers and cortical area patterning.

(a) Schematic diagram of time-dependent expression domains of Sp8 and Fgf8. At E10.5, Sp8 expression (green) is expressed in both progenitor cells in the cortical VZ (cortex) and within the CoP, where it overlaps with the Fgf8 expression domain (overlapping Sp8 and Fgf8 expression in the CoP is colored yellow). Sp8 expression is gradually excluded from the Fgf8 expression domain (red) at E11.5 and later. (b) Domain-dependent regulation of Sp8 TF activity by Emx2. Sp8 forms a reciprocal induction loop with Fgf8 in the CoP. Emx2 (blue) is expressed in cortical progenitors, but not in the CoP. Although Sp8 is expressed in cortex, Emx2 represses the ability of Sp8 to induce Fgf8, thereby restricting Fgf8 expression to the CoP. (c) Electroporation (EP) of Fgf8 and Sp8 results in opposing shifts in cortical area patterning. In early stages of cortical patterning, Sp8 maintains Fgf8 expression in CoP and the Fgf8 signaling pathway, which imposes anterior identity to cortical progenitors. Anterior EP of Fgf8 expression constructs results in enhanced anterior area identities and a corresponding posterior shift in cortical areas. Anterior EP of Sp8 expression constructs has an opposing effect on area patterning to that of Fgf8, and results in an anterior shift in cortical areas. Posterior EP of either Fgf8 or Sp8 have the opposing effect on area patterning compared to their anterior EP. These opposing effects of ectopic expression of Sp8 on area patterning compared to Fgf8 indicate that Sp8 activates a signaling pathway(s) that can overcome the effect of Fgf8 signaling, perhaps by interfering with Fgf8 signaling or dominating it. Furthermore, Sp8 expression in cortical progenitors may trigger distinct signaling pathways that function to facilitate posterior area identity.

Figure 12
Ectopic Sp8 expression affects cortical area patterning in an opposing manner to Fgf8

We show that ectopic expression of Sp8 in either anterior or posterior dTel at embryonic stages early in cortical development has a strong effect on cortical area patterning, both the size and positioning of areas defined by serotonin staining, as well as the expression patterns of areal markers. The timing of our electroporations, and the postnatal stages of our analyses of area patterning are similar to those used by Fukuchi-Shimogori and Grove [18,19] to study the role of anterior ectopic expression of Fgf8 in area patterning. They showed that anterior ectopic expression of Fgf8 causes posterior area shifts, a finding that we have confirmed. Because anterior ectopic expression of Sp8 induces Fgf8, we expected that anterior ectopic expression of Sp8 would exhibit the same phenotype as anterior ectopic expression of Fgf8. Surprisingly, though, we found that anterior ectopic expression of Sp8 results in an anterior shift of areas, the opposite effect reported for Fgf8. We also found that posterior ectopic expression of Fgf8 and Sp8 have opposing effects on area patterning: posterior ectopic expression of Sp8 results in a posterior shift of areas, whereas Fgf8 results in an opposing anterior shift. Since the same ectopic expression of Sp8 not only induces Fgf8, but also induces TFs of the ETS family that are downstream factors of Fgf8 signaling, including Pea3, Erm and Er81, the opposing effects of ectopic expression of Sp8 versus Fgf8 on area patterning is unlikely due to Sp8 triggering an aborted or defective Fgf8 signal cascade.

Thus, we hypothesize that Sp8 can induce a signaling pathway that influences area patterning in a manner that opposes the Fgf8 pathway and competes with or balances the effect of Fgf8 signaling on area patterning. Fgf8 signaling might be balanced delicately, so that small changes of Sp8 activity might result in opposing outputs; consistent with this suggestion are the findings that different dosages of Fgf8 result in opposite effects on cell death/survival and forebrain patterning [27,38]. Sp8 might have dual roles in area patterning. In addition to a role in inducing Fgf8, Sp8 could have a more direct influence on the specification of the area identity of cortical progenitor cells that express it, such as that described for the TF EMX2 [20-22,33]. Indeed, an intriguing study in Drosophila shows that ems, the homolog of vertebrate Emx2, specifies the identity of the head segment, but only when acting with btd, the functional homolog of vertebrate Sp8 [37]. Thus, Sp8 may specify area identities in progenitors that express both it and Emx2, in opposing A-P gradients, potentially through an interaction with Emx2.

Methods

Animals

Timed pregnant ICR mice were used in accordance with Institution guidelines. ROSA26 reporter mice were obtained from Jackson Laboratories, and established homozygous males were crossed with ICR females to obtain heterozygous embryos. The day of insemination and the day of birth are designated as embryonic day 0.5 (E0.5) and postnatal day 0 (P0), respectively.

In situ hybridization

The following S35- or digoxigenin (DIG)-labeled riboprobes were used: Sp5, Sp8, Sp9, Fgf8 [33], Wnt2b (image clone 353765), Shh [39], Pera3, Er81 (RT-PCR), Erm (image clone 5033778), ephrin-A5, RORβ [21]. In situ hybridization was done on 20 μm cryostat sections and DIG whole mount in situ hybridization was performed as described previously [34].

In utero electroporation and expression constructs

Expression constructs were made by fusing cDNA of chicken Fgf8 isoform b, full length Sp8, Sp8-VP16, Sp8-engrailed Sp5 or Sp9, which was then sub cloned into a Bluescript SK(-) vector containing chicken β-actin promoter from pCAGGS vector and a polyA sequence of bovine growth hormone sequences.

Surgery and in utero electroporation were performed according to the method by Saito et al. [40] with some modifications as follows. Plasmid DNA (1 mg/ml for genes of interest and 1 mg/ml for pCAG-nlsEGFP or pCAG-nls-cre), mixed with 0.1% Fast green for visualization, was injected with a glass capillary at E11.5 into the lateral ventricle to transf ect cells in the VZ of dTel. One of two alternative methods were then followed: paddle-type electrodes (Nepagene CUY21, 0.5 cm diameter, 30 V, 50 mS × 5) were used to obtain broad electroporation sites for studies of gene induction and needle type electrodes made of tungsten were used for area analysis to ensure precise control of the electroporation site (80 V, 50 mS × 3). Embryos were later harvested, fixed and processed. The survival rate of embryos was approximately 70%. Approximately 77% exhibited efficient GFP expression (n = 105/137) when analyzed at E13 and E14. Targeting accuracy of the electroporation sites was also evaluated by the site of the GFP domain at E13 or E14: accuracy was 76% for anterior injections (n = 87/115) and 82% for posterior injections (n = 18/22). Only one case of anterior injection resulted in middle expression of the A-P axis, and the rest were negative for GFP signals.

In vitro promoter assay

A 585 bp fragment, containing a 555 bp region immediately upstream of the mouse Fgf8 transcription start site and 30 bp of 5’-UTR of Fgf8, was subcloned into a promoter-less pGL3 basic vector (a firefly luciferase reporter vector; Promega, Madison, WI, USA), and transfected into C3H10T1/2 cells. Luciferase activity was measured after a 48 h transfection period as described previously [29].
Gel retardation assay
Full length Sp8 was subcloned into pcDNA3.1 (Invitrogen, Carlsbad, CA, USA) and incubated in reticulocyte lysates following the manufacture's protocols (Promega) for in vitro translation. For the gel retardation assay, 5 µl of lysate was incubated with 1 ng of the P32-labeled 585 bp fragment of DNA corresponding to the Fgf8 promoter region in binding buffer (20 mM Hepes-KOH, pH 8.0, 5 µg/ml poly(dI-dC), 2% polyvinyl alcohol) for 1 h on ice in the presence or absence of unlabeled fragments as indicated in the figure 6b. Binding products were separated in 4% polyacrylamide gels and analyzed by phosphoimage analyzer (Typhoon 8600, Molecular Dynamics, Sunnyvale, CA, USA).

Pull-down assay using biotinylated oligonucleotides
Biotinylated oligonucleotides containing the putative Sp8 binding site in the Fgf8 promoter (-230 to -190 from the transcription start site) were used. For control experiments, we introduced a mutation into the core region of the Sp1-recognition sequence, replacing GCC with TTT (designated as lower case t in the sequence below), based on a previous study [28]. The sequences of the wild-type (WT) and mutated oligos (Mut) were: WT, 5’ TGCCCAGCGCCGGCCGGGCGGG-CCGGCCCGCCGCGCCGGCG; Mut: 5’, TGCCCCCTGCTCtttCCtttCCG.

A pair of complementary oligonucleotides was annealed and used as a probe. C3H10T1/2 cells were transfected with pCAGGS or pCAGGS-HA-tagged Sp8 with Fugene6 (Roche, Indianapolis, IN, USA) according to the manufacturer’s instruction. Cell lysates were harvested 48 h after transfection and each cell lysate was split in half to react with WT and Mut oligos. A biotin-streptavidin pull down assay was performed for the analysis of Sp8 binding to probes as previously described [41,42].

Histochmistry and quantification of area size
Serotonin immunostaining and X-gal staining of tangential sections were performed as described previously [33]. The ratio of the length of motor areas and total areas of serotonin immunostained flattened brains, or cad8 expression domains of whole mount in situ P2 brains as shown in schematic diagrams in each figure, were measured by Image J software [43].

Competing interests
The author(s) declare that they have no competing interests.

Authors’ contributions
SS conceived of the studies, engineered most of the expression constructs, designed and carried out most of the experimental studies, and helped draft the paper. YK performed the luciferase assays and pull-down assays, and engineered some of the expression constructs. JCIB provided unique reagents and oversaw the assays done by YK. DDMO helped conceive of the studies, coordinated them, analyzed and interpreted data, and drafted the paper. All authors read and approved the final manuscript.

Additional material

Additional File 1
Bmp signaling is not involved in Sp8-mediated Fgf8 upregulation. It is possible that Sp8-mediated Fgf8 upregulation could be due to a suppression of Bmp signaling, because Fukuchi et al. [30] reported that Noggin, a Bmp inhibitor, induces Fgf8 expression in vivo when overexpressed in forebrain. We thus tested whether manipulating Bmp activity can mediate Sp8 transcriptional activation of Fgf8 (see Legend to Additional file 1 below). First, we tested if C3H10T1/2 cells in our assay respond to manipulation of Bmp signaling. For this purpose, we used a Id1-reporter construct that has been shown to be activated by Smad1-dependent manner [41,44] In a condition of culture containing 1% FBS, we detected endogenous Bmp-mediated Id1 reporter activation compared to a deletion construct lacking Smad binding sites. Addition of Noggin reduced the reporter activity to the similar level of mutant reporter, indicating that basal activity of the reporter is due to Bmp-like activity in the 1% FBS and Noggin effectively blocks extrinsic Bmp activity in this system. In addition, co-transfection of constitutively active BMP receptors (CA-Alk3), also induced Id1 reporter activation, that was not blocked by an intracellular antagonist Noggin. Addition of Bmp2 protein, in a dose-dependent manner, stimulated the reporter activity. We thus conclude that C3H10T1/2 cells respond to Bmp signaling. We next tested whether Noggin elicits the expression of the Fgf8 reporter construct in C3H10T1/2 cells. We hypothesized if Sp8 induction of Fgf8 is mediated by the inhibition of Bmp pathways, such as an induction of Noggin as shown by Fukuchi et al in vivo, we expect that Sp8-mediated Fgf8 reporter expression will be affected by the presence of Noggin or Bmp proteins. However, we did not observed a significant upregulation of Fgf8 reporter expression in the presence of Noggin, though it slightly upregulates it (see error bar). If this minor change by Noggin reflects synergistic action of the inhibition of Bmp signaling and Sp8-mediated Fgf8 regulation, we would see that opposite effect of Bmp2 to the addition of to Noggin, i.e., suppression of Sp8 induction of Fgf8 reporter expression by Bmp. However, again, we did not see any significant downregulation of reporter expression by Bmp2 presence. Thus we conclude that altered Bmp signaling in our in vitro reporter assay does not mediate Sp8-mediated Fgf8 upregulation. In support of our conclusion, it was previously shown that Fgf8 upregulation by Noggin is observed when Noggin is overexpressed at E9.5, but not later than this time point [30]. In contrast, we detect Fgf8 upregulation by Sp8 when Sp8 expression construct is introduced at E11.5, the time point that overexpression of Noggin no longer upregulate Fgf8 in forebrain. So we propose that the Bmp signaling restricts Fgf8 expression in early stages, but not in later stages when still Sp8 regulates Fgf8 expression. (A) C3H10T1/2 cells respond to Bmp signaling. C3H10T1/2 cells were transfected with reporter construct of Id1 promoter (Id1-Luc) or mutated construct lacked Smad binding sites that does not respond to either serum in culture medium or Bmp2 protein (100 ng/ml). Transfection of Alk3, constitutively active BMP receptors (CA-Alk3), induces Id1 reporter expression in a Smad-dependent manner. Addition of Noggin (100 ng/ml) inhibits endogenous Bmp signaling. Bmp2 can elicit Id1 reporter expression in a dose dependent manner and saturated at 10 ng/ml. (B) Sp8-mediated Fgf8 upregulation is independent from Bmp signaling. The expression of Fgf8 reporter construct mediated by Sp8, or endogenous factors, is not significantly affected in the presence or the absence of Noggin, or Bmp2 proteins. Click here for file [http://www.biomedcentral.com/content/supplementary/1749-8104-2-10-S1.tif]
Additional File 2

Confirmation of anterior and posterior targeting of electroporated constructs. Tangential sections of ROSA26 reporter mouse brains electroporated with a CAG-VP16 control expression vector (A, C) or a dominant active CAG-Sp8-VP16 expression vector (B, D) together with a CAG-Cre expression vector. Counterstaining of LacZ in sections indicates the position of the cells electroporated with a nuclear-localized-signal (nls)-Cre. Direct detection of GFP signal shows that approximately 80% of cases electroporated at E11.5 and analyzed at E13.5 have accurately positioned electroporations. However, because the GFP reporter co-electroporated at E11.5 with the Sp8 constructs is not detectable at P7, to confirm these results, we repeated these studies using ROSA26 reporter mice by co-electroporating a CAG-Cre Recombinase construct with the Sp8-VP16 dominant active construct and the VP16 control construct. The findings confirmed that in every case (n = 4 of 4), the transfection domains, marked by X-gal labeled cells, is either at the targeted cortical pole (anterior or posterior), and the area shifts are identical to those described above. (see Methods for details). Scale bar: 1.0 mm. [http://www.biomedcentral.com/content/supplementary/1749-8104-2-10-S2.tif]

Acknowledgements

We thank members of the O’Leary lab, particularly T Hamaasaki, S-C Chou, Y Nakagawa, and K Yee for advice, and Berta Higgins and Haydee Gutierrez and Henry Juguilon for technical help. We are grateful to P Soriano for providing the R26R mice and T Matsu for the pull-down assay protocol. This work was supported by NIH grants R37 NS31558 and R01 NS50646 (DDMO) and R01 HD034538 (CBIB). SS was supported by fellowships from the JSPS Fellowships for Research Abroad, and Uehara Memorial Foundation.

References

1. Dalton D, Chadwick R, McGinnis W: Expression and embryonic function of empty spiracles: a Drosophila homeobox gene with two patterning functions on the anterior-posterior axis of the embryo. Genes Dev 1989, 3:1940-1956.
2. Cohen S, Jurgens G: Drosophila headlines. Trends Genet 1991, 7:267-272.
3. Finkelstein R, Boncinelli E: From fly head to mammalian forebrain: the story of otd and Otx. Trends Genet 1994, 10:310-315.
4. Acampora D, Annino A, Tuorto F, Puelles E, Lucchesi W, Papalia A, Simeone A: Otx genes in the evolution of the vertebrate brain. Brain Res Bull 2003, 66:410-420.
5. Cecchi C: Emx2: a gene responsible for cortical development, regionalization and area specification. Gene 2002, 291:1-9.
6. Wimmer EA, Jackle H, Pflicfe C, Cohen SM: A Drosophila homologue of human Sp1 is a head-specific segmentation gene. Nature 1993, 366:690-694.
7. Bouwman F, Philipsen S: Regulation of the activity of Sp1-related transcription factors. Mol Cell Endocrinol 2002, 195:27-38.
8. Kawakami Y, Esteban CR, Matsui T, Rodriguez-Leon J, Kato S, Belmonte JC: Sp8 and Sp9, two closely related buttonhead-like transcription factors, regulate Fgf8 expression and limb outgrowth in vertebrate embryos. Development 2004, 131:4763-4774.
9. Treichel D, Schook F, Jackle H, Gruss P, Mansouri A: mBtd is required to maintain signaling during murine limb development. Genes Dev 2003, 17:2630-2635.
10. Harrison SM, Houzelstein D, Dunwoodie SL, Beddington RS: Sp5, a new member of the Sp1 family, is dynamically expressed during development and genetically interacts with Brachyury. Dev Biol 2000, 227:358-372.
11. Bell SM, Schreiner CM, Waclaw RR, Campbell K, Potter SS, Scott WJ: Sp8 is crucial for limb outgrowth and neuromere closure. Proc Natl Acad Sci U S A 2003, 100:12195-12200.
12. Groves EA, Fukuchi-Shimogori T: Generating the cerebral cortical area map. Annu Rev Neurosci 2003, 26:355-380.
13. O’Leary DD, Nakagawa Y: Patterning centers, regulatory genes and extrinsic mechanisms controlling arealization of the neocortex. Curr Opin Neurobiol 2002, 12:14-25.
14. Crossley PH, Martinez S, Ohkubo Y, Rubenstein JL: Coordinate expression of Fgf8, Otx2, Bmp4, and Shh in the rostral prosencephalon during development of the telencephalic and optic vesicles. Neuroscience 2001, 108:183-206.
15. Bachler M, Neubauer A: Expression of members of the Fgf family and their receptors during midfacial development. Mech Dev 2001, 100:313-316.
16. Groves EA, Tole S, Limon J, Tip L, Ragdale CW: The hem of the embryonic cerebral cortex is defined by the expression of multiple Wnt genes and is compromised in gli3-deficient mice. Development 1998, 125:2315-2325.
17. Furuta Y, Piston DW, Hogan BL: Bone morphogenetic proteins (BMPs) as regulators of dorsal forebrain development. Development 1997, 124:2203-2212.
18. Fukuchi-Shimogori T, Groves EA: Neocortex patterning by the secreted signaling molecule FGF8. Science 2001, 294:1071-1074.
19. Fukuchi-Shimogori T, Groves EA: Emx2 patterns the neocortex by regulating FGF positional signaling. Nat Neurosci 2003, 6:825-831.
20. Bishop KM, Goudreau G, O’Leary DD: Regulation of area identity in the mammalian neocortex by Emx2 and Pax6. Science 2000, 288:344-349.
21. Bishop KM, Rubenstein JL: O’Leary DD: Distinct actions of Emx1, Emx2, and Pax6 in regulating the specification of areas in the developing neocortex. J Neurosci 2002, 22:7627-7638.
22. Mallamaci A, Muzio L, Chan CH, Parnavales J, Boncinelli E: Area identity shifts in the early cerebral cortex of Emx2/- mutant mice. Nat Neurosci 2000, 3:679-686.
23. Nakamura T, Ueda F, de-Vega S, Vilaxa A, Fukumoto S, Yamada KM, Yamada Y: The Kruppel-like factor epipodin is expressed by epithelium of developing teeth, hair follicles, and limb buds and promotes cell proliferation. J Biol Chem 2004, 279:626-634.
24. Nakashima K, Zhou X, Kunkel G, Zhang Z, Deng JM, Behringer RR, de Crombrugghe B: The novel zinc finger-containing transcription factor osterix is required for osteoblast differentiation and bone formation. Cell 2002, 108:17-29.
25. Marín O, Rubenstein JL: A long, remarkable journey: tangential migration in the telencephalon. Nat Rev Neurosci 2001, 2:780-790.
26. Waclaw RR, Allen ZJ 2nd, Bell SM, Erdelyi F, Szabo G, Potter SS, Campbell K: The zinc finger transcription factor Sp8 regulates the generation and diversity of olfactory bulb interneurons. Neuron 2006, 49:503-516.
27. Storm EE, Garel S, Borello U, Hebert JM, Martinez S, McConnell SK, Martin GR, Rubenstein JL: Dose-dependent functions of Fgfr8 in regulating telencephalic patterning centers. Development 2006, 133:1831-1844.
28. Schafer G, Cranker T, Suske G, Kemmer W, Wiedenmann B, Hocker M: Oxidative stress regulates vascular endothelial growth factor-A gene transcription through Sp1- and Sp3-dependent activation of two proximal GC-rich promoter elements. J Biol Chem 2003, 278:8190-8198.
29. Kawakami Y, Rodriguez-Leon J, Koth CM, Buscher D, Isot T, Raya A, Ng JK, Esteban CR, Takahashi S, Henrique D, Schwarz MF, Asahara H, Izpisua Belmonte JC: MKP3 mediates the cellular response to FGFR signalling in the vertebrate limb. Nat Cell Biol 2003, 5:513-519.
30. Shimogori T, Banuchi V, Ng HY, Strauss JB, Groves EA: Embryonic signaling centers expressing BMP, WNT and FGF proteins interact to pattern the cerebral cortex. Development 2004, 131:5639-5647.
31. Raible F, Brand M: Tight transcriptional control of the ETS domain factors Erm and Pea3 by Fgf signaling during early zebrafish development. Mech Dev 2001, 107:105-117.
32. Roehl H, Nusslein-Volhard C: Zebrafish pea3 and erm are general targets of FGF signaling. Curr Biol 2001, 11:503-507.
33. Hamasaki T, Leingartner A, Ringstedt T, O’Leary DD: **EMX2 regulates sizes and positioning of the primary sensory and motor areas in neocortex by direct specification of cortical progenitors.** *Neuron* 2004, 43:359-372.

34. Nakagawa Y, Johnson JE, O’Leary DD: **Graded and areal expression patterns of regulatory genes and cadherins in embryonic neocortex independent of thalamocortical input.** *J Neurosci* 1999, 19:10877-10885.

35. Miyashita-Lin EM, Heyner R, Wassarman KM, Martinez S, Rubenstein JL: **Early neocortical regionalization in the absence of thalamic innervation.** *Science* 1999, 285:906-909.

36. Weidinger G, Thorpe CJ, Wuennenberg-Stapleton K, Ngai J, Moon RT: **The Sp1-related transcription factors sp5 and sp5-like act downstream of Wnt/beta-catenin signaling in mesoderm and neuroectoderm patterning.** *Curr Biol* 2005, 15:489-500.

37. Schock F, Reischl J, Wimmer E, Taubert H, Purnell BA, Jackle H: **Phenotypic suppression of empty spiracles is prevented by buttonhead.** *Nature* 2000, 405:351-354.

38. Storm EE, Rubenstein JL, Martin GR: **Dosage of Fgf8 determines whether cell survival is positively or negatively regulated in the developing forebrain.** *Proc Natl Acad Sci U S A* 2003, 100:1757-1762.

39. Tsukui T, Capdevila J, Tamura K, Ruiz-Lozano P, Rodriguez-Esteban C, Yonei-Tamura S, Magallon J, Chandraratna RA, Chien K, Blumberg B, Evans RM, Belmonte JC: **Multiple left-right asymmetry defects in Shh(-/-) mutant mice unveil a convergence of the shh and retinoic acid pathways in the control of Lefty-1.** *Proc Natl Acad Sci U S A* 1999, 96:11376-11381.

40. Saito T, Nakatsuji N: **Efficient gene transfer into the embryonic mouse brain using in vivo electroporation.** *Dev Biol* 2001, 240:237-246.

41. Suzuki A, Raya A, Kawakami Y, Morita M, Matsui T, Nakashima K, Gage FH, Rodriguez-Esteban C, Izpisua Belmonte JC: **Nanog binds to Smad1 and blocks bone morphogenetic protein-induced differentiation of embryonic stem cells.** *Proc Natl Acad Sci U S A* 2006, 103:10294-10299.

42. Fan W, Jin S, Tong T, Zhao H, Fan F, Antinore MJ, Rajasekaran B, Wu M, Zhan Q: **BRCA1 regulates GADD45 through its interactions with the OCT-1 and CAAT motifs.** *J Biol Chem* 2002, 277:8061-8067.

43. Lopez-Rovira T, Chalaux E, Massague J, Rosa JL, Ventura F: **Direct binding of Smad1 and Smad4 to two distinct motifs mediates bone morphogenetic protein-specific transcriptional activation of Id1 gene.** *J Biol Chem* 2002, 277:3176-3185.