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RESEARCH ARTICLE

Spalt-like 4 promotes posterior neural fates via repression of *pou5f3* family members in *Xenopus*

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ABSTRACT

Amphibian neural development occurs as a two-step process: (1) induction specifies a neural fate in undifferentiated ectoderm; and (2) transformation induces posterior spinal cord and hindbrain. Signaling through the Fgf, retinoic acid (RA) and Wnt/β-catenin pathways is necessary and sufficient to induce posterior fates in the neural plate, yet a mechanistic understanding of the process is lacking. Here, we screened for factors enriched in posterior neural tissue and identify *spalt-like 4* (*sall4*), which is induced by Fgf. Knockdown of Sall4 results in loss of spinal cord marker expression and increased expression of *pou5f3.2* (*oct25*), *pou5f3.3* (*oct60*) and *pou5f3.1* (*oct91*) (collectively, *pou5f3* genes), the closest *Xenopus* homologs of mammalian stem cell factor *Pou5f1* (*Oct4*). Overexpression of the *pou5f3* genes results in the loss of spinal cord identity and knockdown of *pou5f3* function restores spinal cord marker expression in Sall4 morphants. Finally, knockdown of Sall4 blocks the posteriorizing effects of Fgf and RA signaling in the neurectoderm. These results suggest that Sall4, activated by posteriorizing signals, represses the *pou5f3* genes to provide a permissive environment allowing for additional Wnt/Fgf/RA signals to posteriorize the neural plate.

KEY WORDS: Oct4, Sall4, *Xenopus*, Gene regulation, Neural patterning

INTRODUCTION

Nieuwkoop and Eyal-Giladi suggested that development of the amphibian central nervous system arises by 'activation and transformation' (Nieuwkoop, 1952; Nieuwkoop et al., 1952a, 1952b; Eyal-Giladi, 1954) whereby neural tissue is induced as an anterior state by the organizer and then posteriorized by additional signals from the mesoderm to specify the anterior-posterior (A-P) pattern of the neural plate. Activation, or neural induction, requires bone morphogenetic protein (Bmp) antagonists, such as Noggin (Lamb et al., 1993), Chordin (Sasai et al., 1994) and Follistatin (Hemmati-Brivanlou et al., 1994), from the organizer to induce the neural fate (Khokha et al., 2005). Indeed, any manipulation that blocks Bmp signaling in ectoderm results in anterior neural fates (Hemmati-Brivanlou and Melton, 1994). Caudalization, or transformation, occurs via signaling by retinoic acid (RA) (Durston et al., 1989; Sive et al., 1990; Ruiz i Altaba and Jessell, 1991; Blumberg et al., 1997; Kolm et al., 1997), Fgf (Cox and Hemmati-Brivanlou, 1995; Kengaku and Okamoto, 1995; Lamb and Harland, 1995; Ribisi et al., 2000; Fletcher et al., 2006) and Wnt/β-catenin (McGrew et al., 1995; Itoh and Sokol, 1997; Domingos et al., 2001; Erter et al., 2001; Kiecker and Niehrs, 2001). Despite the identification of these secreted factors as mediators of A-P neural patterning, the mechanism by which transduction of these signals results in the adoption of posterior fates remains poorly understood.

Given the interest in axial patterning, a few transcription factors that mediate A-P differentiation have been identified. The homeobox gene *gbx2* is a direct target of canonical Wnt signaling and primarily serves to localize the isthmus and induce neural crest (Simeone, 2000; Li et al., 2009). The *meis3* gene, which is required for hindbrain and neuronal differentiation, is directly activated by Wnt3a from the dorsal lateral marginal zone (Elkouby et al., 2010, 2012). The caudal homologs *Cdx1* and *Cdx4* are direct Wnt targets in the mouse (Prinos et al., 2001; Pilon et al., 2006, 2007) and have overlapping roles in posterior development of the three germ layers (Isaacs et al., 1998; Faas and Isaacs, 2009; van de Ven et al., 2011). In the neural plate of *Xenopus*, simultaneous knockdown of Cdx1, Cdx2 and Cdx4 is required to block adoption of the most posterior neural fates (Faas and Isaacs, 2009).

The Spalt-like (Sall) proteins are vertebrate homologs of the *Drosophila* protein Spalt. The four members of the Sall family of zinc-finger transcription factors in vertebrates contain an N-terminal C2HC zinc-finger domain followed by variable numbers of doublet and triplet C2H2 zinc-finger domains (Sweetman and Münsterberg, 2006; de Celis and Barrio, 2009). Sall1 and Sall4 function as either transcriptional repressors (Lauberth and Rauchman, 2006; Lauberth et al., 2007; Lu et al., 2009; Yang et al., 2012) or activators (Kiefer et al., 2002; Zhang et al., 2006; Yang et al., 2007; Lim et al., 2008). Mutations in human *SALL1* and *SALL4* cause the autosomal dominant Townes-Brocks and Okihiro syndromes, respectively, both characterized by limb and cognitive defects (Kohlhase et al., 1998, 2002). *Sall4* knockout mice fail to maintain a pluripotent inner cell mass (Sakaki-Yumoto et al., 2006); null embryos lack *Pou5f1* (*Oct4*) expression in the ICM, increase *Cdx2* expression and replace epiblast with trophectoderm (Wu et al., 2006; Zhang et al., 2006). Furthermore, knockdown of *Sall4* inhibits induction of induced pluripotent stem cells (iPSCs) (Tsubooka et al., 2009).

Previously, *sall2*, *sall3* and *sall4* were shown to be expressed during early *Xenopus* embryogenesis (Hollemann et al., 1996; Onuma et al., 1999; Onai et al., 2004) and, with the exception of *sall2* (Onai et al., 2004), were expressed in posterior neural regions. Conditional knockouts of *Sall1*, *Sall2* and *Sall4* result in mouse embryos with neural tube closure defects (Böhm et al., 2008), revealing a role for these genes in neural differentiation or morphogenesis. Despite their expression in posterior neural regions of vertebrate embryos, a role for the Sall genes in caudalization has not been elucidated.

Here we describe the results of an expression screen designed to discover targets of canonical Wnt signaling that determine neural posteriorization in *Xenopus*. This screen identified *sall1* and *sall4* as targets in neuralized tissue. We show that *sall4* is required for

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caudalization and, importantly, spinal cord differentiation of neural tissue. Finally, we show that *sall4* represses the stem cell factor *pou5f3* to release cells from an undifferentiated state.

RESULTS

Screen to identify posterior neural patterning genes

We used the inducible β-catenin analog TVGR (TCF/LEF DNA-binding domain fused to both the VP-16 transactivation domain and growth hormone receptor) to mimic a posteriorizing Wnt signal (Darken and Wilson, 2001). Having confirmed that this treatment effectively activates Wnt signaling in response to DEX using ventral vegetal injections (supplementary material Fig. S1A), we tested the activity of TVGR in posteriorizing neural tissue using ectodermal explants treated at later stages. Animal caps overexpressing Noggin expressed the anterior neural marker *otx2* but not *epidermal keratin*, demonstrating that the explants had adopted a neural fate. By contrast, neuralized caps, which were injected with TVGR and induced with DEX, expressed the posterior markers *krox20* and *hoxb9*. Ethanol vehicle did activate the hindbrain marker *krox20*, but DEX was required to induce spinal cord fates as assayed by *hoxb9* expression (supplementary material Fig. S1B). Consistent with these results, activation of TVGR in neuralized animal caps induced convergent extension-like morphogenesis consistent with differentiation into spinal cord (Elul et al., 1997) (supplementary material Fig. S1C).

Next, we validated the use of activated TVGR in neuralized animal caps to enrich for transcriptional targets of Wnt signaling (supplementary material Fig. S2A). Treatment with the translational inhibitor cycloheximide (CHX) did not prevent activation of the direct target *meis3* (Elkouby et al., 2010) but did block the indirect target *hoxb9* (Domingos et al., 2001) (supplementary material Fig. S2B). Thus, these conditions induce neural tissue and posteriorize it via Wnt activation.

To screen for posterior neural genes, we harvested total RNA from animal caps treated with *noggin* alone (anterior neural), neuralized caps with activated TVGR (posteriorized neural), and neuralized animal caps treated with or without CHX prior to TVGR activation (enriched target sample). The RNAs from these samples were used for Illumina sequencing. The resulting reads were mapped to a collection of non-redundant full-length *Xenopus laevis* cDNA sequences (Xenopus Gene Collection, http://xgc.nci.nih.gov). By comparing read quantities between anterior neural tissue and tissue treated to enrich for direct targets, we found over 200 genes with expression that was increased greater than 2-fold (supplementary material Table S2). Importantly, the set included the direct targets *meis3* (Elkouby et al., 2010) and *cdx2* (Wang and Shashikant, 2007). To determine whether the expression of these genes was consistent with a posteriorizing Wnt signal, we stained *Xenopus tropicalis* embryos by *in situ* hybridization to identify candidates expressed in the posterior neurectoderm (Fig. 1A). Several candidates were expressed in dorsal tissues of gastrula embryos and posteriorly in early and mid-neurula stage embryos, consistent with the expression domains of known Wnt targets. Of particular note, the transcription factors *spalt-like 1* (*sall1*) and *sall4* showed strong expression in posterior neurectoderm (Fig. 1A).

We confirmed the results of our screen by qPCR (Fig. 1B). Incubation with CHX prior to activation of TVGR in neuralized caps resulted in increased *cdx2* (supplementary material Fig. S3A), *sall1* (supplementary material Fig. S4A) and *sall4* (Fig. 1B) expression. Incubation with CHX alone did result in an increase in *sall4* expression, but this was not statistically different from caps treated with *noggin* alone. However, injection of *fgf8a* RNA [a posteriorizing spliceform of *fgf8* (Fletcher et al., 2006)] was sufficient to significantly induce *sall4* expression in neuralized animal caps (Fig. 1C).

The activation of *sall4* by TVGR in the presence of CHX prompted us to examine whether β-catenin binds to the genomic locus of *sall4*. We overexpressed a C-terminal FLAG-tagged version of *X. laevis* β-catenin and confirmed expression by immunoblotting (supplementary material Fig. S5A). Co-injection of FLAG-tagged β-*catenin* RNA restored dorsal structures in embryos injected with β-catenin morpholinos (MOs) (Heasman et al., 2000), demonstrating both the specificity and activity of this construct (supplementary material Fig. S2B). Consistent with a previous report (Yost et al., 1996), injection of 500 pg RNA encoding tagged β-catenin did not significantly alter dorsal structures as measured by the dorsoanterior index (Kao and Elinson, 1985) (supplementary material Fig. S2C). The *sall4* locus in *X. laevis* contains four exons and three introns (supplementary material Fig. S2D), with six putative TCF/LEF binding sites (Elkouby et al., 2010; McKendry et al., 1997) within the first intron (supplementary material Fig. S6). Three of these sites are tightly clustered within a 150 bp span at positions +2347, +2387 and +2456 (relative to the predicted transcription start site) and are conserved in *X. tropicalis*. Using FLAG antibodies for ChIP, this region was found to be significantly enriched compared with a negative control (*Xmlc2*) region (supplementary material Fig. S2E). A −2.7 kb region upstream of *meis3* was used as a positive control for β-catenin binding (Elkouby et al., 2010). Anti-FLAG pulldowns in uninjected control embryos resulted in negligible enrichment of any loci assayed. A 500 bp fragment containing these three TCF/LCF sites was cloned and used in luciferase reporter assays. This fragment was not sufficient to enhance expression upon Wnt activation alone (supplementary material Fig. S2F) but was found to be significantly responsive to Fgf (Fig. 1D). Additional experiments demonstrated that Fgf and Wnt did not result in synergistic activation of this fragment.

Taken together, these results suggest that *sall4* is likely to be primarily regulated by the posterior Fgf signal and that Wnt signaling may play a minor or negligible role in its regulation.

sall4 and *sall1* expression in *X. laevis*

During gastrulation, *sall4* is expressed throughout the marginal zone and the animal pole (Fig. 2A). At stage 10, *sall4* is restricted to the sensorial neurectodermal cells in animal dorsal regions (Fig. 2E). At the onset of neurulation, *sall4* continues to be expressed in the sensorial neurectoderm (Fig. 2B,F,G). Neural expression of *sall4* in stage 15 (mid-neurula) embryos is in the hindbrain and spinal cord anlage (Fig. 2C,H,I). In later stage neurulae (stage 18), *sall4* spreads through the posterior neural tube, hindbrain, developing placodes and epidermis (Fig. 2D,J,K).

Similarly, *sall1* is expressed in the dorsal ectoderm and involuting mesoderm during gastrulation (supplementary material Fig. S4B,B′). Expression becomes restricted to the notochord and circumblastoporal collar at the early neurula stage (supplementary material Fig. S4C-C″). Like *sall4*, *sall1* is expressed in the spinal cord anlage at mid- and late neurula stages (supplementary material Fig. S4D-E″).

Sall4 is required for posterior neural differentiation but not for induction or maintenance of neural identity

Given its neural expression, we hypothesized that loss of Sall4 would affect neural patterning. To test this, we knocked down Sall4 function with MOs. Morphant embryos had neural tube

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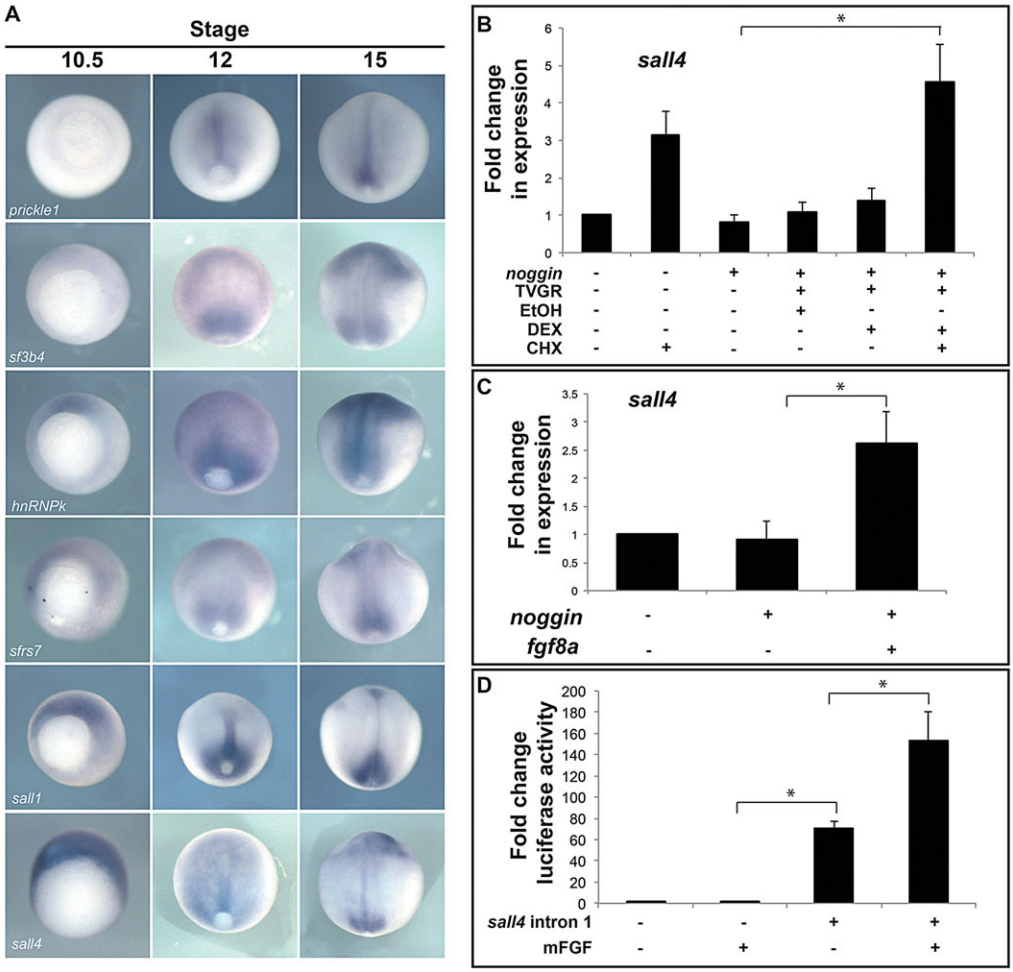

Fig. 1. Expression screen for direct transcriptional targets of Wnt signaling in neural tissue. (A) *X. tropicalis* embryos stained for transcripts identified as Wnt targets by RNA-seq. Stage 10.5 embryos show dorsovegetal views with the dorsal lip toward the top. Stage 12 and 15 embryos show dorsal views with anterior toward the top. (B,C) qPCR on 15-25 animal caps treated as indicated on the *x*-axis. The *y*-axis shows expression relative to *odc*. *n*=4 experiments. (D) Luciferase reporter assays in HEK293 cells treated with or without mouse Fgf. *n*=3 experiments. Error bars indicate s.e.m. All means were compared by one-way ANOVA followed by Tukey post-hoc analyses (**P*<0.05).

closure defects and began to disintegrate at mid-tailbud stages. The closure defect is consistent with defects in neural patterning, so we assayed several markers of neural differentiation. The pan-neural marker *sox2* was expressed in the neural plate in uninjected and Sall4 morphants, demonstrating that the dorsal ectoderm of morphants still retained a neural identity (Fig. 3A,B). Conversely, the expression of *n-tub*, a marker for differentiating neurons, was markedly reduced although still present in the morphants, suggesting that Sall4 is required for the second wave of neurogenesis in the tailbud tadpole (Fig. 3C,D). Another marker for early motor neuron differentiation, *nkx6.1*, was expressed in the central nervous system of morphants, and neural crest cells were still induced as determined by the expression of *snai2* (Fig. 3E,F). Although present, these markers were expressed in a pattern more similar to that of early neurulae, suggesting either a delay or failure of terminal differentiation. Sall4 morphants expressed the dorsal

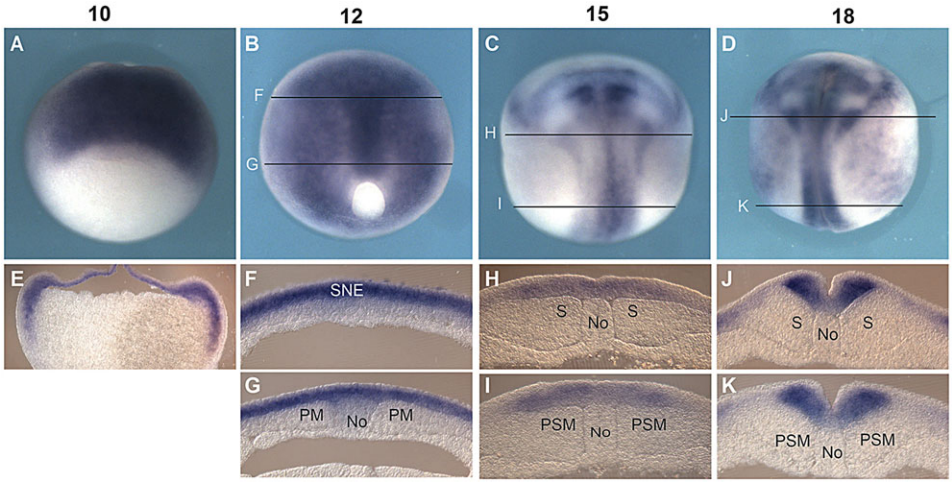

Fig. 2. *sall4* is expressed in the neurectoderm. (A) Stage 10 embryo stained for *sall4* RNA; dorsovegetal view with the dorsal lip of the blastopore toward the top. (B-D) Dorsal views of neurula stage embryos with anterior toward the top. (E) Sagittal section of stage 10 embryo stained for *sall4* expression; animal pole is to the top and dorsal to the right. (F-K) Transverse sections at anterior (F,H,J) or posterior (G,I,K) of embryos stained for *sall4* as indicated in B-D. (E-K) 50 μm sections, with (F-K) dorsal uppermost. SNE, sensorial neurectoderm; No, notochord; S, somite; PM, paraxial mesoderm; PSM, presomitic mesoderm.

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mesoderm marker *myoD* in a similar pattern to uninjected control embryos, and therefore the neural defects were not secondarily due to a loss of paraxial mesoderm (Fig. 3G,H).

As *sall4* was identified in a screen for posterior neural genes, we predicted that Sall4 morphants would lose posterior neural identity. To test for this, we injected Sall4 MOs into one animal dorsal (A/D) cell of 4-cell stage embryos to allow for comparison between injected and uninjected sides. The injected side of embryos showed a posterior shift in expression of the hindbrain markers *gbx2* (Fig. 4A,B), *mafb* (Fig. 4D,E) and *pax2* (Fig. 4J,K). Sall4 loss resulted in loss of *meis3* rhombomere expression and a reduction of its spinal cord expression domain (Fig. 4J,K). Surprisingly, overexpression of *sall4* did not result in a change or shift in any of these markers (Fig. 4C,F,I,L), nor was it sufficient to rescue defects associated with Dkk1 overexpression (supplementary material Fig. S7).

The posterior shift of brain markers observed in Sall4 morphants suggested that knockdown of Sall4 results in an expansion of anterior neural identity at the expense of posterior neural differentiation. Accordingly, *otx2* is expanded and *krox20* is significantly shifted relative to the control side (Fig. 5A-C). Strikingly, the injected side had a significant reduction in the expression domain of the spinal cord markers *hoxb9* (Fig. 5D-F), *hoxc10* (Fig. 5G-I) and *hoxd10* (Fig. 5J-L). However, Sall4 knockdown does not reduce expression of the Wnt target *cdx2* to the same extent (supplementary material Fig. S3B,C).

Loss of Sall4 in the neural plate increases expression of the *Pou5f1* homologs *pou5f3.1*, *pou5f3.2* and *pou5f3.3*

The failure of Sall4 morphants to induce posterior neural identity suggested that the caudal tissue remained in an undifferentiated state. In mouse embryos, Sall4 positively regulates the stem cell factor *Pou5f1* (*Oct4*) to maintain pluripotency (Zhang et al., 2006). One explanation for our results is that Sall4 negatively regulates the *Pou5f1* homologs in neural tissue. In *Xenopus*, there are three class 5 Pou-domain genes that show similar sequence and ancient synteny to mammalian *Pou5f1* (Morrison and Brickman, 2006). However, eutherian mammals and frogs retain different copies of the locus from the last tetrapod whole-genome duplication, and their Pou5 genes are not the simple orthologs of *Pou5f1*. Here, we use the term *pou5f3* (as used by Xenbase.org, zfin.org) (Morrison and Brickman, 2006; Frankenberg et al., 2010).

If Sall4 negatively regulates *pou5f3*, then morphants should increase their expression. Indeed, knockdown of Sall4 in unilateral and bilateral injections resulted in ectopic expression of *pou5f3.2* (*oct25*) (Fig. 6A-C), *pou5f3.3* (*oct60*) (Fig. 6D-F)

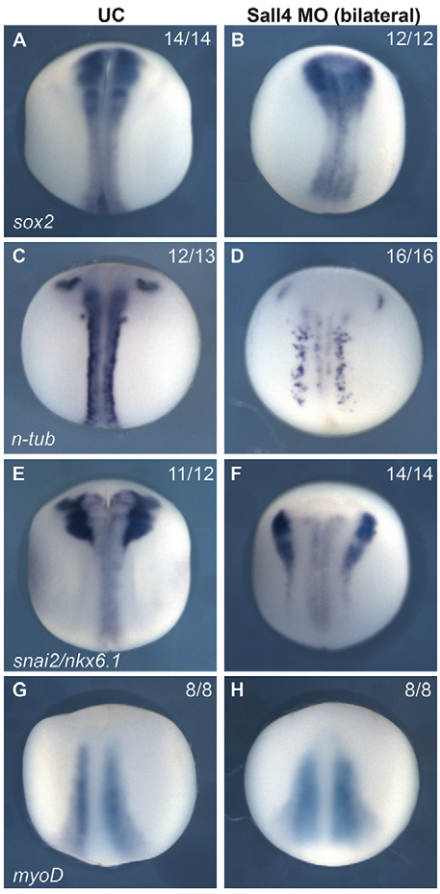

Fig. 3. Loss of Sall4 results in a loss of neural differentiation. Whole-mount *in situ* hybridizations of (A,C,E,G) uninjected control (UC) embryos and (B,D,F,H) embryos injected bilaterally with 40 ng Sall4 MO (20 ng/blastomere at the 2-cell stage), showing expression of *sox2* (A,B), *n-tub* (C,D), *snai2* and *nkx6.1* (E,F) and *myoD* (G,H). Dorsal views with anterior to the top. The number of embryos showing the illustrated expression pattern among the total examined is indicated top right.

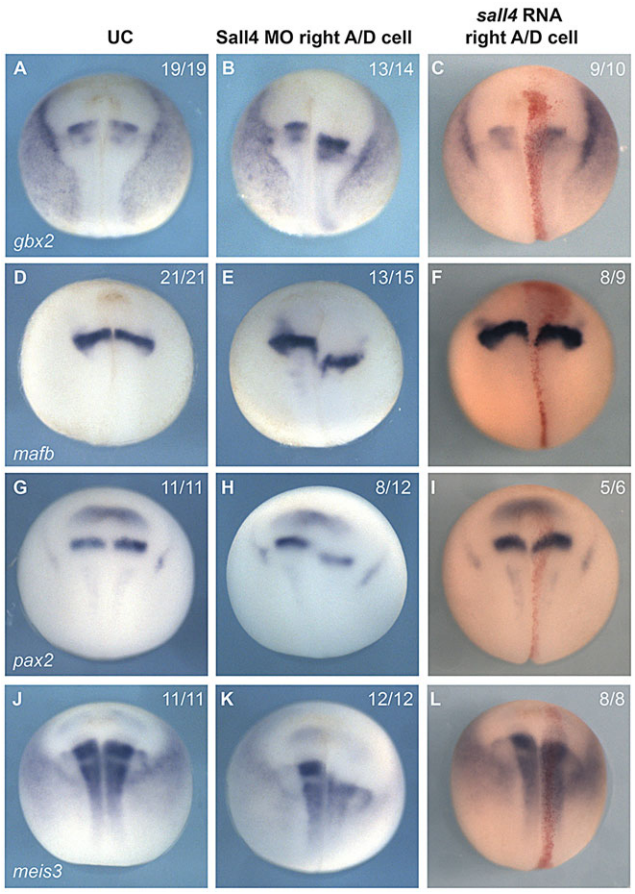

Fig. 4. Expression of hindbrain markers is altered in Sall4 morphants. Whole-mount *in situ* hybridization of (A,D,G,J) uninjected control embryos, (B,E,H,K) embryos injected with 20 ng Sall4 MO into the right animal-dorsal (A/D) blastomere, and (C,F,I,L) embryos injected with 250 pg *sall4* RNA into the right A/D blastomere, showing expression of *gbx2* (A-C), *mafb* (D-F), *pax2* (G-I) and *meis3* (J-L). Dorsal views with anterior to the top.

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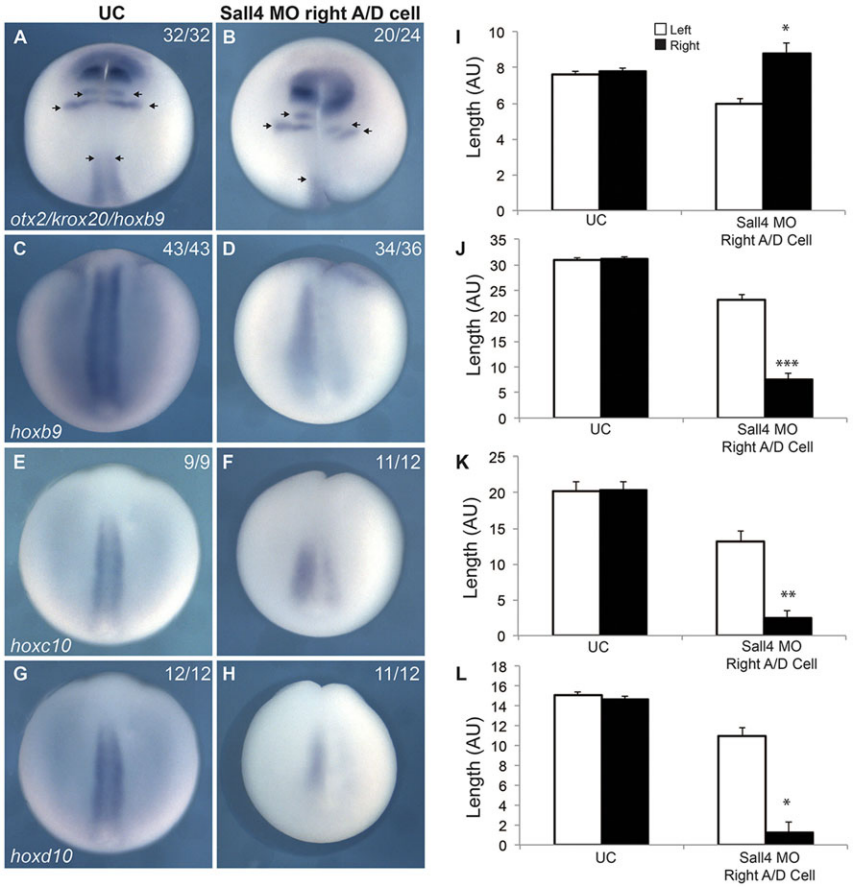

Fig. 5. Sall4 knockdown results in a loss of spinal cord differentiation. (A-H) Whole-mount *in situ* hybridization of (A,C,E,G) uninjected control embryos and (B,D,F,G) embryos injected with 20 ng Sall4 MO into the right A/D blastomere. (A,B) Expression of *otx2*, *krox20* and *hoxb9*. Arrows indicate the relative anterior-posterior (A-P) position of *krox20* and the anterior limit of *hoxb9*. Dorsal views with anterior to the top. (C-H) Posterior views of *hoxb9* (C,D), *hoxc10* (E,F) and *hoxd10* (G,H) expression. (I-L) Quantification of A-P patterning defects associated with Sall4 knockdown. (I) Distance between the anteriormost expression of *otx2* and the first *krox20* stripe in arbitrary units (AU). (J-L) Length of the *hoxb9* (J), *hoxc10* (K) and *hoxd10* (L) expression domains (AU). Error bars indicate s.e.m. Means were compared between left and right sides by Student's *t*-test (**P*<0.05, ***P*<0.01, ****P*<0.001). Data were generated from analyzing all embryos shown in A-H.

and *pou5f3.1* (*oct91*) (Fig. 6G-I). Accordingly, the increase in expression of *pou5f3.2* and *pou5f3.1* was greatest in the neural tube, where *sall4* is normally expressed. *pou5f3* expression in Sall4 morphants relative to control embryos was quantified by qPCR and displayed a significant increase in all three *pou5f3* genes (Fig. 6J-L). Co-injection of *X. tropicalis sall4* RNA that is not targeted by the Sall4 MO resulted in a partial rescue of the *pou5f3.2* expression level and a full rescue of the *pou5f3.3* and *pou5f3.1* expression levels.

Next, we asked whether ectopic *pou5f3* expression is sufficient to block posterior neural differentiation by injecting RNA for the three *pou5f3* genes unilaterally into embryos and assaying A-P neural gene expression. Neural plate cells expressing ectopic *pou5f3* (as traced by β-galactosidase) had altered *otx2* expression and failed to express *krox20* (Fig. 7A,B), *hoxb9* (Fig. 7C,D) and *hoxc10* (Fig. 7E,F). This loss in A-P neural marker expression cannot be attributed to a loss of neural identity as the *pou5f3*-injected side of embryos broadly expresses *sox2* (Fig. 7G,H).

The loss of spinal cord identity in Sall4 morphants is attributable to the overexpression of *pou5f3*

The observed *pou5f3* increase following knockdown of Sall4 suggested a mechanism for the loss of posterior neural identity whereby the ectopic *pou5f3* expression prevents differentiation of neural tissue into spinal cord. We reasoned that knocking down *pou5f3* in Sall4 morphants would restore posterior neural identity. To this end, we co-injected MOs targeting the three *pou5f3* homologs (Morrison and Brickman, 2006; Livigni et al., 2013) along with Sall4 MOs. Consistent with the results described above, knockdown of Sall4 resulted in loss of posterior *hoxb9* (Fig. 8A,E), *hoxc10* (Fig. 8B,F) and *hoxd10* (Fig. 8C,G) but not in a loss of pan-neural *sox2* (Fig. 8D,H). Co-injection of the Pou5f3 MOs with Sall4 MOs restored the spinal cord marker expression lost by Sall4 knockdown alone (Fig. 8I-K). Knockdown of Pou5f3 in Sall4 morphants did not restore *krox20* stripe expression, consistent with previous work showing that Pou5f3 MOs inhibit *krox20* expression (Morrison and Brickman, 2006). Although reduced, *sox2* was expressed in the neural plate of Pou5f3 morphants (Fig. 8N) and Sall4-Pou5f3 double morphants (Fig. 8L). Finally, measuring the Hox gene expression domains of Sall4 and Sall4-Pou5f3 morphants revealed a significant rescue of all three spinal cord markers (Fig. 8M).

Sall4 is required for neural posteriorization by the caudalizing factors Fgf and RA

Our results demonstrate that posteriorizing factors induce Sall4 expression, which represses *pou5f3*, thereby allowing posterior neural differentiation. Fgf and RA signaling also posteriorize the neural plate. Therefore, we tested whether repression of *pou5f3* via Sall4 is required for both Fgf- and RA-induced caudalization. We treated embryos with either *fgf8a* RNA or incubation in RA. Again, Sall4 knockdown resulted in loss of *hoxb9* (Fig. 9A,B) without major alterations to *sox2* (Fig. 9E,F). Overexpression of *fgf8a* in the dorsal ectoderm resulted in expansion of *sox2* and *hoxb9*, a lateral expansion of *krox20*, and repression of *otx2* (Fig. 9C) (Fletcher et al., 2006). These expansions are due to the long-range effects of overexpressing the secreted Fgf ligand. However, overexpressing *fgf8a* in Sall4 morphants still resulted in *otx2* (brain) repression, but *hoxb9* (spinal cord) was lost (Fig. 9D). *krox20* expression in rhombomere 5 was severely reduced in the Sall4 morphants despite *fgf8a* overexpression,

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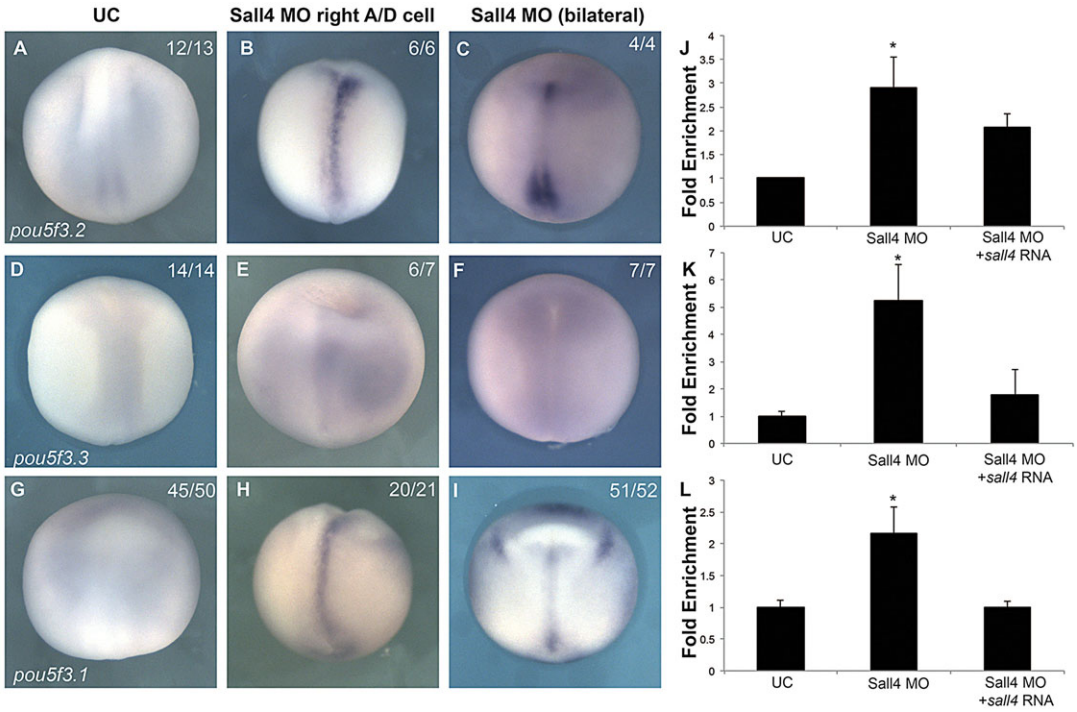

Fig. 6. Knockdown of Sall4 causes an increase in *pou5f3* expression. (A-I) Whole-mount *in situ* hybridization of (A,D,G) uninjected control embryos, (B,E,H) embryos injected with 20 ng Sall4 MO into the right A/D blastomere, and (C,F,I) embryos injected bilaterally with 40 ng Sall4 MO (20 ng/blastomere at the 2-cell stage), showing expression of (A-C) *pou5f3.2*, (D-F) *pou5f3.3* and (G-I) *pou5f3.1*. Dorsal views, anterior to the top. (J-L) qPCR for *pou5f3.2* (J), *pou5f3.3* (K) or *pou5f3.1* (L) in uninjected embryos, embryos injected with 40 ng Sall4 MO (20 ng/blastomere at the 2-cell stage), and embryos injected with 40 ng Sall4 MO (20 ng/blastomere at the 2-cell stage) plus 500 pg *X. tropicalis sall4* RNA (250 pg/animal-dorsal blastomere at the 4-cell stage). The expression is relative to *odc*. Error bars indicate s.e.m. Means compared with uninjected control by one-way ANOVA followed by Tukey post-hoc analyses (*P<0.05). n=4 experiments.

whereas rhombomere 3 expression remained expanded, probably owing to the specific posteriorizing effects of Sall4. Morphants typically had a posterior shift and reduction in rhombomere 5 *krox20* expression, whereas expression in rhombomere 3 was shifted but not reduced (Fig. 5B).

Increasing RA signaling results in severe loss of anterior neural tissue and expansion of posterior identities (Durston et al., 1989; Sive et al., 1990; Ruiz i Altaba and Jessell, 1991; Blumberg et al., 1997; Shiotsugu et al., 2004). To test whether Sall4 is required for posteriorization via RA, we treated control embryos and Sall4 morphants with all-trans retinoic acid (ATRA). Uninjected control embryos treated with 1 µM ATRA lacked *otx2* and *krox20* but sustained *hoxb9* expression (Fig. 9I). However, 1 µM ATRA treatment of Sall4 morphant embryos repressed *otx2* and *krox20* but also failed to induce the caudally expressed marker *hoxb9* (Fig. 9J). The reduction of these markers was not due to a loss of neural tissue as *sox2* expression was similar between control embryos, embryos treated with ATRA, and Sall4 morphant embryos treated with ATRA (Fig. 9E,K,L).

DISCUSSION

Wnt, Fgf and RA signaling are caudalizing factors required for posteriorization of the neural plate. However, the transcription factors identified as mediating the patterning signals from these pathways have largely been restricted to those specifying midbrain and hindbrain fates. In this study, we identify *sall4* as a posteriorizing factor target required for spinal cord differentiation. The primary role of Sall4 in neural patterning is to repress *pou5f3* (*oct4*). This repression is necessary for spinal cord differentiation; Sall4 knockdown (Fig. 5E,H,K), as well as *pou5f3* overexpression (Fig. 7D,F), results in loss of spinal cord fate. Furthermore, the posterior defects in Sall4 morphants can be rescued via *pou5f3* knockdown (Fig. 8I-K). We suggest that repression of *pou5f3* via Sall4 provides a permissive environment allowing cells in the neural plate to respond to instructive signals from Fgf, RA and Wnt. This model fits with the observation that overexpression of *sall4* did not result in a perturbation of A-P hindbrain marker expression (Fig. 4). If the main role of Sall4 in neural patterning is to repress *pou5f3*, then overexpression is unlikely to have a significant effect on otherwise normal embryos. Further, this model predicts that Sall4 would not rescue a Wnt loss-of-function phenotype since it is functioning as a permissive and not as an instructive signal. Another prediction is that Sall4 is required for adoption of posterior fates by multiple posteriorizing signals. Therefore, an increase in *pou5f3* expression after Sall4 knockdown would inhibit differentiation induced by other caudalizing factors. Indeed, we found Sall4 knockdown prevented induction of *hoxb9* by Fgf or RA (Fig. 9D,J).

Our findings build upon previously described mechanisms of posterior neural patterning. Wnt activates *cdx1* (Prinos et al., 2001; Pilon et al., 2007) and, in frogs, Cdx1 represses *pou5f3* gene expression at the onset of gastrulation (Rousso et al., 2011). However, knockdown of Cdx1 does not result in a loss of spinal cord differentiation, and combinatorial knockdown of Cdx1/2/4 is required before *hoxb9* and *hoxc10* are reduced (Faas and Isaacs, 2009). There is, however, a dramatic loss of *hoxb9*, *hoxc10* and *hoxd10* in Sall4 morphants. In the absence of Sall4, *pou5f3* expression remains high, resulting in neural cells being unable to commit to a posterior neural fate and differentiate into spinal cord. Several studies have shown that Cdx factors regulate posterior Hox gene expression in vertebrates (Isaacs et al., 1998; van den

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Akker et al., 2002; Gaunt et al., 2004, 2008). Therefore, Wnt acts as an instructive signal through the activation of Cdx genes to induce posterior Hox genes and thereby transform the neural precursors into a posterior fate. Here, we find that *sall4* represses *pou5f3*, providing a parallel, permissive signal for posterior Hox gene expression. Wnt still signals in the posterior neural regions of Sall4 morphants, activating Cdx genes (supplementary material Fig. S3B,C), but the prolonged expression of *pou5f3* prevents Hox gene expression. Conversely, it is likely that *sall4* is still expressed in Cdx morphants, priming the neural plate to respond to other instructive signals. This could explain why knockdown of individual Cdx homologs results in unexpectedly mild phenotypes.

Posteriorizing signals regulate *sall4* expression

Our work found *sall4* to be activated by Fgf signaling in the neurectoderm. However, our finding that a 500 bp fragment in the first intron of *sall4* is enriched in β-catenin ChIP is consistent with it being a Wnt target (supplementary material Fig. S2E). However, this region does not mediate a Wnt-induced signal. Interestingly, we found that this region does show responsiveness to Fgf signaling. Taken together, these experiments show that Fgf is the primary posteriorizing signal that regulates *sall4* expression and that Wnt either plays a minor role or does not regulate *sall4* during early neural patterning.

The broad expression of *sall4* at early neurula stages (Fig. 2B) and later in limbs during *Xenopus* development and regeneration (Neff et al., 2011) suggests regulation through different enhancers, each responsible for discrete expression domains. This is the case with the neural expression of Sox2 in the chick, which is regulated by five different enhancers, each responsible for a portion of the full expression domain (Uchikawa et al., 2003). Fgf signaling is sufficient to posteriorize neurectoderm (Kengaku and Okamoto, 1995; Lamb and Harland, 1995; Christen and Slack, 1997; Fletcher et al., 2006), and we found that this activity requires Sall4. Therefore, it is possible that Fgf and Wnt signaling converge on other, as yet unidentified, enhancers to regulate *sall4* expression. Indeed, Fgf and Wnt signaling converge on one enhancer in the chick *sox2* gene to mediate the most posterior expression of *sox2* in the neural plate (Takemoto et al., 2006). Likewise, Wnt and Fgf response elements in the enhancers of *pax3* and *zic* genes cooperatively regulate their expression (Garnett et al., 2012), and both pathways mediate expression of these genes at the neural plate border (Monsoro-Burq et al., 2005).

A-P neural patterning requires downregulation of pluripotency factors

In amphibians, caudalization of the neural plate via Fgf and canonical Wnt signaling induces undifferentiated neural precursors to commit to posterior fates. This induction requires repression of stem cell factors and the activation of differentiation factors. *pou5f3* (*oct4*) genes are first expressed animally in cleavage stages and throughout the mesoderm and ectoderm of amphibian gastrulae (Frank and Harland, 1992; Morrison and Brickman, 2006). Knockdown of Pou91 (Pou5f3.1), Pou60 (Pou5f3.3) and Pou25 (Pou5f3.2) results in precocious cell fate commitment in the three germ layers (Morrison and Brickman, 2006; Snir et al., 2006). Accordingly, *pou5f3* overexpression prolongs the undifferentiated state (Morrison and Brickman, 2006; Archer et al., 2011). Our results suggest that *pou5f3* expression must be downregulated in the neurectoderm to allow for cells to respond to instructive Wnt/Fgf/RA signals and commit to posterior fates.

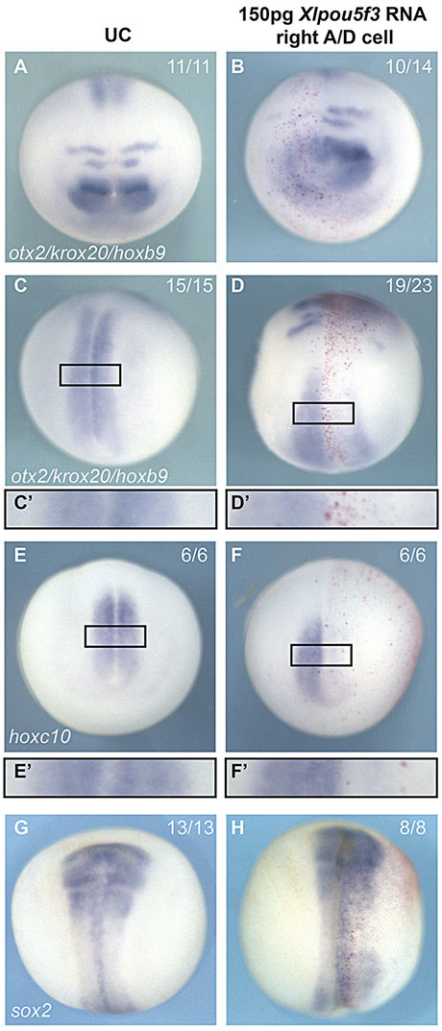

Fig. 7. Overexpression of *pou5f3* represses neural differentiation. Whole-mount *in situ* hybridization of (A,C,E,G) uninjected control embryos and (B,D,F,H) embryos injected with 150 pg *pou5f3* RNA (50 pg each of *pou5f3.2*, *pou5f3.3* and *pou5f3.1* RNAs) into the right A/D blastomere. Red staining is β-galactosidase used as a tracer for RNA injection. (C′-F′) Higher magnification views of the boxed regions in C-F. (A-D) *otx2*, *krox20* and *hoxb9* expression in anterior (A,B) or dorsal (C,D) view. (E-H) *hoxc10* (E,F) and *sox2* (G,H) expression in dorsal view.

Several studies have demonstrated the role for the *pou5f3* genes in maintaining pluripotency in *Xenopus*. In the early embryo, Oct25 (Pou5f3.2) and Oct60 (Pou5f3.3) were found to antagonize VegT and Wnt/β-catenin signaling to prevent precocious germ layer fates (Cao et al., 2007) and overexpression of Oct25 activates *Xvent-2B*, resulting in a failure of neurectoderm to differentiate (Cao et al., 2004). Further, the histone methyltransferase Suv4-20h has been demonstrated to directly repress *oct25* to allow for neural differentiation in *Xenopus* eye development (Nicetto et al., 2013). These studies all support a conserved role of *pou5f3* genes in pluripotency (Morrison and Brickman, 2006; Cao et al., 2007). Our results are consistent with the model; we find that the ectopic expression of *pou5f3* following knockdown of Sall4 results in the neurectoderm failing to differentiate in response to transforming signals.

Injection of *pou5f3* RNA results in more severe anterior defects than does Sall4 knockdown. This is likely to be due to higher levels of *pou5f3* expression following RNA injection (Fig. 7). Indeed, since the Pou5f3 family inhibits Fgf signaling (Cao et al., 2006; Snir

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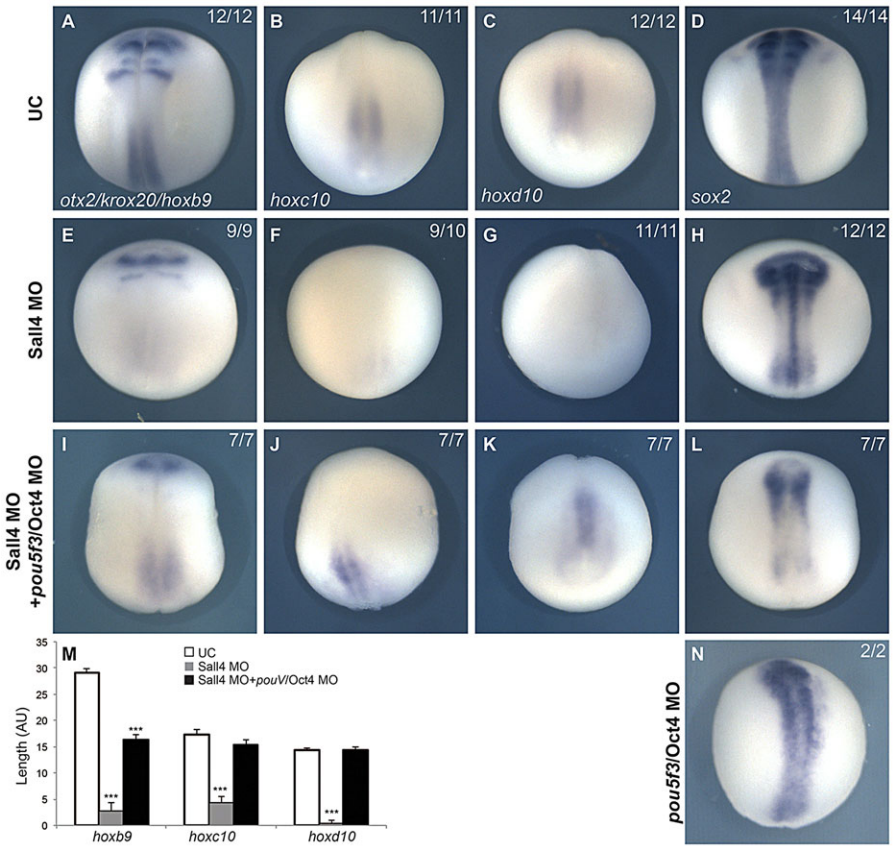

Fig. 8. Loss of spinal cord in Sall4 morphants is due to an increase in *pou5f3* expression. (A-L) Whole-mount *in situ* hybridization of (A-D) uninjected control embryos, (E-H) embryos injected with 40 ng Sall4 MO (20 ng/blastomere at the 2-cell stage), or (I-L) embryos injected with 40 ng Sall4 MO, 20 ng Pou5f3.2 MO, 10 ng Pou5f3.3a MO, 10 ng Pou5f3.3b MO, and 20 ng Pou5f3.1 MO. (N) Embryos injected with 20 ng Pou5f3.2 MO, 10 ng Pou5f3.3a MO, 10 ng Pou5f3.3b MO and 20 ng Pou5f3.1 MO. Expression is shown for (A,E,I) *otx2*, *krox20* and *hoxb9*, (B,F,J) *hoxc10*, (C,G,K) *hoxd10* and (D,H,L,N) *sox2*. Dorsal views with anterior to the top. (M) Quantification of posterior neural gene expression as measured by expression domain length in arbitrary units (AU). White, uninjected control embryos. Gray, embryos injected with 40 ng Sall4 MO (20 ng/blastomere at the 2-cell stage). Black, embryos injected with 40 ng Sall4 MO, 20 ng Pou5f3.2 MO, 10 ng Pou5f3.3a MO, 10 ng Pou5f3.3b MO and 20 ng Pou5f3.1 MO. Error bars indicate s.e.m. Means compared with uninjected control by one-way ANOVA followed by Tukey post-hoc analyses (***P<0.001). Data were generated from analyzing all embryos shown in A-C, E-G, and I-K.

et al., 2006), the disruption of *krox20* expression in the *pou5f3*-injected embryos is likely to be due to ectopic Pou5f3 inhibiting hindbrain patterning mediated by Fgf from the isthmus.

The class 5 Pou-domain factors play a conserved role in maintaining pluripotency in *Xenopus*. Here, we show that Sall4 mediates the transition between pluripotency maintenance and differentiation in the neural plate via repression of *pou5f3*. How Sall4 regulates *pou5f3*and whether this is a general role for Sall4 or specific to the neurectoderm remains to be elucidated.

MATERIALS AND METHODS

Embryo and explant culture

X. laevis embryos were obtained (Sive et al., 2010) and staged (Nieuwkoop and Faber, 1967) as described previously. Ectodermal explants (animal caps) were cut using fine watchmaker's forceps from stage 9 embryos and cultured in 0.75× NAM (Sive et al., 2010).

Cloning and DNA constructs

A cDNA clone of *X. tropicalis sall4* (CT025472) was identified in a full-length cDNA collection generated from gastrula embryos (Gilchrist et al., 2004). The coding sequence was subcloned into CS-108 (DQ649433.1) with *Sal*I and *Xho*I using primers (5′-3′): forward, CGATGTCGACGGACCATG-TCGAGGCGAAAGCAGCC; and reverse, ATCGATCCTCGAGTTA-cttatcgtcgtcatccttgtaatcGTTCACCGCAATATTTT. The coding sequence of *X. laevis* β-*catenin* was amplified with a FLAG epitope using: forward, GCATGAATTCCCACCATGGCAACTCAAGCAGATCT; and reverse, GCTAGCGGCCGCTTActtatcgtcgtcatccttgtaatcCAAGTCAGTGTCAAA-CCAGG; it was then subcloned into CS-108 with *Eco*RI and *Not*I. Lowercase sequence delineates the FLAG epitope and underlined sequences are restriction sites. *X. laevis sall4* was PCR amplified with primers: forward, CTTGGTGCGCACTTATCTCA; and reverse, GCCTCAGATTGTGTGG-GACT; it was then cloned into pCR TOPO II (Invitrogen) for the generation of antisense RNA probes.

RNA and MO microinjections

Capped RNAs were synthesized using mMessage mMachine (Ambion). *sall4* CS-108, *fgf8a* CS-108, *noggin* CS-108 and β-*catenin* CS-108 were linearized with *Asc*I and transcribed with SP6 RNA polymerase. The *pou5f3* plasmids (a gift from Joshua Brickman, University of Copenhagen), TVGR (Darken and Wilson, 2001) and nuclear β-galactosidase CS2+ were linearized with *Not*I and transcribed with SP6. All RNAs were injected in 5 or 10 nl bursts along with *GFP* and *lacZ* RNAs, which served as tracers.

The Sall4 MO oligonucleotide (5′-GCCAATTATTCCCTTTCTCCACC-AC-3′; Gene Tools) and the Pou5f3 MOs (a gift from Joshua Brickman) (Livigni et al., 2013; Morrison and Brickman, 2006) were injected in 5 or 10 nl along with fluoresceinated control MO (Gene Tools) to serve as a tracer.

Cycloheximide and dexamethasone treatments

noggin (10 pg) and TVGR (4 pg), an inducible Wnt agonist (Darken and Wilson, 2001), RNAs were injected animally into both blastomeres of 2-cell embryos (Fig. 1A). At stage 9, animal caps were cultured with or without 10 μM dexamethasone (DEX) (Sigma) to activate Wnt signaling (Darken and Wilson, 2001). To block translation, caps were pre-treated with 5 μM cycloheximide (CHX) (Sigma) for 1.5 h prior to DEX addition (Obrig et al., 1971). Animal caps were cultured until stage 15 equivalent and total RNA was harvested using Trizol (Invitrogen).

Whole-mount *in situ* hybridization

Embryos were stained by *in situ* hybridization as described (Harland, 1991). β-galactosidase staining was as described (Fletcher et al., 2006). Embryos for sectioning were mounted in a PBS solution containing 20% sucrose, 30% BSA and 4.9% gelatin, and fixed with 1.5% glutaraldehyde. Embedded embryos were sectioned on a Pelco 101 vibratome.

RT-PCR and qPCR

RNA was isolated from whole embryos or animal caps using Trizol and 1 μg total RNA was reverse transcribed with either MMLV reverse transcriptase

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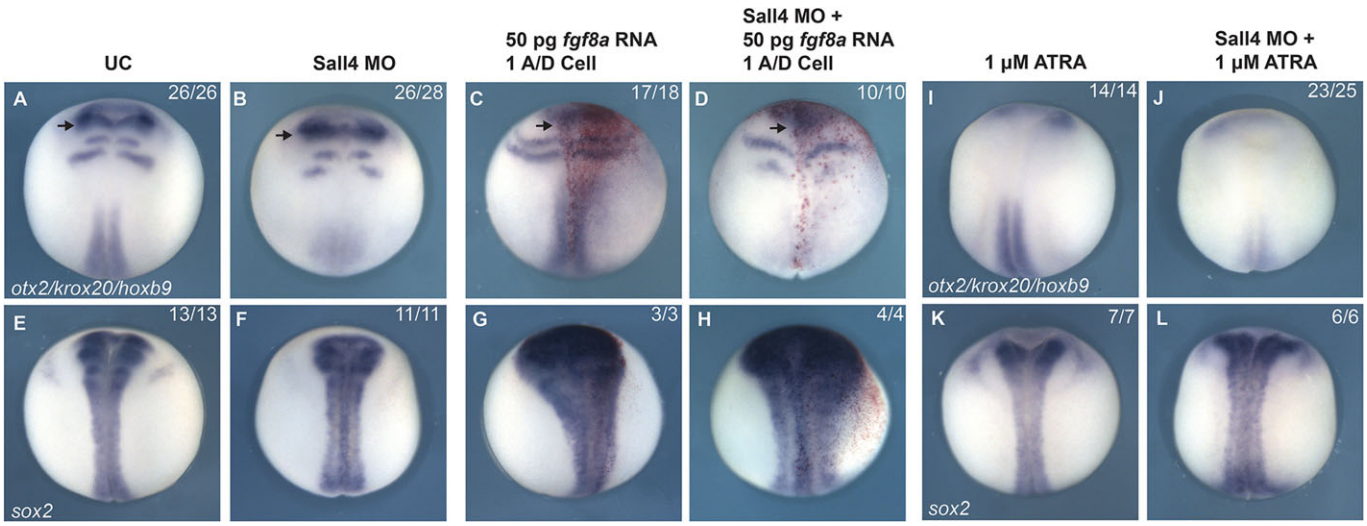

Fig. 9. Fgf and RA signaling fail to posteriorize Sall4 morphants. Whole-mount *in situ* hybridization of (A,E) uninjected control embryos, (B,F) embryos injected with 40 ng Sall4 MO (20 ng/blastomere at the 2-cell stage), (C,G) embryos injected with 50 pg *fgf8a* RNA into the right A/D blastomere, (D,H) embryos injected with 40 ng Sall4 MO (20 ng/blastomere at the 2-cell stage) and 50 pg *fgf8a* RNA into the right A/D blastomere, (I,K) embryos treated with 1 μM all-trans retinoic acid (ATRA) and (J,L) embryos injected with 40 ng Sall4 MO (20 ng/blastomere at the 2-cell stage) and treated with 1 μM ATRA. Expression is shown for (A-D,I,J) *otx2*, *krox20* and *hoxb9* or (E-H,K,L) *sox2*. Arrowheads indicate the posterior limit of *otx2* expression. Dorsal views, anterior to the top.

(Promega) for semi-quantitative PCR or iScript (Bio-Rad) for quantitative PCR (qPCR). Semi-quantitative PCRs included [^{32}P]dCTP (PerkinElmer) in the reaction and were analyzed during the log phase of amplification. qPCR reactions were amplified on a CFX96 light cycler (Bio-Rad). *ornithine decarboxylase* (*odc*) and *elongation factor 1a1* (*eef1a1*) were used for internal controls. All primers annealed at 60°C and are listed in supplementary material Table S1.

RNA-seq

RNA-seq was performed as described (Dichmann and Harland, 2012). Single-end 76-bp reads were sequenced on an Illumina Genome Analyzer II. All reads were mapped to an index created from a collection of full-length *X. laevis* mRNA sequences (NCBI, http://xgc.nci.nih.gov) using TOPHAT and BOWTIE (Langmead et al., 2009; Trapnell et al., 2009). Analysis of transcript abundance employed CUFFDIFF (Trapnell et al., 2010).

Chromatin immunoprecipitation (ChIP)

FLAG-β-catenin RNA-injected embryos were prepared for ChIP as described (Blythe et al., 2009). Chromatin shearing used a Branson Model 450 digital sonifier with a Model 102C probe for 24 ten-second bursts set at 30% amplitude. ChIP DNA was quantified with SYBR Green PCR mix (Bio-Rad) on a CFX96 light cycler (Bio-Rad). Enrichment was calculated by comparing the percentage input among ChIP samples. Uninjected embryos served as a control for non-specific binding. *Xmlc2* (Blythe et al., 2009) and *meis3* (Elkouby et al., 2010) served as negative and positive controls, respectively, for β-catenin binding.

Luciferase assays and mutagenesis

A 500 bp fragment containing three putative TCF/LEF sites in *sall4* intron 1 (Scaffold 1115: 234, 269-234, 644) was cloned into the pGL4.23 luciferase reporter (Promega) with *Sac*I and *Xho*I (NEB). Each of the three sites was mutagenized using *Pfx* polymerase (Invitrogen) according to the manufacturer's instructions. HEK293 cells were transfected with 0.1 μg each of pGL4.23 and pLR-CMV (Promega) and treated with 0.1 μg mouse Fgf or 50 μM BIO (Cayman). Relative luciferase units were measured on a Turner Design TD-20/20 luminometer using the Dual Luciferase Assay Kit (Promega).

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Competing interests

The authors declare no competing financial interests.

Author contributions

J.J.Y. and R.M.H. designed the experiments with contributions from R.A.S.K. and N.R.K. J.J.Y., R.A.S.K., N.K.R. and S.D.M. carried out all experiments. J.J.Y., R.A.S.K., N.R.K., S.D.M. and R.M.H. analyzed all data generated from the experiments. J.J.Y. and R.M.H. wrote the paper incorporating comments from R.A.S.K., N.R.K. and S.D.M.

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Supplementary material

Supplementary material available online at
http://dev.biologists.org/lookup/suppl/doi:10.1242/dev.099374/-/DC1

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