Epigenetic activation of Sox2 gene in the developing vertebrate neural plate

Santiago O. Bouzas†, Melisa S. Marini†, Eliana Torres Zelada, Ailín L. Buzzi, David A. Morales Vicente, and Pablo H. Strobl-Mazzulla*

Laboratory of Developmental Biology, Instituto de Investigaciones Biotecnológicas-Instituto Tecnológico de Chascomús (CONICET-UNSAM), 7130 Chascomús, Argentina

ABSTRACT One of the earliest manifestations of neural induction is onset of expression of the neural marker Sox2, mediated by the activation of the enhancers N1 and N2. By using loss and gain of function, we find that Sox2 expression requires the activity of JmjD2A and the Msks1 kinase, which can respectively demethylate the repressive H3K9me3 mark and phosphorylate the activating H3S10ph mark. Bimolecular fluorescence complementation reveals that the adaptor protein 14-3-3, known to bind to H3S10ph, interacts with JmjD2A and may be involved in its recruitment to regulatory regions of the Sox2 gene. Chromatin immunoprecipitation reveals dynamic binding of JmjD2A to the Sox2 promoter and N-1 enhancer at the time of neural plate induction. Finally, we show a clear temporal antagonism on the occupancy of H3K9me3 and H3S10ph modifications at the promoter of the Sox2 locus before and after the neural plate induction. Taken together, our results propose a series of epigenetic events necessary for the early activation of the Sox2 gene in neural progenitor cells.

INTRODUCTION

Neural induction involves a series of molecular and structural events mediated by multiple signaling molecules and transcription factors. The outcome results in segregation of the neural plate from the non-neural ectoderm, the first step in formation of the CNS. In vertebrates, the transcription factor Sox2 is one of the earliest definitive markers for neural plate cells (Streit and Stern, 1997; Linker and Stern, 2004; Albazerchi and Stern, 2007). Thus activation of the Sox2 gene plays an essential role in vertebrates as a readout of neural induction.

The regulatory elements mediating the early spatiotemporal expression of the Sox2 gene have been characterized by in depth cis-regulatory analysis in the chick embryo. Uchikawa et al. (2003) demonstrated that two distinct conserved regulatory elements, N2 and N1, recapitulate endogenous Sox2 expression during early neural plate development. The N2 enhancer mediates Sox2 activation in the early anterior neural plate and is regulated by Zic, Pou, and Otx factors (Iwafuchi-Doi et al., 2011, 2012). On the other hand, the N1 enhancer is responsible for Sox2 activation in the caudal lateral epiblast adjacent to the primitive streak and is regulated by the synergetic action of Wnt and Fgf signals (Takemoto et al., 2006; Kondoh and Takemoto, 2012; van Rooijen et al., 2012).

To explore how these enhancers might be activated in the nascent neural plate, Papanayotou et al. (2008) performed elegant experiments demonstrating that a series of molecular events results in Sox2 activation via the N-2 enhancer. In this model, a competitive interaction between three proteins (ERNI, BERT, and Geminin) occurs on this enhancer, which modulates the capacity of the Heterochromatin protein HP1 to repress premature and ectopic activation of Sox2 expression.

Recruitment of HP1 proteins to certain sites in the genome involves interactions with multiple chromatin components. In particular, the repressive epigenetic mark H3K9me3 is important for HP1 binding to specific chromosomal areas (Peters et al., 2003; Thiru et al., 2004; Stewart et al., 2005). Indeed, HP1 proteins have a chromoshadow domain, necessary for interaction with other proteins,
and an amino-terminal chromodomain essential for the HP1-H3K9me3 interaction (Bannister et al., 2001; Lachner et al., 2001; Fischle et al., 2003, 2005).

The H3K9me3 demethylase JMJD2A (also known as KDM4A) increases chromatin accessibility by antagonizing HP1 occupancy (Black et al., 2010). Moreover, the interaction between HP1 and

**FIGURE 1:** JmjD2A expression precedes that of Sox2 in the neural territory. Expression pattern in the early chick embryo by whole-mount ISH at stages 3+, 4, and 5 revealed that JmjD2A precedes that of Sox2 in the nascent neural plate.

and H3K9me3 is displaced when the adjacent serine on the histone 3 is phosphorylated (H3S10ph) (Fischle et al., 2005), facilitating accessibility for the H3K9me3 demethylation (Metzger et al., 2008).

Of interest, we previously showed that the JMJD2A demethylase was expressed at gastrulation stages and plays a central role in activating neural crest specification (Strobl-Mazzulla et al., 2010), an event that closely follows neural induction. To explore whether there was an earlier role for JMJD2A in HP1 displacement during neural induction, we analyzed its possible function in Sox2 activation at the embryonic neural plate territory.

**RESULTS**

**JmjD2A expression precedes that of Sox2 at the embryonic neural plate territory**

To determine the temporal relationship between JMJD2A and Sox2 activation, we first characterized their relative spatiotemporal expression patterns during neural plate induction. JmjD2A transcripts were expressed before Sox2, initially detected at stage 3+ in a small population of cells around the Hensen’s node. Expression then spreads throughout the neural plate, in a notched horseshoe shape surrounding Hensen’s node, by stages 4 and 5 (Figure 1). On the other hand, we first detected the Sox2 expression at stage 4, in the presumptive cephalic neural plate, as previously described by Uchikawa et al., (2004). Thus the expression profiles of both transcripts in early neural territory are consistent with a possible regulatory relationship between JmjD2A and Sox2.

**Loss of JmjD2A causes decreased expression of Sox2**

To test whether JmjD2A might influence the expression of endogenous Sox2, we tested the effects of its loss of function using a translation-blocking morpholino (JmjD2A-tbMO) or a splicing-blocking morpholino (JmjD2A-sbMO), which yielded similar results. These were introduced onto one side of the embryo, with the contralateral side serving as an internal control. The specificity of these MOs and ability to reduce expression of JMJD2A protein were previously demonstrated (Strobl-Mazzulla et al., 2010).

After electroporation at stage 4, embryos were grown until stage 7, and Sox2 expression on the experimental side (right) was compared with that on the control side (left). As expected, control MO–treated embryos had no obvious phenotype (Figure 2A). Electroporation of either JmjD2A target MO profoundly reduced Sox2 expression throughout the entire neural plate (Figure 2, B and C) on the MO-treated side. Both MOs caused a significant increase in the number of embryos with reduced Sox2 expression compared with control MO–treated embryos (Figure 2E).

To further demonstrate specificity, we performed rescue experiments in which JmjD2A-spMO was coelectroporated with JmjD2A full-length vector (pCI-JmjD2A). This experiment demonstrated that JmjD2A full length attenuated the loss of Sox2 expression on MO-treated embryos (Figure 2D), showing a lack of significant differences with respect to the control embryos (Figure 2E).

Taken together, these results demonstrate that JmjD2A is required for early Sox2 activation in the neural plate territory.

**Jmd2A and MSK1 gain of function induce ectopic Sox2 expression**

To test whether JMJD2A activity might function by releasing the constitutive repression of HP1 protein via interaction with H3K9me3, we misexpressed JmjD2A lateral to the neural plate territory. However, this failed to induce significant ectopic Sox2 expression compared with that observed in embryos electroporated with an empty vector (control; Figure 3, A and B).
Previous studies demonstrated that accessibility of H3K9me3 demethylation (Metzger et al., 2008) is facilitated when the interaction with HP1 is displaced by phosphorylation of the adjacent serine on the histone 3 (H3S10ph) by the kinase mitogen and stress-activated kinases 1 (MSK1; Fischle et al., 2005). Therefore we tested by in situ hybridization whether the Msk1 gene was expressed in the early neural plate territory at the time of Sox2 activation (Supplemental Figure S1). On the basis of its presence, we coelectroporated the JmjD2A expression vector with one containing the kinase Msk1 responsible for the H3S10ph in vertebrates. Of interest, unilateral electroporation of both JmjD2A- and Msk1-containing vectors resulted in ectopic Sox2 expression in 87.5% of the analyzed embryos (Figure 3, D and E), which is significantly different (p < 0.05) from that for embryos electroporated with a control or Msk1 vector alone (Figure 3E). These observations suggest that H3S10 phosphorylation via MKS1 might help to dissociate the interaction between HP1 and H3K9me3 (Fischle et al., 2005; Hirota et al., 2005), thus allowing JmjD2A to demethylate this mark and induce the ectopic expression of Sox2.

**JmjD2A directly interacts in vivo with the adaptor protein 14-3-3**

It is well known that the 14-3-3 proteins bind to chromatin-modifying proteins and transcriptional regulators, such as histone acetyltransferase 1 (Imhof and Wolffe, 1999), histone deacetylases (Bertos et al., 2001), p53 (Waterman et al., 1998), and TATA-binding proteins (Pan et al., 1999). Moreover, 14-3-3 proteins are able to interact with H3S10ph during gene activation in mammalian cells (Chen and Wagner, 1994; Meek et al., 2004; Macdonald et al., 2005). On the basis of these precedents, we performed a bimolecular fluorescence complementation assay (BiFC) to test whether the 14-3-3 protein might serve as an adaptor protein that interacts with JmjD2A. To this end, we cloned the coding regions of JmjD2A and 14-3-3 genes to the N- and C-termini of the VENUS proteins, respectively (Figure 4A). After electroporation of these constructs into chick embryos, Venus signal is detectable mostly in the cytoplasm but also in the nuclei (Figure 4, B–D) of electroporated cells, demonstrating direct protein interaction. This is in agreement with the bipartite localization observed by immunohistochecmistry using anti-JmjD2A on electroporated embryos with a pCI-JmjD2A-ires-H2BFP-overexpressing vector (Supplemental Figure S3). Individual electroporation of JmjD2A-VC or 14-3-3-VN containing vector failed to generate VENUS fluorescence (Supplemental Figure S4). Moreover, when JmjD2A-VN was coelectroporated with a MOCK protein fused to the VENUS C-terminus, no interaction was observed (Figure 4, E–G). These results show that the 14-3-3 protein interacts with JmjD2A and may function as an adaptor protein to bring JmjD2A to the H3S10ph sites in order to activate the target gene via H3K9me3 demethylation.

**JmjD2A is recruited to the Sox2 regulatory regions in vivo**

Sox2 expression in the neural plate is driven by enhancers N2 and N1, which are activated in the anterior and posterior domains, respectively, of the early neural primordium (Uchikawa et al., 2003; Kamachi et al., 2009). To demonstrate the in vivo association of JmjD2A protein with regulatory regions of Sox2 gene, we performed in vivo chromatin immunoprecipitation (ChiP) experiments in conjunction with quantitative PCR (qPCR) on dissected neural plate tissue from stage 3 and stage 5 embryos (Figure 5B). At stage 3, we failed to detect association of JmjD2A protein with any of the analyzed regulatory regions. However, at stage 5, a very consistent JmjD2A interaction was observed in the promoter region (−0.5 kb from the transcriptional start site [TSS]). On the other hand, the interaction with the N1 enhancer was more variable since it was detected in two of the three independent experiments. The Sox10 promoter region (sox10−0.5 kb from the TSS) was used as a positive control for JmjD2A binding (Supplemental Figure S5) since we previously described this interaction at these developmental stages (Strobl-Mazzulla et al., 2010). These data demonstrate that JmjD2A interacts in vivo with regulatory regions at the time of initial Sox2 activation at the neural plate territory. Finally, we performed ChIP-qPCR to assess the occupancy of H3K9me3 and H3S10ph modifications at the promoter (−0.5 kb from the TSS) and N1 and N2 enhancers of Sox2 before and after neural plate induction (Figure 5C). We found at the promoter region that H3K9me3 abundance is reduced from stage 3 to stage 5 in a reciprocal manner to the observed increase in the H3S10ph mark. Moreover, we observed on the N2 enhancer a consistent increase in H3S10ph but no variation for the H3K9me3 mark. Finally, we detected at the N1 enhancer a slight decrease or nonvariation for the H3K9me3 and H3S10ph marks, respectively.

On the basis of these findings, we propose the model in Figure 6, in which a sequential series of epigenetic events is necessary for early activation of Sox2 expression. The initial step is phosphorylation of H3S10 by the kinase MSK1 to dissociate the HP1–H3K9me3 interaction that maintains repression of the Sox2 gene. Then the adaptor protein 14-3-3 interacts with JmjD2A and may be involved in nuclear translocation of the complex and recruitment to the H3S10ph mark, which is necessary for H3K9me3 demethylation and concomitant Sox2 activation in the nascent neural plate. Our model does not rule out additional mechanisms.
In vertebrates, development of the CNS begins with the specification of neural progenitor cells in the developing midline to form the neural plate. There are several families of transcription factors that define and specify the neural plate territory. However, Sox2 is the earliest definitive marker for the neural plate territory (Linker and Stern, 2004; Albazerchi and Stern, 2007). As a consequence, understanding the mechanisms that regulate early activation in the neural plate is of great interest in development.

Previous studies have shown that the protein HP1 maintains the conformation of the chromatin around the N2 enhancer in a closed configuration, thus maintaining the repressive state of the Sox2 gene and preventing premature or ectopic activation (Papanayotou et al., 2008). A series of mutually inhibitory interactions between several proteins then occurs, resulting in activation of the N2 enhancer.

Here we demonstrate a parallel sequence in vivo of the epigenetic events necessary for earlier activation of the Sox2 gene (Figure 6). In particular, we demonstrate that the histone demethylase JMJD2A directly interacts with regulatory regions during early Sox2 activation by demethylating the H3K9me3 repressive mark. This is intriguingly similar to its later function in activation of the neural crest specifier gene, Sox10 (Strobl-Mazzulla et al., 2010). This role of JMJD2A in activating Sox2 expression is consistent with the idea that HP1 interacts, via its chromodomain, with the epigenetic mark H3K9me3. Of interest, our results demonstrate a requirement for the kinase MSK1, which can phosphorylate the adjacent serine (H3S10ph) of the H3K9me3 in order to release the interaction between HP1 and H3K9me3, as proposed in other systems (Fischle et al., 2005). Moreover, it is well known that H3S10ph marks are related to mitotic progression, as well as to transcriptional activation, in organisms ranging from yeast to humans (Mahadevan et al., 1991; Nowak and Corces, 2000; Lo et al., 2001; Berger, 2007; Zippo et al., 2009). Of interest, we found in vivo that the H3S10ph mark is highly enriched at the promoter of the Sox2 locus at the onset of its activation. The resultant permissive state of the chromatin likely enables JMJD2A to demethylate the H3K9me3 mark. It is interesting to note that the ectopic Sox2 expression observed in our overexpression experiments was always in proximity to the normal Sox2 expression territory. This observation suggests there may be other, perhaps diffusible, factors necessary for Sox2 activation.
The 14-3-3 proteins are members of a well-characterized family of ubiquitous phospho-Ser/Thr–binding protein that function as adaptors/chaperones (Yaffe and Elia, 2001), and they have also been found to bind to H3S10ph during gene activation (Macdonald et al., 2005). Moreover, 14-3-3 proteins are able to interact with and recruit chromatin-modifying proteins, such as histone acetyltransferase 1 (Imhof and Wolffe, 1999) and histone deacetylases (Bertos et al., 2001), to regulate target genes. Of interest, the interaction with 14-3-3 proteins may regulate both cytoplasmic localization (Kumagai and Dunphy, 1999) and nuclear translocation (Neasta et al., 2012). Consistent with this, the present study shows that JMJD2A protein is detected and directly interacts with 14-3-3 in both the cytoplasm and the nucleus. This observation is in agreement with dual nucleus and cytoplasm localization of JMJD2A described in rat brain and medulla and with the existence of a potential bipartite localization signal spanning residues 999–1015 (Gray et al., 2005). Accordingly, it has been shown that the JmjN, Tudor, and PHD domains of JMJD2A contribute to its efficient nuclear translocation (Klose et al., 2006). Thus we propose that the interaction of 14-3-3 with the H3S10ph mark may help to position JMJD2A in proximity to H3K9me3 after HP1 displacement, enabling it to demethylate this lysine and activate Sox2 expression. A very similar regulation was described in which phosphoacetylation of histone H3 (H3S10phK14ac) at the HDAC1 promoter leads to the recruitment of 14-3-3 proteins concomitant with dissociation of HP1 (Winter et al., 2008).

An exhaustive analysis of the genomic regions of the chicken Sox2 gene revealed the existence of 25 distinct conserved enhancers (Uchikawa et al., 2003). Five of them (N1–N5) are involved in the spatiotemporal regulation of Sox2 expression on the neural plate and CNS (Uchikawa et al., 2003; Takemoto et al., 2006; Iwafuchi-Doi et al., 2012). The model proposed by Papanayotou et al. (2008) described the onset of Sox2 expression on the nascent neural plate via the N2 enhancer. They described a series of protein and chromatin-remodeling interactions (BRM-GEMININ-ERNI-HP1) necessary for early Sox2 activation using in vivo approaches in chick embryos. They further demonstrated the physical association of Geminin, which indirectly interacts with HP1, with the N2 enhancer. Here we extend that analysis by demonstrating epigenetic mechanisms underlying activation of the Sox2 gene. Our demonstration of the in vivo interaction of JMJD2A protein and the epigenetic changes observed at the promoter region of the Sox2 gene highlights the central role of epigenetic regulation at the onset of Sox2 expression on the nascent neural plate. Thus our model is complementary to that of Papanayotou et al. (2008) by adding a new level of complexity. Because we observed changes in JMJD2A binding and H3K9me3 abundance only at the promoter and not at the N2 enhancer, we speculate that HP1 might act as a molecular bridge, interacting on one side with proteins such as Geminin-ERNI located on the N2 enhancer and on the other side with the H3K9me3 mark at the Sox2 promoter. This dual specificity might underlie the fine-tuning that controls the dynamic expression of Sox2 in the neural plate.

**MATERIALS AND METHODS**

**Embryos**

Fertilized chicken (*Gallus gallus domesticus*) eggs were obtained from local commercial sources and incubated at 37°C to the desired stages according to the criteria of Hamburger and Hamilton.
Electroporation of morpholinos and vectors
We used previously described and characterized JmjD2A antisense MOs (Strobl-Mazzulla et al., 2010), one near the ATG codon blocking their translation (JmjD2A-tbMO: 5′-TAAGGCTTCTCGAGCTC-GAGGCCTAT-3′), and a second one at the boundary of exon 3–intron 3 blocking the splicing and producing a frameshift and a premature stop (JmjD2A-spMO: 5′-CGGGCAGGCCTACTTTGCTGTGTT-3′). Injections of fluorescein-tagged MO (0.75 mM plus 0.3 ng of plasmidic DNA used as carrier) and pCI-H2BRFP, pCI-JmjD2A-H2BRFP, and pCI-MSK-GFP vectors were performed by air pressure using a glass micropipette targeted to the presumptive neural plate region at the desired stages. Electroporation was made with five pulses of 5.5 V in 50 ms at 100-ms intervals. Embryos were cultured in 0.5 ml of albumen in tissue culture dishes until the desired stages. Embryos were then removed and fixed overnight in 4% paraformaldehyde (PFA) at 4°C. They were then placed in phosphate-buffered saline (PBS), viewed, and photographed as whole mounts using a fluorescence stereomicroscope to determine electroporation efficiency. Embryos used for in situ hybridization (ISH) were dehydrated in a MeOH/PBS–TWEEN 20 series at room temperature before being stored at −20°C in 100% MeOH.

In situ hybridization
Whole-mount ISH was carried out as described previously (Kee and Bronner-Fraser, 2001). Digoxigenin-labeled probes were synthesized from the full-length chicken CDNA of JmjD2A, Msx1, and Sox2 using linearized plasmids. Hybridized probes were detected using an alkaline phosphatase–conjugated anti-digoxigenin antibody (1:2000; Roche, Buenos Aires, Argentina) in the presence of nitro blue tetrazolium/5-bromo-4-chloro-3-indolylphosphate substrates (Roche). Embryos were photographed as a whole mount.

Bimolecular fluorescence complementation
For the BiFC assay on chick embryos, we used previously described vectors containing the N- and C-terminal halves of Venus (amino acids 1–172 and 173–239; Strobl-Mazzulla et al., 2010). Full-length JmjD2A, 14-3-3, and SAP18 (used as a MOCK protein) were cloned in-frame upstream of the Venus halves–containing vector. Chick embryos were electroporated with the vectors at stage 5 and then cultured in 0.5 ml of albumen in tissue culture dishes for 16 h. Embryos were fixed in 4% PFA for 20 min at room temperature, washed in PBS, and visualized as a whole mount. Some embryos were subsequently embedded in gelatin, cryostat sectioned at 16 μm, cover slipped using Permafluor (Beckman Coulter), and photographed.

Chromatin immunoprecipitation
Three independent samples were made by dissecting neural plates from 30 embryos collected at stages 3 and 5. Cells were cross-linked and sonicated to yield 300– to 800–base pair fragments. Samples were evenly split for rabbit anti-JmjD2A (Abcam, Buenos Aires, Argentina), anti-H3K9me3 (Abcam), anti-H3S10ph (Epigentek, Farmingdale, NY), control rabbit anti–immunoglobulin G (IgG; Abcam), and input. Antibodies were preincubated with protein A magnetic beads (Invitrogen, Buenos Aires, Argentina) before incubation with a sonicated protein–DNA complex. The complexes were magnetically isolated, eluted, and cross-link reversed. The DNA was purified, precipitated, and finally used as a template for qPCR analysis (see Supplemental Table S1 for a list of primers). Every sample was loaded by three replicates each. The results were quantified using the ΔΔCt method, calculated according to manufacturer’s instructions (Applied BioSystems), and expressed as fold enrichment with respect to IgG.

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