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 *Msk1* kinase, which can respectively demethylate the repressive H3K9me3 mark and phosphorylate the activating H3S10 (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.**

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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 synergic 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,

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†These authors contributed equally.

*Address correspondence to: Pablo H. Strobl-Mazzulla (strobl@intech.gov.ar).

Abbreviations used: BiFC, bimolecular fluorescence complementation; ChIP, chromatin immunoprecipitation; ISH, in situ hybridization; MO, morpholino; PAF, paraformaldehyde; PBS, phosphate-buffered saline; TSS, transcription start site.

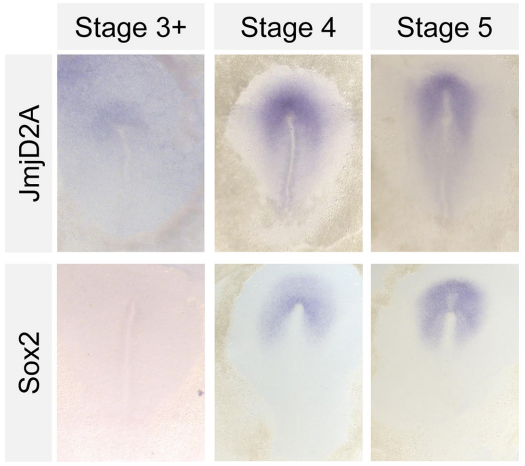

FIGURE 1: *JmjD2A* expression precedes that of *Sox2* in the neural territory. Expression pattern in the early chick embryo by whole-mount ISH at stages 3+, 4, and 5 revealed that *JmjD2A* precedes that of *Sox2* in the nascent neural plate.

and 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

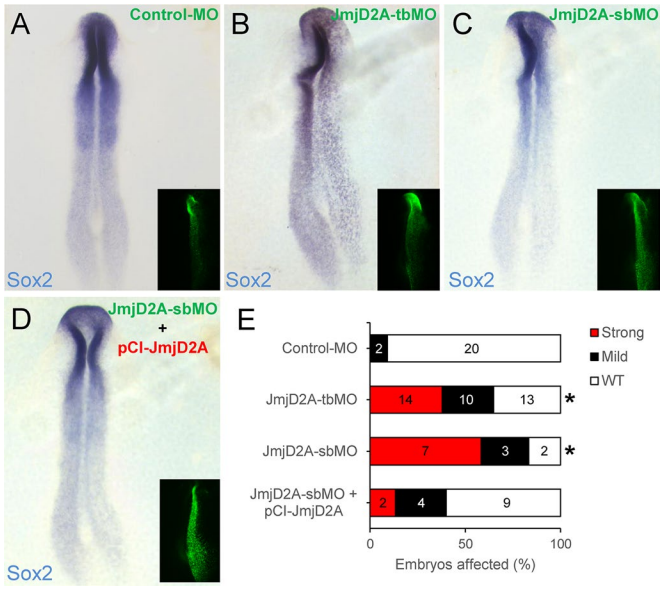

FIGURE 2: Loss of JmjD2A causes reduced expression of *Sox2*. *Sox2* ISH of chick embryos electroporated with control-MO (A), JmjD2A-tbMO (B), or JmjD2A-sbMO (C). Loss of JmjD2A causes a clear reduction in the expression of *Sox2* all along the neural plate. (D) Electroporation of JmjD2A-sbMO together with a vector containing the coding region of JmjD2A (pCI-JmjD2A) rescues the depletion of *Sox2* expression as assayed by ISH. Insets, the distribution of fluorescently labeled MO (green). (E) Quantification of embryos showing wild-type (WT; white), mild (black), or strong (red) *Sox2* reduction on electroporated embryos. Asterisk indicates significant difference ($p < 0.01$) by contingency table followed by chi-square test. Numbers represent individual embryos. See Supplemental Figure S2 for phenotype description.

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).

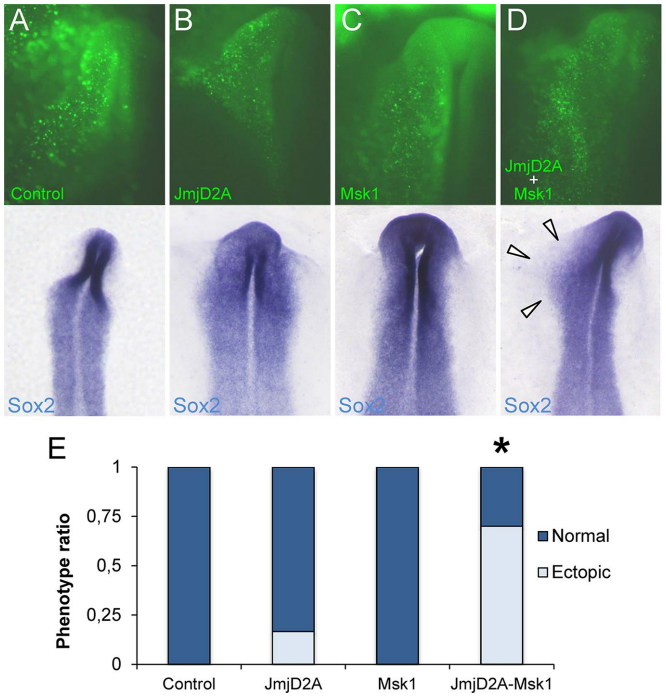

FIGURE 3: *JmD2A* and *Msk1* gain of function induces ectopic *Sox2* expression. ISH of *Sox2* gene expression after electroporation with control empty vector (A), JmjD2A (B), MSK1 (C), or a combination of both *JmjD2A* + *Msk1* (D) overexpression vectors. Ectopic *Sox2* expression (white arrowhead) was clearly evident when both *JmjD2A* and *Msk1* overexpression vectors were coelectroporated. Top, left fluorescent electroporated area. (E) Ratio of embryos with normal or ectopic *Sox2* expression phenotype. *$p < 0.05$ vs. control MO by contingency table followed by chi-square.

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 immunohistochemistry using anti-JMJD2A on electroporated embryos with a pCI-JmjD2A-IRES-H2BRFP–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 (Stroble-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.

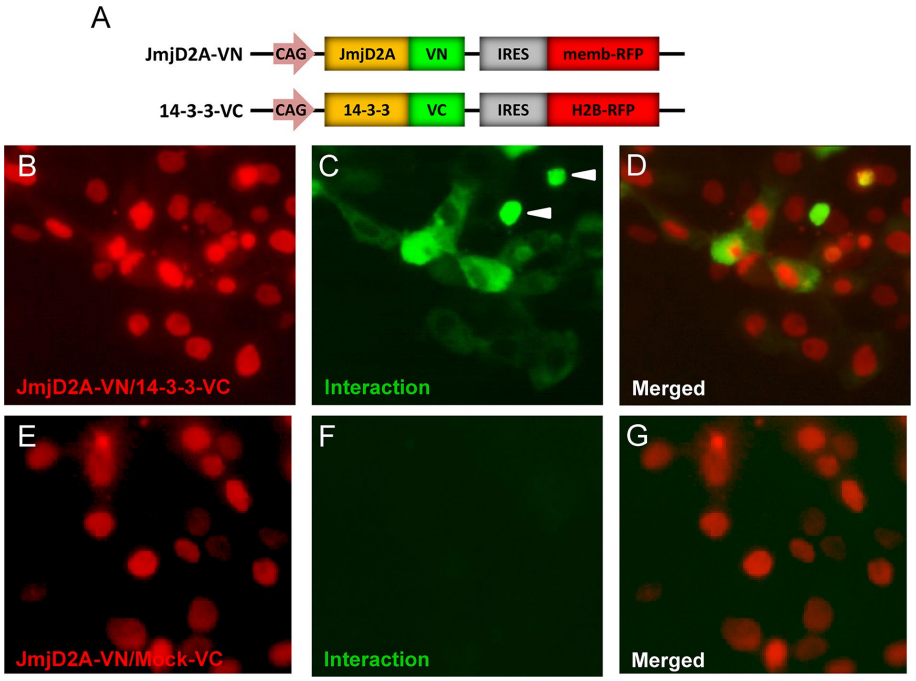

FIGURE 4: *JmjD2A* directly interacts with the adaptor protein *14-3-3* as revealed by BiFC. (A) Schematic representation of *JmjD2A* and *14-3-3* constructs used for the BiFC assay. (B–D) Chick embryos were coelectroporated with JmjD2A-VN/14-3-3-VC or (E-G) JmjD2A-VN/mock-VC vectors at stage 5 and collected 16 h later. Embryos were then cryostat sectioned, and the electroporated cells (red) were analyzed for Venus fluorescence, reflecting positive protein interaction (green). White arrowheads indicate nuclear interaction. VC, C-terminus of Venus; VN, N-terminus of Venus; IRES, internal ribosome entry site.

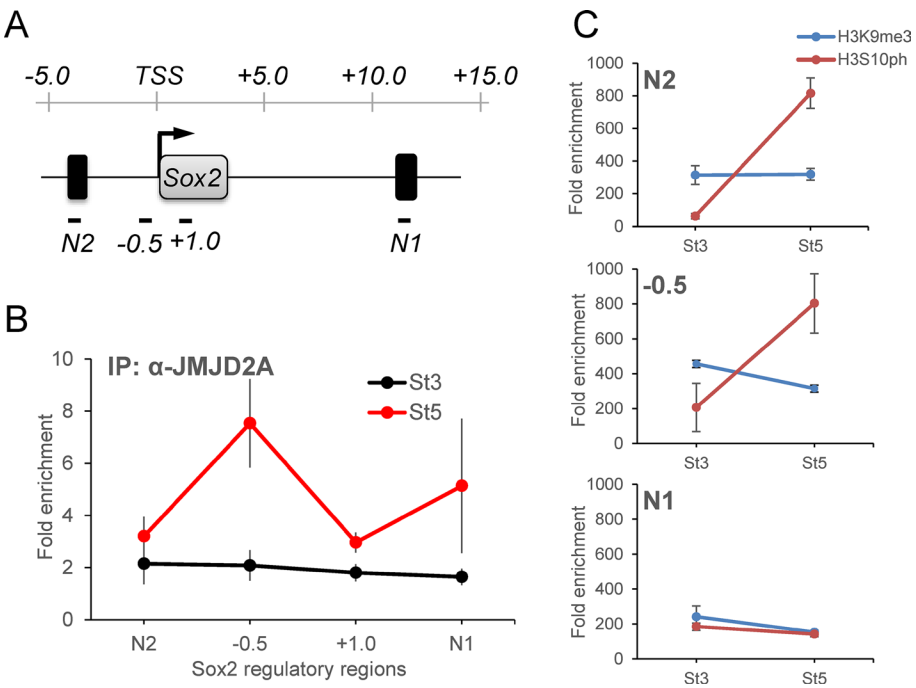

FIGURE 5: Endogenous JMJD2A protein is recruited to *Sox2* promoter concomitantly with dynamic and opposite H3K9me3 and H3S10ph occupancy. ChIP-qPCR on dissected neural plate tissue from stage 3 and stage 5 embryos. (A) Schematic diagram representing the position where the primers were designed on the *Sox2* locus: N1 and N2 enhancers, the promoter region at –0.5 kb, and the gene body at +1.0 kb from the TSS. (B) Endogenous JMJD2A binds to *Sox2* promoter and N1 enhancer at stage 5 (St5) but was undetected at stage 3 (St3). (C) In vivo ChIP-qPCR for H3K9me3 and H3S10ph at Sox2 promoter (–0.5) and N1 and N2 enhancers before (St3) and after (St5) neural plate induction. IgG antibody was used as a mock antibody. Graphs represent the fold enrichment with respect to IgG immunoprecipitation, and error bars show SDs.

DISCUSSION

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 sowed 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 it activation. The resultant permissive state of the chromatin likely enables JMJD2A to demethylate the H3K9m3 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.

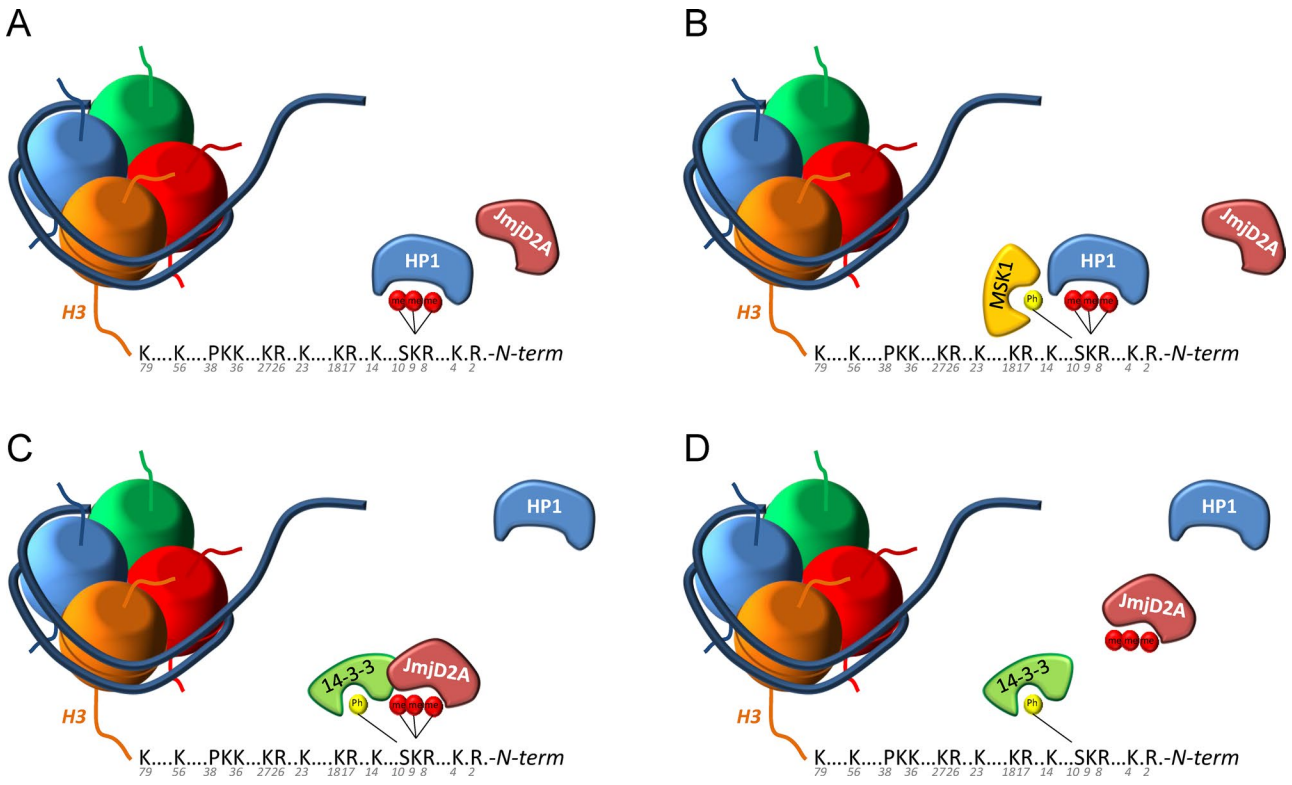

FIGURE 6: Model for *Sox2* enhancer activation on the neural plate. (A) The interaction between HP1 and H3K9me3 maintains the repressive state of Sox2. (B) The phosphorylation of H3S10 by the kinase MSK1 destabilizes the interaction between HP1 and H3K9me3. (C) The adaptor protein 14-3-3 interacts with JmjD2A and recruits it to the H3S10ph mark. (D) JMJD2A demethylates the H3K9me3 mark, activating *Sox2* expression in the nascent neural plate.

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′-TGAGGCTCTCCAGCTCCGAGGCCAT-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′-CGGCCCGCGCCTACTTGTCGCTGTT-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*, *Msk1*, 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′-indolyphosphate 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 $\Delta\Delta Ct$ method, calculated according to manufacturer's instructions (Applied BioSystems), and expressed as fold enrichment with respect to IgG.

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