FoxM1-driven cell division is required for neuronal differentiation in early Xenopus embryos

Hiroyuki Ueno1, Nobushige Nakajo1,*, Minoru Watanabe2, Michitaka Isoda1 and Noriyuki Sagata1,3,*

In vertebrate embryogenesis, neural induction is the earliest step through which the fate of embryonic ectoderm to neuroectoderm becomes determined. Cells in the neuroectoderm or neural precursors actively proliferate before they exit from the cell cycle and differentiate into neural cells. However, little is known about the relationship between cell division and neural differentiation, although, in Xenopus, cell division after the onset of gastrulation has been suggested to be nonessential for neural differentiation. Here, we show that the Forkhead transcription factor FoxM1 is required for both proliferation and differentiation of neuronal precursors in early Xenopus embryos. FoxM1 is expressed in the neuroectoderm and is required for cell proliferation in this region. Specifically, inhibition of BMP signaling, an important step for neural induction, induces the expression of FoxM1 and its target G2–M cell-cycle regulators, such as Cdc25B and cyclin B3, thereby promoting cell division in the neuroectoderm. Furthermore, G2–M cell-cycle progression or cell division mediated by FoxM1 or its target G2–M regulators is essential for neuronal differentiation but not for specification of the neuroectoderm. These results suggest that FoxM1 functions to link cell division and neuronal differentiation in early Xenopus embryos.

KEY WORDS: Xenopus, Neural differentiation, Cell division, FoxM1, Cdc25B, BMP

INTRODUCTION
During vertebrate embryogenesis, the nervous system is formed from embryonic ectoderm through complex processes. Neural induction is the earliest step through which the fate of embryonic ectoderm to neuroectoderm becomes determined. In many species, inhibition of bone morphogenetic protein (BMP) signaling plays an important role in neural induction; FGF and Wnt signaling are also involved in this process (Muñoz-Sanjuán and Brivanlou, 2002; Stern, 2005). During gastrulation in Xenopus, BMP inhibition induces the expression of Sox2, an initial-stage neural marker that specifies the formation of neuroectoderm (Kishi et al., 2000). Subsequently, XngnR1 (a Xenopus neurogenin-related bHLH factor) is expressed in a subset of the neuroectodermal cells to generate primary neurons, which form the simple nervous system of early embryos (Ma et al., 1996). XngnR1 activates certain neurogenic regulators, such as NeuroD, in primary neuronal precursors and thereby promotes neuronal differentiation (Lee et al., 1995; Ma et al., 1996). Other factors, such as Notch and Delta, are also expressed in the neuroectoderm and play positive or negative roles in neuronal differentiation (Bertrand, 2002).

Neuroectodermal cells have a higher mitotic activity than non-neuroectodermal cells in Xenopus embryos (Saka and Smith, 2001), and neural precursors actively proliferate in both chick and mouse embryos (Graham et al., 2003; Hollyday, 2001). Neural precursors exit from the cell cycle and differentiate into functional neural cells, owing in part to the actions of Cdk inhibitors, such as p27Xic1 in Xenopus (Vernon et al., 2003) and p27Kip1/p57Kip2 (Cdkn1b/Cdkn1c – Mouse Genome Informatics) in mouse (Cremisi et al., 2003).

However, it remains unclear how cell proliferation is initially stimulated in the neuroectoderm or neural precursors. Furthermore, it is unknown whether preceding cell division or proliferation is required for terminal differentiation of neural precursors, although, in Xenopus, cell division after the onset of gastrulation has long been thought to be nonessential for neural differentiation (Harris and Hartenstein, 1991; Rollins and Andrews, 1991; Yeo and Gautier, 2003).

The Fox gene family encodes transcription factors containing a conserved Forkhead DNA-binding motif (Katoh and Katoh, 2004). FoxM1 has been isolated from both mammals and Xenopus (Pohl et al., 2005; Ye et al., 1997). In mammalian cultured cells, FoxM1 activates the expression of many genes, particularly those encoding G2–M cell-cycle regulators, such as cyclin B and Cdc25B, and thereby promotes cell division (Laoukili et al., 2005; Wang et al., 2005). Although Foxn1 is expressed in actively proliferating neural precursors in mouse (Karsten et al., 2003), little is known about whether this expression makes any contribution to the proliferation or differentiation of neural precursors (Krupczak-Hollis et al., 2004; Schüller et al., 2007).

To investigate the possible relationship between cell division and neural differentiation, we have analyzed the role of FoxM1 in early Xenopus development. We show that FoxM1 is expressed in the neuroectoderm and is required for cell division in this region. In addition, BMP inhibition induces cell division by augmenting the expression of FoxM1 and its target G2–M regulators. Furthermore, and importantly, preceding FoxM1-dependent cell division is required for neuronal differentiation but not specification. Thus, our results reveal the primary mechanism of proliferation of neural precursors, and link cell division and neuronal differentiation in early Xenopus embryos.

MATERIALS AND METHODS
Embryo culture and antisense morpholino oligos (MOs)
Embryos were prepared, cultured, staged and microinjected as described (Shimuta et al., 2002). Antisense MOs were obtained from Gene Tools (Philomath, OR). MO sequences were as follows (5’ to 3’): FoxM1-MO,
cDNAs and in vitro transcription

cDNAs encoding *Xenopus* FoxM1 (Pohl et al., 2005) (accession number AJ853462) and cyclin B3 (Hochegger et al., 2001) (AJ853462) were isolated by RT-PCR from *Xenopus* neurula RNA and subcloned into either the pT7-G (UKII+) or pCS2+ transcriptional vector (Nakajo et al., 2000; Watanabe and Whitman, 1999). The FoxM1 (ΔN) cDNA encoding amino acids 215-759 of *Xenopus* FoxM1 protein was as previously described (Lüscher-Firzlaff et al., 2006). A cDNA encoding *Xenopus* Cdc25B (AB363840) was isolated by PCR from a tailbud cDNA library and subcloned into the pT7-G (UKII+) or pCS2+ transcriptional vector. cDNAs encoding dnBMPR and Noggin were as previously described (Graff et al., 1994; Smith and Harland, 1992). In vitro transcription of cDNAs was performed as described (Nakajo et al., 2000).

RT-PCR

RT-PCR of RNA from whole embryos or animal caps was performed essentially as described (Watanabe and Whitman, 1999). The primer sets used for PCR were (5’ to 3’; U, upstream and D, downstream): FoxM1 U, CCGACCACCTTTCTCCTCCAGC and D, GATGACGCAAGTTTGTCTACTGCTG; Cdc25B U, ACGTTGAGATCATGCTGGTAAG and D, TCTCCTGTCTTTCTTCCG; cyclin B1 U, GATGTGGATATATGATTG and D, CCATTCCACACACATCT; cyclin B3 U, CTTCCTGCGCAGATTTGCTA and D, TGTGAGTATTTGCTCGTGGATTATGATATGG and D, CCATTCCACACACATCT; cyclin C G (UKII+) or pCS2+ transcriptional vector. cDNAs encoding dnBMPR and Noggin were as previously described (Graff et al., 1994; Smith and Harland, 1992). In vitro transcription of cDNAs was performed as described (Nakajo et al., 2000).

Whole-mount in situ hybridization, β-Gal staining and pH3 staining

Whole-mount in situ hybridization was performed essentially as described (Sive et al., 2000); the constructs used were for Sox2 (Mizuseki et al., 1998), Xngnr1 (Ma et al., 1996), N-tubulin (Chitnis et al., 1995) and MyoD (Sive et al., 1992). In vitro transcription of cDNAs was performed as described (Graff et al., 1994; Smith and Harland, 1992). In vitro transcription of cDNAs was performed as described (Nakajo et al., 2000).

Hoechst staining

Neural plates were isolated from embryos at stage (st.) 14, fixed with MEMFA for 20 minutes, stained for β-Gal and then re-fixed with Fixative 1 [10% formaldehyde, 60 mM HEPES-KOH (pH 7.5)] for 1 hour. They were then treated with Fixative 2 [10% formaldehyde, 60 mM HEPES-KOH (pH 7.5), 50% glycerol] containing 5 μg/ml Hoechst for 15 minutes, washed twice with Fixative 2 (without Hoechst) and then mounted for fluorescence microscopy.

Antibodies and immunoblotting

For immunoblotting, whole embryos or animal caps were homogenized with an extraction buffer (80 mM β-glycerophosphate, 15 mM MgCl₂, 20 mM EGTA, 10 μM pepstatin A, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 0.2 mM PMSF, 1 mM NaF, 1 μM microcystin, 1 mM sodium orthovanadate). Proteins equivalent to one embryo or ten animal caps were analyzed by immunoblotting using antibodies against histone H3 phospho-Ser10 (Upstate Biochemistry) or ERK1 (Santa Cruz Biotechnology).

RESULTS

FoxM1 is expressed and required for cell proliferation in the neural plate

The temporal expression pattern of FoxM1 was first investigated by RT-PCR during early *Xenopus* development. Transcripts of FoxM1 were detected throughout the stages of early embryogenesis examined, i.e. from the unfertilized egg until the tailbud stage (their expression before the gastrula stage being maternal, data not shown) (Fig. 1A, upper panel). This expression pattern was highly reproducible, although it is significantly different from that reported previously (Pohl et al., 2005). Using whole-mount in situ hybridization (WISH), FoxM1 mRNA was detected principally in the animal hemisphere at the initial gastrula stage (st. 10), in the neural plate at the early neurula stage (st. 14), in the neural tube at the late neurula stage (st. 19), and in the head region and eye primordium at the tailbud stage (st. 25) (Fig. 1A, lower panels). Given these results and the major role of FoxM1 in G2–M cell-cycle progression (Laoukili et al., 2005; Wang et al., 2005), it might also do so in the neural plate on the injected side, whereas injection of control MO caused no appreciable reduction (Fig. 1B). We also counted the number of cells in the neural plate (isolated from st. 14 embryos) by staining their nuclei with Hoechst. Injection of FoxM1-MO, but not of control MO, caused a significant (~25%) reduction in cell numbers in the neural plate, as well as causing an enlargement and a stronger staining of nuclei (probably reflecting G2-phase arrest of the cell cycle) (Fig. 1C). Thus, intriguingly, FoxM1 is essential for normal cell proliferation in the neural plate, consistent with it being expressed in this region (Fig. 1A).

FoxM1 activates expression of G2–M cell-cycle regulators in the neural plate

Because FoxM1 activates many genes encoding G2–M cell-cycle regulators, such as Cdc25B and cyclin B, in cultured cells (Laoukili et al., 2005; Wang et al., 2005), it might also do so in the neural plate, thereby promoting cell proliferation in this region. When analyzed by WISH at the mid-neurula stage (st. 15-16), both Cdc25B and cyclin B3, like FoxM1, were found to be expressed in the neural plate (Fig. 1D, control MO). More importantly, pre-injecting FoxM1-MO into two-cell embryos significantly suppressed the expression of Cdc25B (in 81% of the injected embryos, n=52) and cyclin B3 (88%, n=51) in the neural plate (Fig. 1D, FoxM1-MO; see the right-hand, injected side of the embryos). To confirm these results, we also performed RT-PCR analysis of Cdc25B and cyclin B3 using whole embryos. FoxM1-MO injection at the one-cell stage caused a dramatic reduction in the expression of Cdc25B and cyclin B3 at the initial neurula stage (st. 13) (Fig. 1E). Furthermore, immunoblotting analysis, like immunostaining analysis (Fig. 1B), revealed a substantial decrease in pH3 levels in FoxM1-MO-treated embryos (Fig. 1E). Importantly, all of these effects of FoxM1-MO injection were rescued by co-injection of FoxM1-MO-resistant mRNA encoding wild-type FoxM1 (but not a transcriptionally inactive FoxM1 mutant, data not shown) (Fig. 1E). Thus, these results suggest that FoxM1 promotes cell proliferation in the neural plate, most probably by transcriptionally activating the expression of G2–M cell-cycle regulators (see also Fig. 4C).
Fig. 1. Requirement of FoxM1 for both cell proliferation and expression of G2–M cell-cycle regulators in the Xenopus neural plate. (A) Embryos were analyzed for FoxM1 expression by either RT-PCR (upper panel) or WISH (lower panels). In RT-PCR analysis, EF1-α was used as a loading control; ODC was also used as a loading control and was confirmed to be expressed throughout embryogenesis (data not shown). UFE, unfertilized egg; NF, Nieuwkoop-Faber. (B) Embryos injected with both lacZ mRNA (100 pg) and control MO or FoxM1-MO (18 ng) at one blastomere at the two-cell stage were cultured, fixed at st. 13, and analyzed by immunostaining with anti-pH3 antibody. A dorsal view of the embryos is shown (left panels, anterior up), with the injected side (β-Gal, light blue) being on the right of the midline (dotted). The area boxed in black is enlarged in the lower panels. (Right panel) Relative numbers of pH3-positive cells on the injected and uninjected sides of the neural plate (the area boxed in red) are shown, with the number on the uninjected side set at 1.0. Error bars indicate s.d. (n=10); *P<0.01. (C) Embryos co-injected with lacZ mRNA and either control MO or FoxM1-MO, as in B, were cultured until st. 14. Neural plates were isolated from these embryos, stained with Hoechst, and photographed (left panels) for Hoechst-stained nuclei (the injected side of the neural plate being on the right). (Right panel) Relative numbers of cells on the injected and uninjected sides of the neural plate are shown, with the number on the uninjected side set at 1.0. Error bars indicate s.d. (n=10); *P<0.01. (D) Embryos co-injected with lacZ mRNA and either control MO or FoxM1-MO, as in B, were fixed at st. 15-16 and analyzed by WISH for Cdc25B and cyclin B3. The injected side (β-Gal, red) is on the right. (E) Embryos pre-injected with control MO or FoxM1-MO (36 ng) at the one-cell stage were analyzed at st. 13 by either RT-PCR (upper panel) or immunoblotting (lower panel) for the indicated transcripts or proteins (EF1-α and ERK1 being loading controls). For a rescue experiment, embryos were co-injected with FoxM1-MO (36 ng) and FoxM1-MO-resistant FoxM1 mRNA (200 pg).

BMP inhibition induces cell proliferation in the neuroectoderm

Although cell proliferation is pronounced in the proneural region in many species (Hollyday, 2001; Saka and Smith, 2001) (Fig. 1B), little is known about the identity of the signaling that leads to this proliferation. In vertebrates, including Xenopus, neural induction involves signaling induced by FGF, Wnt, or by inhibition of BMP (Muñoz-Sanjuán and Brivanlou, 2002, Stern, 2005). To test whether any of these signaling pathways could induce cell proliferation in the neural plate (Fig. 1B), we performed animal cap assays, in which any of these signaling pathways could induce cell proliferation in any of these signaling pathways could induce cell proliferation in any of these signaling pathways could induce cell proliferation in the neural plate (or neuroectoderm) of Xenopus embryos.

BMP inhibition induces expression of FoxM1 and G2–M regulators in the neuroectoderm

We next asked whether BMP inhibition could induce the expression of FoxM1 and of any other direct cell-cycle regulators in animal caps. RT-PCR analysis revealed that BMP inhibition by Noggin greatly enhanced the expression of FoxM1, various G2–M cell-cycle regulators, such as Cdc25B, cyclin B1 and cyclin B3, and also of cyclin D1 in the animal cap (Fig. 2C, control MO). BMP inhibition by Chordin or dnBMPR (but not Wnt or FGF signaling, data not shown) could also do so (see Fig. S2A in the supplementary material), whereas forced activation of BMP signaling by ectopic BMPs or a constitutively active form of BMP receptor suppressed the expression of FoxM1 (and of the cell-cycle regulators, data not shown) in the Noggin-treated animal caps (see Fig. S2B in the supplementary material). Importantly, when co-treated with FoxM1-MO, Noggin failed to enhance not only the expression of the G2–M cell-cycle regulators but also cell proliferation, in the neural plate (or neuroectoderm) of Xenopus embryos.
regulator that would not be targeted by FoxM1 (see Fig. 2D). Finally, even ectopic expression of a transcriptionally active form of FoxM1 [FoxM1ΔN] (Lüscher-Firzlaff et al., 2006) alone was able to induce both the expression of the G2–M regulators (but not of cyclin D1) and cell proliferation in the animal cap, albeit significantly less strongly than Noggin could (Fig. 2D). Taken together, these results strongly suggest that BMP inhibition activates the expression of FoxM1 and hence that of G2–M cell-cycle regulators, thereby promoting cell proliferation in the neuroectoderm.

**FoxM1 is required for neural but not epidermal or muscular development**

The spatial expression pattern (Fig. 1A) and its expression and function after BMP inhibition (Fig. 2) suggest that FoxM1 might be involved in neural development. To test this, we injected FoxM1-MO into one-cell embryos and looked at FoxM1 loss-of-function phenotypes at the late tailbud stage (st. 33). These embryos showed severe defects, principally in head formation and eye development, whereas those treated with control MO were apparently normal (Fig. 3A, left). Furthermore, when examined by RT-PCR analysis, expression of N-tubulin, a definitive neuronal marker (Chitnis et al., 1995), was markedly reduced in FoxM1-MO-treated (but not control) embryos, whereas expression of M-actin (a muscle marker) and E-keratin (an epidermal marker) were unaffected (Fig. 3A, right). Importantly, all the effects observed with FoxM1-MO injection were rescued by co-injection of FoxM1-MO-resistant FoxM1 mRNA (Fig. 3A). Thus, these results show that FoxM1 is required specifically for neural development, but not for epidermal or muscular development.

**FoxM1 is required for neuronal differentiation but not specification**

To investigate the role of FoxM1 in neurogenesis in more detail, we next examined expression of several neural markers in mid-neurula embryos (st. 14 or 16) by WISH. Treating embryos with FoxM1-MO, but not control MO, markedly inhibited the expression of N-CAM as a pan-neural marker (91%, n=51) and of N-tubulin (which was expressed in stripes) (90%, n=60) (Fig. 3B). However, expression of Xngnr1, a proneural marker specifying primary neurons (Ma et al., 1996), was not affected by FoxM1-MO treatment (7%, n=43), whereas that of Sox2, an initial neural marker specifying neuroectoderm (Kishi et al., 2000), was slightly expanded (79%, n=63) (Fig. 3B). As a control, expression of MyoD, a mesodermal marker, was not affected by FoxM1-MO (4%, n=46). In these experiments, the decrease in N-tubulin (and N-CAM) expression induced by FoxM1-MO could have been due to the decrease in cell number. However, double staining of nuclei and N-tubulin mRNA revealed that, in the FoxM1-MO-treated (primary) neuronal region, the cell number was only moderately reduced (by about 25%), whereas the expression of N-tubulin was totally suppressed (see Fig. 1C and Fig. S3 in the supplementary material), indicating that the decrease in N-tubulin expression induced by the FoxM1-MO was not a matter of cell number. Thus, the present data seemed to suggest that FoxM1 is required for (primary) neuronal differentiation, but not for neuronal specification, in early embryos.

To confirm the requirement of FoxM1 for neuronal differentiation (but not specification), we also performed RT-PCR analysis of various marker genes using animal caps treated with Activin or Noggin. Activin treatment of animal caps induced the expression of M-actin, N-CAM and N-tubulin, as previously reported (Hemmati-Brivanlou and Melton, 1992) (Fig. 3C, control MO); notably, however, co-treatment with FoxM1-MO suppressed the expression of N-CAM and N-tubulin but not of M-actin (Fig. 3C, FoxM1-MO). By contrast, Noggin treatment induced the expression of Sox2 and N-CAM, as previously reported (Stern, 2005), and also of N-tubulin (Fig. 3D, control MO); however, co-treatment with FoxM1-MO suppressed the expression of N-CAM.
and N-tubulin but not of Sox2 (Fig. 3D, FoxM1-MO). Ectopic expression of FoxM1(ΔN) alone was not able to induce expression of the neural markers, including Sox2 (data not shown, but see Fig. 2D). Finally, and as expected, the onset of FoxM1 expression in Noggin-treated animal caps coincided with that of Sox2 but preceded that of N-CAM and N-tubulin (which occurred in this order) (Fig. 3E; see also Fig. 3E legend). Together with the results shown in Fig. 3B, these results strongly suggest that FoxM1 is required (albeit not sufficient) for neuronal differentiation, but not specification, in early neurogenesis.

**FoxM1-dependent G2–M cell-cycle progression is required for neuronal differentiation**

Given the requirement of FoxM1 (which targets several G2–M cell-cycle regulators) for both the proliferation and neuronal differentiation of neuroectodermal cells, FoxM1 might be involved in neuronal differentiation via its function in promoting G2–M cell-cycle progression. To test this possibility, we examined the requirement for G2–M cell-cycle regulators in neuronal differentiation. First, we examined the temporal expression patterns of cyclin B3 and Cdc25B, both of which are targets for FoxM1 (Figs 1, 2). RT-PCR analyses revealed that both cyclin B3 and Cdc25B mRNAs were expressed only zygotically after the blastula stage (Fig. 4A; see also Fig. S4A in the supplementary material). We then attempted to knock down cyclin B3 and Cdc25B mRNA, which strongly inhibited the expression of Cdc25B protein at the gastrula stage (see Fig. S4B in the supplementary material), caused obvious defects in head formation and eye development at the late tailbud stage (st. 32) (Fig. 4B). Specifically, at the initial neurula stage (st. 13), Cdc25B-MO caused a significant reduction in cell division in the neural plate (and in Noggin-treated animal caps, data not shown) (Fig. 4C), consistent with Cdc25B being expressed in this region (Fig. 1D). More importantly, Cdc25B-MO caused a significant reduction in cell division in the neural plate (and in Noggin-treated animal caps, data not shown) (Fig. 4C), consistent with Cdc25B being expressed in this region (Fig. 1D). More importantly, Cdc25B-MO caused a significant reduction in cell division in the neural plate (and in Noggin-treated animal caps, data not shown) (Fig. 4C), consistent with Cdc25B being expressed in this region (Fig. 1D). More importantly, Cdc25B-MO caused a significant reduction in cell division in the neural plate (and in Noggin-treated animal caps, data not shown) (Fig. 4C), consistent with Cdc25B being expressed in this region (Fig. 1D). 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More importantly, Cdc25B-MO caused a significant reduction in cell division in the neural plate (and in Noggin-treated animal caps, data not shown) (Fig. 4C), consistent with Cdc25B being expressed in this region (Fig. 1D). More importantly, Cdc25B-MO caused a significant reduction in cell division in the neural plate (and in Noggin-treated animal caps, data not shown) (Fig. 4C), consistent with Cdc25B being expressed in this region (Fig. 1D). More importantly, Cdc25B-MO caused a significant reduction in cell division in the neural plate (and in Noggin-treated animal caps, data not shown) (Fig. 4C), consistent with Cdc25B being expressed in this region (Fig. 1D).
Indeed, ectopic expression of Cdc25B efficiently rescued the effects of FoxM1-MO on both the external morphology and the expression of N-CAM and N-tubulin (Fig. 4F). Thus, these results indicate that FoxM1 is involved in neuronal differentiation via its function in promoting G2–M cell-cycle progression in neuroectodermal cells. This would in turn suggest that preceding, FoxM1-dependent cell division is required for differentiation of neuronal precursors in early Xenopus embryos (see Fig. 5 and Discussion).

DISCUSSION
In this study, we found that FoxM1 is expressed, and required for cell proliferation, in the neuroectoderm of Xenopus embryos. FoxM1 activated the expression of genes encoding G2–M cell-cycle regulators, such as Cdc25B and cyclin B3, thereby promoting cell proliferation in the neuroectoderm (Fig. 1). Furthermore, and importantly, inhibition of BMP signaling, which is central to neural induction in Xenopus (Stern, 2005), induced cell proliferation in the neuroectoderm by augmenting the expression of FoxM1 and its target G2–M cell-cycle regulators (Fig. 2). BMP is a member of the transforming growth factor-β (TGF-β) superfamily (Feng and Derynck, 2005), and TGF-β can inhibit proliferation of epithelial cells in mammals (Massagué et al., 2000). Interestingly, BMP (inhibition), like TGF-β (Ko et al., 1995), influenced the expression of the G1–S regulator cyclin D1 (Fig. 2C and see Fig. S2A in the supplementary material). More interestingly, forced activation of BMP signaling (in Noggin-treated animal caps) suppressed the expression of FoxM1 and G2–M cell-cycle regulators (see Fig. S2B in the supplementary material). Thus, it seems that active proliferation of (prospective) neuroectodermal cells after BMP inhibition is due, at least in part, to inhibition of the ability of BMP to inhibit FoxM1 expression and hence cell proliferation. Given the various roles of BMPs in embryogenesis (Hogan, 1996), our findings may imply that BMP signaling regulates cell proliferation, in addition to cell fate, in many situations. In any event, our results reveal that BMP inhibition induces not only neural induction, but also FoxM1-dependent cell proliferation, in the neuroectoderm.

We also found that FoxM1 is required for neural but not epidermal or muscular development (Fig. 3A). More specifically, FoxM1 was required for primary neuronal differentiation but not specification (Fig. 3B-E). Furthermore, and interestingly, FoxM1 was involved in neuronal differentiation via its function in promoting G2–M cell-cycle progression, or primarily via activating Cdc25B expression (Fig. 4). According to the temporal expression patterns of neural induction and differentiation markers in Xenopus (Chitnis et al., 1995; Ma et al., 1996; Mizuseki et al., 1998) (see also Fig. S5 in the supplementary material), neural induction (or specification) occurs during gastrulation (st. 10-12), whereas
primary neuronal differentiation occurs during the subsequent neurula stages (st. 13-16) (see Fig. 5). Moreover, according to previous reports (Hartenstein, 1989; Howe et al., 1995; Lamborghini, 1980), primary neuronal precursors presumably undergo only one or two rounds of cell division between the onset of gastrulation (st. 10) and neuronal differentiation (which is accompanied by cell-cycle exit) (st. 13-16). Therefore, our findings that Cdc25B, the FoxM1 target, begins to be expressed at st. 10 (see Fig. S4A in the supplementary material) and that FoxM1/Cdc25B-dependent cell division before or at st. 13 (Fig. 1B-E, Fig. 4C) is required for neuronal differentiation at st. 14 (Fig. 3B, Fig. 4D), would seem to suggest that FoxM1 functions to drive the immediate, preceding cell division(s) of primary neuronal precursors for their terminal differentiation (Fig. 5). Furthermore, the continued expression of FoxM1 (and its target G2–M cell-cycle regulators) in later neural tissues (Fig. 1A), as well as its requirement for head formation and eye development (Fig. 3A, Fig. 4B), suggests that FoxM1-driven cell division is also required to generate secondary neurons (Harris and Hartenstein, 1991; Hartenstein, 1989). Thus, it seems that FoxM1 functions to link cell division and neuronal differentiation in early Xenopus embryos.

In contrast to our study, previous studies showed that preceding cell division, after the onset of gastrulation, is not essential for neuronal differentiation in Xenopus embryos (Harris and Hartenstein, 1991; Rollins and Andrews, 1991; Yeo and Gautier, 2003). In these studies, however, cell division was inhibited using hydroxyurea and/or aphidicolin, either of which causes S-phase arrest (Dasso and Newport, 1990). Hence, it seems likely that a prolonged S phase caused by hydroxyurea/aphidicolin might allow, in some way, the expression of some crucial neurogenic regulator(s) that would normally be expressed just prior to, and function for, neuronal differentiation. Such a neurogenic regulator(s), however, would not be expressed or function in FoxM1-depleted embryos, in which neuroectodermal cells, although specified to neuronal precursors, cannot differentiate into neurons, most likely owing to G2-phase arrest. In any event, in normally developing embryos, cell division occurs and neuronal precursors advance to the postmitotic phase, only within which can the precursors differentiate into neurons (Hartenstein, 1989; Lamborghini, 1980). Our results suggest that FoxM1 is essential for such cell division and, hence, for neuronal differentiation (Fig. 5). Thus, it appears that under normal conditions, preceding cell division is required for neuronal differentiation.

The requirement of BMP inhibition for neural induction is highly conserved from Drosophila to mammals (Muñoz-Sanjuán and Brivanlou, 2002; Stern, 2005). Furthermore, in many species, neural precursors actively proliferate prior to their terminal differentiation (Graham et al., 2003; Hollyday, 2001). Thus, given our results, FoxM1 or some other functionally equivalent transcription factor(s) might also play an important role in the proliferation and differentiation of neural precursors in other species.

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Supplementary material
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References

Fig. 5. Model for the role of FoxM1 in primary neuronal differentiation in Xenopus embryos. BMP inhibition induces not only neural induction but also FoxM1 expression during gastrulation. FoxM1 activates the expression of G2–M cell-cycle regulators, such as Cdc25B and cyclin B3, and thereby drives the immediate, preceding cell division(s) of neuronal precursors for their differentiation. The temporal expression patterns of Cdc25B and cyclin B3 are from Fig. 4A and Fig. 5A in the supplementary material, whereas those of Sox2, Xngnr1, N-CAM and N-tubulin are from the published literature (as cited in the text) and Fig. 5S in the supplementary material. The final division (*) of neuronal precursors presumably occurs between st. 13 and 16, depending on the cells (Hartenstein, 1989), but mainly around st. 13 (Lamborghini, 1980). MBT, midblastula transition; G2/M, G2–M transition of the cell cycle; Hpf, hours post-fertilization.


