Development 135, 2023-2030 (2008) doi:10.1242/dev.019893

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

Hiroyuki Ueno[1], Nobushige Nakajo[1,*], Minoru Watanabe[2], Michitaka Isoda[1] and Noriyuki Sagata[1,3,*]

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 p27^{Xic1} in *Xenopus* (Vernon et al., 2003) and p27^{Kip1}/p57^{Kip2} (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 Foxm1 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,

[1]Department of Biology, Graduate School of Sciences, Kyushu University, Hakozaki 6-10-1, Fukuoka 812-8581, Japan. [2]Faculty of Integrated Arts and Sciences, The University of Tokushima, Minamijyousanjima-cho 1-1, Tokushima 770-8502, Japan. [3]CREST, Japan Science and Technology Agency, Nihonbashi, Tokyo 103-0027, Japan.

*Authors for correspondence (e-mails: nnakascb@mbox.nc.kyushu-u.ac.jp; nsagascb@mbox.nc.kyushu-u.ac.jp)

Accepted 7 April 2008

DEVELOPMENT

GCTTGTTCTCATGGTGTGACGGCTC; Cdc25B-MO, TGTGTGAGGCTCTGGCCTGGGAAC; and control MO, CCTCTTACCTCAGTTACAATTTATA.

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, CCGACCACCTCTTCCACTCCCAGC and D, GTCCAGCAGAATTTTGCTTAGACTGTCGT; Cdc25B U, ACGTGGAAGACTTTCTGCTGAAG and D, TCTCGCTTGCTCTTGTCTCCGG; cyclin B1 U, GATGGTGGATTATGATATGG and D, CCATTTCCACAACAACATCT; cyclin B3 U, CTTCCTGCGCAGATTTGCTA and D, TGTGAGTATTTGCTCCTCAC; cyclin D1 U, ACTGACTGAGGATACCAAGC and D, GGAGATGTCCACTTCATCCA; Sox2 U, GCTGCCCATGCACCGCTATGATG and D, TCACATGTGCGACAGAGGCAGCG.

Primer sets for N-CAM, N-tubulin, E-keratin, M-actin and EF1-α are described in Xenbase (http://www.xenbase.org/common).

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 (Hopwood et al., 1989). Staining for β-galactosidase (β-Gal) and phosphorylated histone H3 (pH3) were as described (Saka and Smith, 2002; Sive 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 $MgCl_2$, 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), in post-gastrula embryos FoxM1 might be involved in cell proliferation, particularly in the neural region. To test this possibility, we knocked down *FoxM1* using antisense morpholino oligos (MO), which, upon injection, were able to effectively suppress translation of ectopic *FoxM1* mRNA in early embryos (see Fig. S1 in the supplementary material). Injecting FoxM1-MO into one-cell embryos had no appreciable effects on the external morphology of embryos, at least until the mid-neurula stage (data not shown, but see Fig. 3B). Then we analyzed mitotic cells in the neural plate of early neurula embryos (st. 13) by immunostaining for mitotic Ser10-phosphorylated histone H3 (pH3) (Saka and Smith, 2001). Injection of FoxM1-MO into one blastomere of a two-cell embryo caused a ~70% reduction in the number of pH3-positive cells 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).

DEVELOPMENT

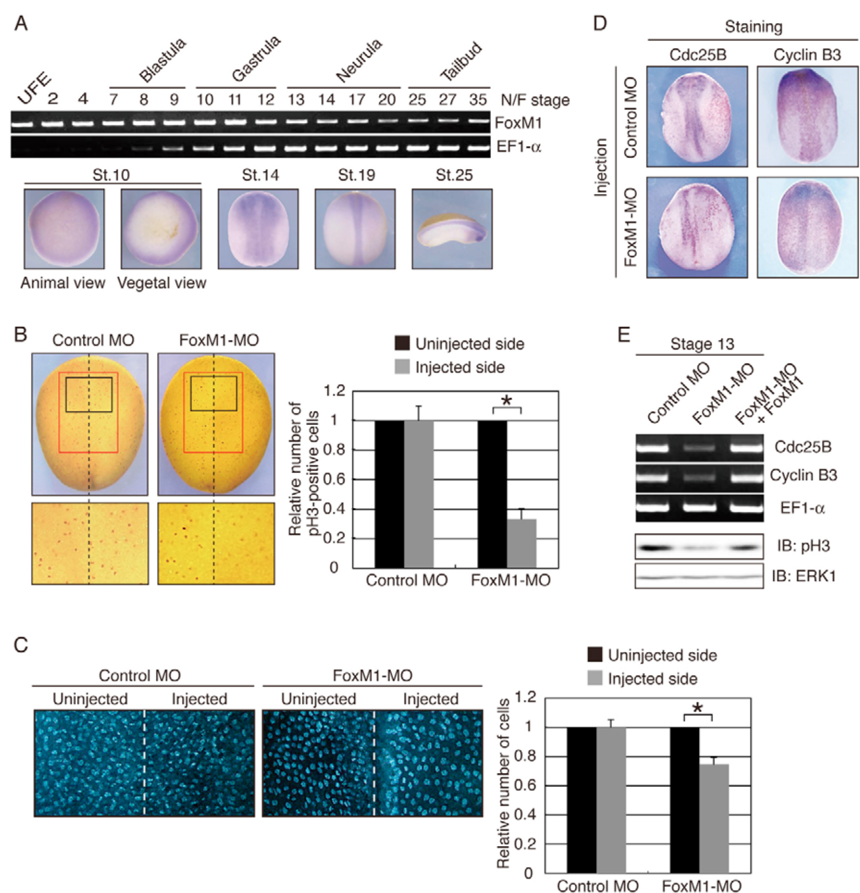

Fig. 1. Requirement of FoxM1 for both cell proliferation and expression of G2–M cell-cycle regulators in the *Xenopus* neural plate. (**A**) Embryos were analyzed for *FoxM1* expression by either RT-PCR (upper panel) or WISH (lower panels). In RT-PCR analysis, *EF1-α* was used as a loading control; *ODC* was also used as a loading control and was confirmed to be expressed throughout embryogenesis (data not shown). UFE, unfertilized egg; N/F, 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 the effect of a single signaling pathway on neural induction or differentiation can be tested (Stern, 2005). As revealed by immunoblotting of pH3, BMP inhibition by ectopically expressed Noggin [a BMP antagonist (Muñoz-Sanjuán and Brivanlou, 2002)], but not FGF or Wnt signaling, strongly induced cell proliferation in the animal cap, although the three signaling pathways (induced by Noggin, FGF or Wnt) were all effectively activated, as judged by expression of their downstream genes (*N-CAM*, *Xbra* and M-actin, respectively) (Fig. 2A). Similar to BMP inhibition by Noggin, BMP inhibition by Chordin (another BMP antagonist) or a dominant-negative BMP receptor (dnBMPR), also induced cell proliferation in the animal cap (see Fig. S2A in the supplementary material). Moreover, even overexpression of dnBMPR in early neurula embryos was able to significantly enhance cell proliferation in the ventral region (Fig. 2B). In *Xenopus*, inhibition of BMP signaling is initiated during gastrulation and is central to neural induction (Muñoz-Sanjuán and Brivanlou, 2002; Stern, 2005) (see also below). Thus, these results strongly suggest that BMP inhibition induces not only neural induction, but also 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 (analyzed by pH3 immunoblotting) (Fig. 2C, FoxM1-MO). FoxM1-MO did not affect the Noggin-induced expression of cyclin D1, a G1–S cell-cycle

regulator that would not be targeted by FoxM1 (see Fig. 2D). Finally, even ectopic expression of a transcriptionally active form of *FoxM1* [*FoxM1(ΔN)* (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.

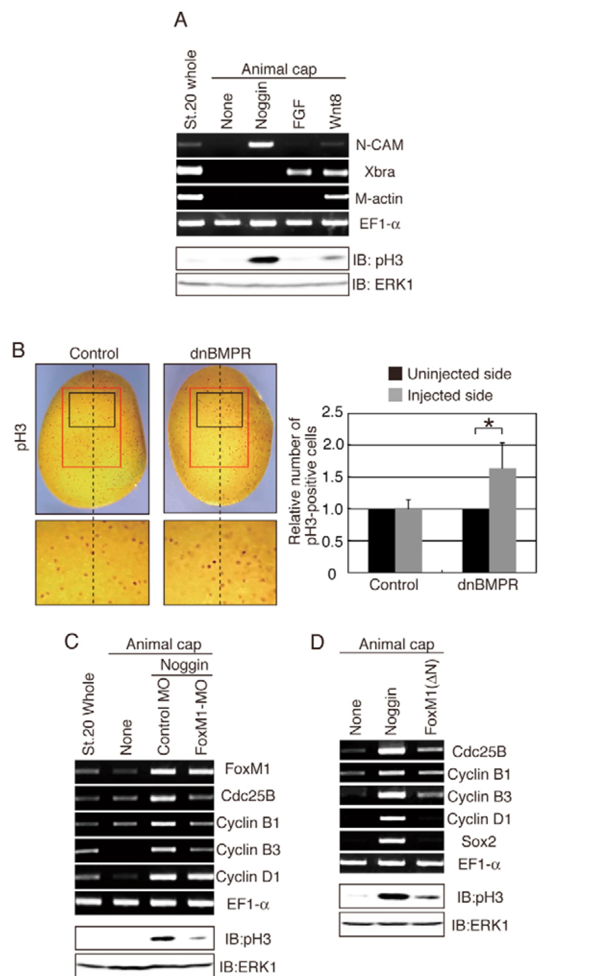

Fig. 2. Induction of both cell proliferation and expression of *FoxM1* and its target G2–M cell-cycle regulators by BMP inhibition. (**A**) Animal caps were isolated from late blastula *Xenopus* embryos (st. 9) pre-injected with Noggin mRNA (100 pg) or *Wnt8* mRNA (100 pg) at the one-cell stage; for FGF signaling, animal caps (from uninjected embryos) were treated with bFGF (100 ng/ml). The animal caps were cultured until sibling control embryos reached st. 20 and were then analyzed by RT-PCR (upper panel) or immunoblotting (lower panel). *N-CAM*, *Xbra* and M-actin are downstream markers of Noggin, FGF and Wnt, respectively. (**B**) Embryos pre-injected with *lacZ* mRNA (100 pg) together with or without *dnBMPR* mRNA (500 pg) at one animal-ventral blastomere at the eight-cell stage were cultured until st. 14. Embryos were then processed and analyzed as in Fig. 1B, except that the numbers of pH3-positive cells on the injected (β-Gal, light blue) and uninjected sides of the ventral region (the area boxed in red) were counted. *$P<0.01$. (**C**) Animal caps from the late blastula embryos pre-injected with Noggin mRNA (100 pg) and either control MO or FoxM1-MO (36 ng) at the one-cell stage were cultured as in A and analyzed by RT-PCR (upper panel) or immunoblotting (lower panel). (**D**) Animal caps pre-treated with Noggin mRNA (100 pg) or *FoxM1(ΔN)* mRNA (1 ng) were processed as in C.

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*

DEVELOPMENT

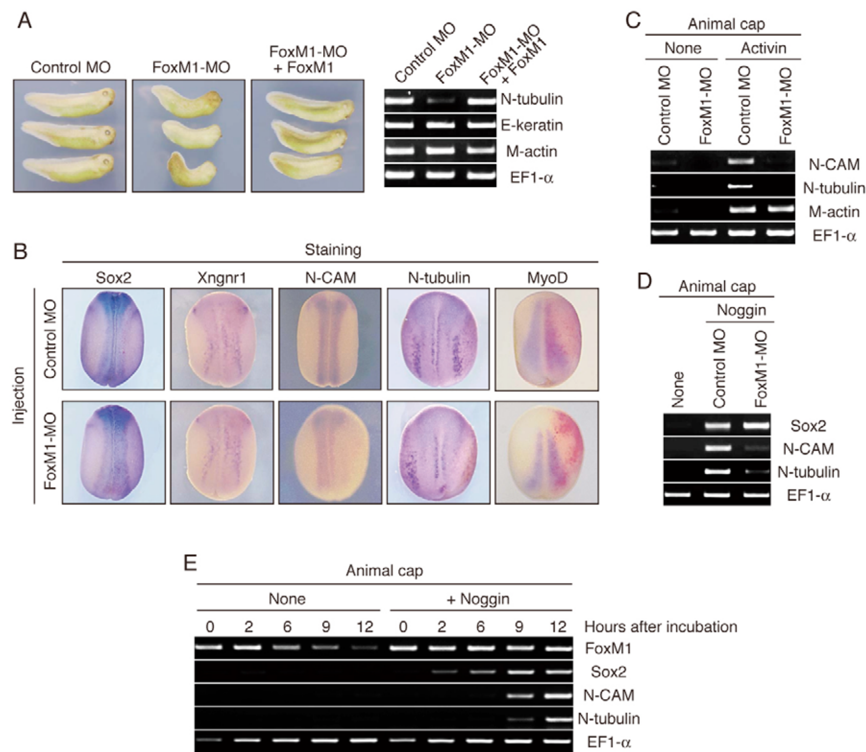

Fig. 3. Requirement of FoxM1 for neuronal differentiation but not specification. (**A**) *Xenopus* embryos pre-injected with control MO or FoxM1-MO (36 ng) together with or without *FoxM1* mRNA (200 pg) at the one-cell stage were cultured until st. 33 and photographed (left panels). For RT-PCR analysis, embryos were collected at st. 28 (right panel). (**B**) Embryos injected with *lacZ* mRNA (100 pg) and either control MO or FoxM1-MO (18 ng) at one blastomere at the two-cell stage were cultured until st. 14 for WISH analysis of *Xngnr1*, N-tubulin and *MyoD*, or until st. 16 for analysis of *Sox2* and *N-CAM*. In each panel, the injected side (β-Gal, red) of the embryo is on the right (dorsal view, anterior up). (**C**) Animal caps from the late blastula embryos pre-injected with control MO or FoxM1-MO (36 ng) at the one-cell stage were cultured with or without Activin (200 pM) until sibling control embryos reached st. 20 and then analyzed by RT-PCR. (**D**) Animal caps pre-treated with Noggin mRNA (100 pg) and either control MO or FoxM1-MO (36 ng) were cultured and analyzed as in C. (**E**) Animal caps pre-treated or not with Noggin mRNA (100 pg) were incubated for the indicated times and analyzed by RT-PCR. Whereas *FoxM1* expression in control animal caps decreased after 2 hours of incubation, that in Noggin-treated animal caps remained constant until 12 hours of incubation, indicating that the induction of *FoxM1* expression by Noggin began after 2 hours of incubation. Note that *Sox2* began to be expressed coincidently with *FoxM1*, whereas expression of *N-CAM* and N-tubulin began after *FoxM1*, in Noggin-treated animal caps.

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* by MO. MO against cyclin B3 mRNA had no appreciable effects on early embryogenesis (data not shown), probably owing to the presence of cyclin B1 and cyclin B2 (Hochegger et al., 2001). However, MO against *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 markedly inhibited the expression of N-tubulin (88%, n=60) in early neurula embryos (st. 14) (Fig. 4D); it also inhibited the expression of *N-CAM* and N-tubulin, but not of *Sox2* or M-actin, in the animal caps treated with Noggin or Activin (Fig. 4E). Clearly, all of these results (Fig. 4B-E) are very similar to those obtained with *FoxM1* knockdown (Fig. 1B, Fig. 3A-D), suggesting that Cdc25B acts downstream of FoxM1 not only for proliferation, but also for differentiation, of neuroectodermal cells.

DEVELOPMENT

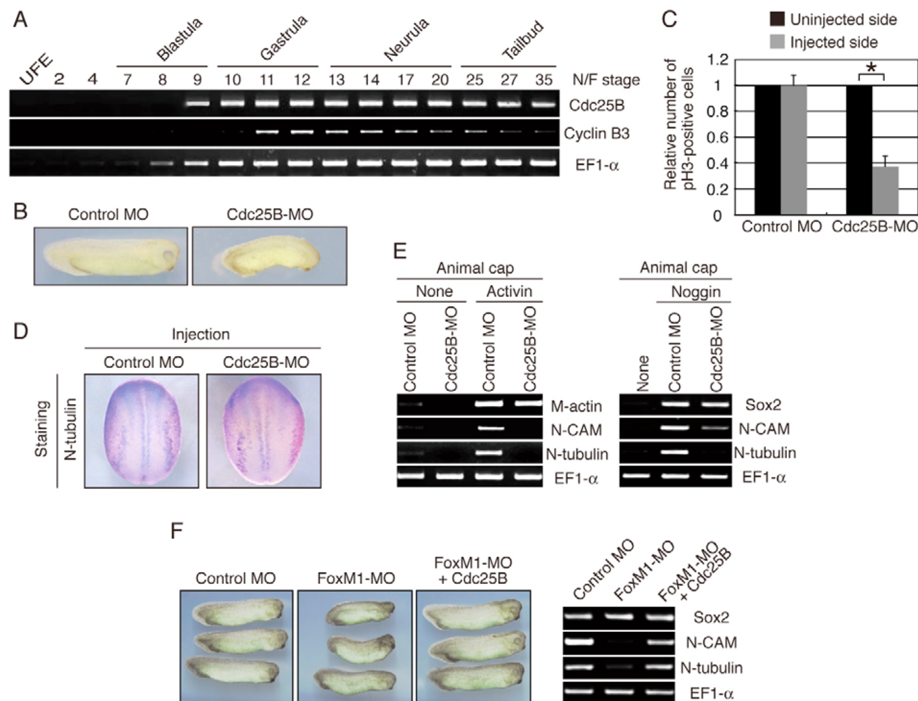

Fig. 4. Requirement of FoxM1-dependent G2–M progression for neuronal differentiation. (**A**) *Xenopus* embryos were analyzed for *Cdc25B* and cyclin B3 by RT-PCR. (**B**) Embryos pre-injected with control MO or Cdc25B-MO (18 ng) at the one-cell stage were cultured until st. 32 and photographed to show morphological phenotypes. (**C**) Embryos pre-injected with both *lacZ* mRNA (100 pg) and control MO or Cdc25B-MO (18 ng) at one blastomere at the two-cell stage were fixed at st. 13, immunostained for pH3, and analyzed as in Fig. 1B. *$P<0.01$. (**D**) Embryos were co-injected with *lacZ* mRNA (100 pg) and either control MO or Cdc25B-MO (18 ng) at one blastomere at the two-cell stage, cultured until st. 14, and analyzed by WISH. In each panel, the injected side (β-Gal, red) of the embryo is on the right (dorsal view, anterior up). (**E**) Animal caps from the late blastula embryos pre-injected with control MO or Cdc25B-MO (36 ng) at the one-cell stage were cultured with or without Activin (200 pM) until sibling embryos reached st. 20 and were then analyzed by RT-PCR (left panel). Animal caps pre-treated with Noggin mRNA (100 pg) and either control MO or Cdc25B-MO (36 ng) were cultured and analyzed by RT-PCR (right panel). (**F**) Embryos pre-injected with control MO or FoxM1-MO (36 ng) together with or without *Cdc25B* mRNA (200 pg) at the one-cell stage were cultured until st. 32 and photographed (left panels). For RT-PCR analysis (right panel), embryos were collected at st. 15.

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 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

DEVELOPMENT

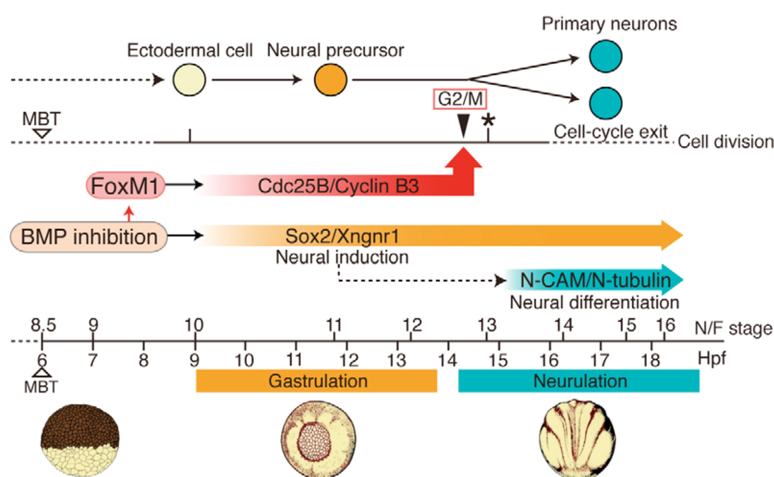

Fig. 5. Model for the role of FoxM1 in primary neuronal differentiation in *Xenopus* embryos. BMP inhibition induces not only neural induction but also *FoxM1* expression during gastrulation. FoxM1 activates the expression of G2–M cell-cycle regulators, such as *Cdc25B* and cyclin B3, and thereby drives 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. S4A 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. S5 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.

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.

We thank T. Kinoshita, Y. Sasai, M. Taira and the Xenopus Developmental Biology Database at NIBB for cDNA clones (N-tubulin, Sox2, Xngnr1 and N-CAM, respectively); members of the Sagata laboratory for discussions; and K. Gotoh for typing the manuscript. This work was supported by grants from the CREST Research Project of the Japan Science and Technology Agency and from the Ministry of Education, Culture, Sports, Science and Technology of Japan to N.S.

Supplementary material

Supplementary material for this article is available at http://dev.biologists.org/cgi/content/full/135/11/2023/DC1

References

Bertrand, N., Castro, D. S. and Guillemot, F. (2002). Proneural genes and the specification of neural cell types. *Nat. Rev. Neurosci.* **3**, 517-530.
Chitnis, A., Henrique, D., Lewis, J., Ish-Horowicz, D. and Kintner, C. (1995). Primary neurogenesis in *Xenopus* embryos regulated by a homologue of the *Drosophila* neurogenic gene Delta. *Nature* **375**, 761-766.
Cremisi, F., Philpott, A. and Ohnuma, S. (2003). Cell cycle and cell fate interactions in neural development. *Curr. Opin. Neurobiol.* **13**, 26-33.
Dasso, M. and Newport, J. W. (1990). Completion of DNA replication is monitored by a feedback system that controls the initiation of mitosis *in vitro*: studies in *Xenopus*. *Cell* **61**, 811-823.
Feng, X. H. and Derynck, R. (2005). Specificity and versatility in TGF-β signaling through Smads. *Annu. Rev. Cell Dev. Biol.* **21**, 659-693.
Graff, J. M., Thies, R. S., Song, J. J., Celeste, A. J. and Melton, D. A. (1994). Studies with a *Xenopus* BMP receptor suggest that ventral mesoderm-inducing signals override dorsal signals *in vivo*. *Cell* **79**, 169-179.
Graham, V., Khudyakov, J., Ellis, P. and Pevny, L. (2003). SOX2 functions to maintain neural progenitor identity. *Neuron* **39**, 749-765.
Harris, W. A. and Hartenstein, V. (1991). Neuronal determination without cell division in *Xenopus* embryos. *Neuron* **6**, 499-515.
Hartenstein, V. (1989). Early neurogenesis in *Xenopus*: the spatio-temporal pattern of proliferation and cell lineages in the embryonic spinal cord. *Neuron* **3**, 399-411.
Hemmati-Brivanlou, A. and Melton, D. A. (1992). A truncated activin receptor inhibits mesoderm induction and formation of axial structures in *Xenopus* embryos. *Nature* **359**, 609-614.
Hochegger, H., Klotzbücher, A., Kirk, J., Howell, M., Guellec, K. L., Fletcher, K., Duncan, T., Sohail, M. and Hunt, T. (2001). New B-type cyclin synthesis is required between meiosis I and II during *Xenopus* oocyte maturation. *Development* **128**, 3795-3807.

DEVELOPMENT

Hogan, B. L. (1996). Bone morphogenetic proteins: multifunctional regulators of vertebrate development. *Genes Dev.* **10**, 1580-1594.
Hollyday, M. (2001). Neurogenesis in the vertebrate neural tube. *Int. J. Dev. Neurosci.* **19**, 161-173.
Hopwood, N. D., Pluck, A. and Gurdon, J. B. (1989). MyoD expression in the forming somites is an early response to mesoderm induction in *Xenopus* embryos. *EMBO J.* **8**, 3409-3417.
Howe, J. A., Howell, M., Hunt, T. and Newport, J. W. (1995). Identification of a developmental timer regulating the stability of embryonic cyclin A and a new somatic A-type cyclin at gastrulation. *Genes Dev.* **9**, 1164-1176.
Karsten, S. L., Kudo, L. C., Jackson, R., Sabatti, C., Kornblum, H. I. and Geschwind, D. H. (2003). Global analysis of gene expression in neural progenitors reveals specific cell-cycle, signaling, and metabolic networks. *Dev. Biol.* **261**, 165-182.
Katoh, M. and Katoh, M. (2004). Human FOX gene family. *Int. J. Oncol.* **25**, 1495-1500.
Kishi, M., Mizuseki, K., Sasai, N., Yamazaki, H., Shiota, K., Nakanishi, S. and Sasai, Y. (2000). Requirement of Sox2-mediated signaling for differentiation of early *Xenopus* neuroectoderm. *Development* **127**, 791-800.
Ko, T. C., Sheng, H. M., Reisman, D., Thompson, E. A. and Beauchamp, R. D. (1995). Transforming growth factor-β1 inhibits cyclin D1 expression in intestinal epithelial cells. *Oncogene* **10**, 177-184.
Krupczak-Hollis, K., Wang, X., Kalinichenko, V. V., Gusarova, G. A., Wang, I. C., Dennewitz, M. B., Yoder, H. M., Kiyokawa, H., Kaestner, K. H. and Costa, R. H. (2004). The mouse Forkhead Box m1 transcription factor is essential for hepatoblast mitosis and development of intrahepatic bile ducts and vessels during liver morphogenesis. *Dev. Biol.* **276**, 74-88.
Lamborghini, J. E. (1980). Rohon-Beard cells and other large neurons in *Xenopus* embryos originate during gastrulation. *J. Comp. Neurol.* **189**, 323-333.
Laoukili, J., Kooistra, M. R. H., Brás, A., Kauw, J., Kerkhoven, R. M., Morrison, A., Clevers, H. and Medema, R. H. (2005). FoxM1 is required for execution of the mitotic programme and chromosome stability. *Nat. Cell Biol.* **7**, 126-136.
Lee, J. E., Hollenberg, S. M., Snider, L., Turner, D. L., Lipnick, N. and Weintraub, H. (1995). Conversion of *Xenopus* ectoderm into neurons by NeuroD, a basic helix-loop-helix protein. *Science* **268**, 836-844.
Lüscher-Firzlaff, J. M., Lilischkis, R. and Lüscher, B. (2006). Regulation of the transcription factor FOXM1c by Cyclin E/CDK2. *FEBS Lett.* **580**, 1716-1722.
Ma, Q., Kintner, C. and Anderson, D. J. (1996). Identification of neurogenin, a vertebrate neuronal determination gene. *Cell* **87**, 43-52.
Massagué, J., Blain, S. W. and Lo, R. S. (2000). TGF-β signaling in growth control, cancer, and heritable disorders. *Cell* **103**, 295-309.
Mizuseki, K., Kishi, M., Matsui, M., Nakanishi, S. and Sasai, Y. (1998). *Xenopus* Zic-related-1 and Sox-2, two factors induced by chordin, have distinct activities in the initiation of neural induction. *Development* **125**, 579-587.
Muñoz-Sanjuán, I. and Brivanlou, A. H. (2002). Neural induction, the default model and embryonic stem cells. *Nat. Rev. Neurosci.* **3**, 271-280.
Nakajo, N., Yoshitome, S., Iwashita, J., Iida, M., Uto, K., Ueno, S., Okamoto, K. and Sagata, N. (2000). Absence of Wee1 ensures the meiotic cell cycle in *Xenopus* oocytes. *Genes Dev*. **14**, 328-338.
Pohl, B. S., Rössner, A. and Knöchel, W. (2005). The Fox gene family in *Xenopus laevis*: FoxI2, FoxM1 and FoxP1 in early development. *Int. J. Dev. Biol.* **49**, 53-58.
Rollins, M. B. and Andrews, M. T. (1991). Morphogenesis and regulated gene activity are independent of DNA replication in *Xenopus* embryos. *Development* **112**, 559-569.
Saka, Y. and Smith, J. C. (2001). Spatial and temporal patterns of cell division during early *Xenopus* embryogenesis. *Dev. Biol.* **229**, 307-318.
Schüller, U., Zhao, Q., Godinho, S. A., Heine, V. M., Medema, R. H., Pellman, D. and Rowitch, D. H. (2007). Forkhead transcription factor FoxM1 regulates mitotic entry and prevents spindle defects in cerebellar granule neuron precursors. *Mol. Cell. Biol.* **27**, 8259-8270.
Shimuta, K., Nakajo, N., Uto, K., Hayano, Y., Okazaki, K. and Sagata, N. (2002). Chk1 is activated transiently and targets Cdc25A for degradation at the *Xenopus* midblastula transition. *EMBO J.* **21**, 3694-3703.
Sive, H. L., Grainger, R. M. and Harland, R. M. (2000). *Early Development of Xenopus laevis: A Laboratory Manual*. New York: Cold Spring Harbor Laboratory Press.
Smith, W. C. and Harland, R. M. (1992). Expression cloning of noggin, a new dorsalizing factor localized to the Spemann organizer in *Xenopus* embryos. *Cell* **70**, 829-840.
Stern, C. D. (2005). Neural induction: old problem, new findings, yet more questions. *Development* **132**, 2007-2021.
Vernon, A. E., Devine, C. and Philpott, A. (2003). The cdk inhibitor p27Xic1 is required for differentiation of primary neurones in *Xenopus*. *Development* **130**, 85-92.
Wang, I. C., Chen, Y. J., Hughes, D., Petrovic, V., Major, M. L., Park, H. Y., Tan, Y., Ackerson, T. and Costa, R. H. (2005). Forkhead Box M1 regulates the transcriptional network of genes essential for mitotic progression and genes encoding the SCF (Skp2-Cks1) ubiquitin ligase. *Mol. Cell. Biol.* **25**, 10875-10894.
Watanabe, M. and Whitman, M. (1999). FAST-1 is a key maternal effector of mesoderm inducers in the early *Xenopus* embryo. *Development* **126**, 5621-5634.
Ye, H., Kelly, T. F., Samadani, U., Lim, L., Rubio, S., Overdier, D. G., Roebuck, K. A. and Costa, R. H. (1997). Hepatocyte nuclear factor 3/fork head homolog 11 is expressed in proliferating epithelial and mesenchymal cells of embryonic and adult tissues. *Mol. Cell. Biol.* **17**, 1626-1641.
Yeo, W. and Gautier, J. (2003). A role for programmed cell death during early neurogenesis in *Xenopus*. *Dev. Biol.* **260**, 31-45.