Morphogenesis during *Xenopus* gastrulation requires Wee1-mediated inhibition of cell proliferation

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Summary

Major developmental events in early *Xenopus* embryogenesis coincide with changes in the length and composition of the cell cycle. These changes are mediated in part through the regulation of CyclinB/Cdc2 and they occur at the first mitotic cell cycle, the mid-blastula transition (MBT) and at gastrulation. In this report, we investigate the contribution of maternal Wee1, a kinase inhibitor of CyclinB/Cdc2, to these crucial developmental transitions. By depleting Wee1 protein levels using antisense morpholino oligonucleotides, we show that Wee1 regulates M-phase entry and Cdc2 tyrosine phosphorylation in early gastrula embryos. Moreover, we find that Wee1 is required for key morphogenetic movements involved in gastrulation, but is not needed for the induction of zygotic transcription. In addition, Wee1 is positively regulated by tyrosine autophosphorylation in early gastrula embryos and this upregulation of Wee1 activity is required for normal gastrulation. We also show that overexpression of Cdc25C, a phosphatase that activates the CyclinB/Cdc2 complex, induces gastrulation defects that can be rescued by Wee1, providing additional evidence that cell cycle inhibition is crucial for the gastrulation process. Together, these findings further elucidate the developmental function of Wee1 and demonstrate the importance of cell cycle regulation in vertebrate morphogenesis.

Key words: Wee1, Cell cycle, Gastrulation

Introduction

The generation of a complex multicellular organism from a single fertilized egg involves the precise control of cell division, cell-fate specification and morphogenesis. Specialized cell division cycles are a prominent feature of early embryogenesis in many organisms. In *Xenopus*, the predominant embryonic cell cycle is a rapid 30-minute cycle comprised solely of S and M phases (cell cycles 2-12). Modifications to this minimal cell cycle occur at crucial points during development (Kirschner et al., 1981; Newport and Kirschner, 1982; Maller et al., 2001). After fertilization, the first mitotic cell cycle contains two atypical gap phases that are thought to be necessary for the events unique to this cell cycle: rotation of the cortex, which determines the dorsal/ventral axis; and fusion of the sperm and egg pronuclei (Kirschner et al., 1981; Vincent et al., 1986). The subsequent eleven cell divisions (cell cycles 2-12) are 30-minute cell cycles that rapidly cellularize the embryo. These cell cycles are devoid of gap phases and have no growth or transcriptional requirements. Gap phases are re-introduced into the cell cycle at the mid-blastula transition (MBT; cell cycle 12) when zygotic transcription begins (Newport and Kirschner, 1982; Kimelman et al., 1987). During gastrulation (cycles 13-15), the cell cycle is expanded further, from 55 minutes to 4 hours, as the embryo undergoes a dramatic morphological transformation (Howe et al., 1995). Although these cell cycle modifications are known to occur at the same time as key developmental events, the role of these developmentally regulated cell cycle modifications (hereafter referred to as developmental transitions) in cell fate specification and morphogenesis is unclear.

In *Xenopus*, the regulation of M-phase entry by the Cyclin B/Cdc2 complex appears to be a major control point in the generation of these developmental transitions, as each transition is marked by an increase in the level of inactive tyrosine-phosphorylated Cdc2 (Ferrell et al., 1991). The Wee1 tyrosine kinase and the Myt1 dual-specificity kinase mediate the inhibitory phosphorylation of the Cdc2 subunit on Tyr 15 (Wee1) or Thr14/Tyr15 (Myt1). Dephosphorylation of these sites by the Cdc25 phosphatase family is required for activation of the complex. The balance between the inhibitory kinase activities and the activating phosphatase activities, in addition to the synthesis and degradation of the Cyclin B subunit, regulates CyclinB/Cdc2 activity and, consequently, M-phase entry (Dunphy, 1994; O'Farrell, 2001).

The expression pattern of various cell cycle regulators provides some insight into Cdc2 regulation during early *Xenopus* embryogenesis. In particular, members of the Cdc25 phosphatase and Wee-like kinase families display prominent changes in expression that coincide with the timing of the developmental cell cycle transitions. For example, even though the level of Cdc25C remains relatively constant throughout embryogenesis, maternal Cdc25A is not translated until cell

cycle 2 and is then degraded at the MBT (Kim et al., 1999; Izumi and Maller, 1995; Hartley et al., 1996). Of the Wee-like kinases, Myt1 is expressed throughout embryogenesis (Leise and Mueller, 2002), whereas the maternal Wee1 protein is translated at meiosis II and is degraded at mid-late gastrulation (Murakami and Vande Woude, 1998). A zygotic isoform of Wee1 (Wee1B/Wee2) is then expressed in late gastrula embryos at approximately the same time as the maternal Wee1 protein is degraded (Leise and Mueller, 2002; Okamoto et al., 2002).

In addition to the developmentally regulated pattern of synthesis and degradation, a further level of Wee1 regulation occurs through phosphorylation events. Both the MAPK and Chk1 kinases have been shown to positively regulate Wee1 (Walter et al., 2000; Lee et al., 2001). The identity of the MAPK phosphorylation site(s) are unknown; however, the Chk1-mediated phosphorylation of Wee1 occurs on S549 and confers binding to 14-3-3 proteins (Lee et al., 2001). Wee1 activity is also positively regulated by tyrosine autophosphorylation (Murakami et al., 1999). Tyrosine-phosphorylated Wee1 is observed in the first cell cycle, but not in the rapid cell cycles that follow (cycles 2-12). Consequently, in the first mitotic cell cycle, Cdc2 is maintained in an inactive tyrosine-phosphorylated state because of the increased biological activity of Wee1. Cdc2 is then dephosphorylated when Cdc25A is translated in cell cycle 2, triggering the transition to the rapid cleavage cell cycles (Murakami and Vande Woude, 1998; Kim et al., 1999; Murakami et al., 1999; Walter et al., 2000).

Previous studies have established that maternal Wee1 is an important cell cycle regulator during the first mitotic cell cycle; however, the fact that the protein persists until mid-gastrulation suggests that Wee1 may have additional roles at later times in development. In this report, we use a combination of biochemical and in vivo developmental approaches to examine the function of maternal Wee1 in later *Xenopus* embryogenesis. By using antisense morpholino oligonucleotides to deplete maternal Wee1 protein levels, we find that Wee1 is a crucial regulator of M-phase entry and is an essential component of vertebrate morphogenesis.

Materials and methods

Oocyte and embryo preparation

Oocytes, eggs and embryos were isolated as previously described (Murakami and Vande Woude, 1998) and staged according to Nieuwkoop and Faber (Nieuwkoop and Faber, 1994). Lysates were prepared in either modified EB (80 mM β-glycerolphosphate, 20 mM HEPES pH 7.5, 20 mM EGTA, 15 mM $MgCl_2$, 1 mM sodium vanadate, 50 mM NaF, 20 mM sodium pyrophosphate, 2 mM dithiothreitol, 1 mM pefabloc, 10 μg/ml aprotinin, 50 μg/ml leupeptin, 2 μg/ml pepstatin, 1 μM microcystin and 2.5 μM okadaic acid) or RIPA buffer (20 mM Tris pH 8.0, 137 mM NaCl, 10% glycerol, 1% NP-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate, 1 mM pefabloc, 10 μg/ml aprotinin, 50 μg/ml leupeptin, 2 μg/ml pepstatin, 1 μM microcystin and 2.5 μM okadaic acid) using 10 μl/oocyte or embryo.

Antibodies

The Wee1 antibody is described elsewhere (Murakami and Vande Woude, 1998). Cdc25 antibodies were generous gifts from W. Dunphy, A. Kumagai (CalTech, Cdc25C) and J. Maller (University of Colorado, Cdc25A). Anti-Flag was purchased from Sigma, anti-phospho-Cdc2 from Cell Signaling Technology, anti-Cdc2 and anti-His antibody from Santa Cruz Biotechnology, and anti-phospho-histone H3 from Upstate.

Antisense morpholino, RNA preparation and embryo injection

Antisense morpholino oligonucleotides (MO; Gene Tools LLC) were generated based on the *Xenopus* Wee1 genes isolated previously [GCCGTCCTCATTGCCGACACCTGGG (Mueller et al., 1995) and GCCATTCTCATTGTCACCACCTTGG (Murakami and Vande Woude, 1998)]. A 3:1 mixture of Mueller and Murakami MOs gave optimal Wee1 depletion and was used for all experiments. Control MO was obtained from Gene Tools, LLC. The MOs were resuspended at a concentration of 6.25 ng/nl and 2×20 ng were injected into two-cell embryos. For MO+RNA rescue experiments, 2 ng of in vitro transcribed RNA encoding the Wee1 isoform isolated by Murakami was co-injected with the MO-Wee1 mixture. For the Cdc25C+Wee1 rescue experiments, the indicated amounts of in vitro transcribed Wee1 RNA were co-injected with 4 ng Cdc25C RNA. WT, KD and YYY-FFF Wee1 have been previously described (Murakami et al., 1999); however, the versions used here contain the FLAG epitope tag at the C terminus. The Stop-Wee1 and Shift-Wee1 constructs encode either a stop codon or a frame shift mutation at amino acid positions 4 or 22, respectively. The His-tagged Cdc25C construct was a gift from T. Stukenberg (University of Virginia). In vitro transcribed RNA was prepared using the mMessage Machine kit (Ambion). For morphological analysis, 5-6 ng (1 ng/nl) of RNA was injected into the equatorial region of the dorsal blastomeres at the four-cell stage. Some injections also included 100 pg β-gal RNA. For biochemical analysis, animal cap assays and PH3 staining, both cells of two-cell embryos were injected with MO and/or RNA. For fate mapping analysis, both cells of two-cell embryos containing a clearly discernable dorsal/ventral pattern were injected with MO. At the 32-cell stage, 1 ng of β-gal RNA was injected into the B1 blastomeres. When the embryos reached stage 11-12, they were stained for β-gal activity using Red-Gal® substrate (Research Organics) and were bleached for clear visualization of stained cells (Sive et al., 2000).

Animal cap assay

Embryos were injected with MO at the two-cell stage and animal caps were isolated at stage 8.5-9. The caps were placed in 0.5×MBS (±50 ng/ml activin) and fixed in formaldehyde or MEMPA buffer at stage 22-23.

Analysis of zygotic transcription

Animal cap explants or whole embryos were harvested at stage 10.5. RNA was isolated using Trizol Reagent (Invitrogen) and cDNAs were synthesized with Superscript (Invitrogen). PCR reactions were performed using primers and conditions described at www.xenbase.org.

In situ hybridization and immunohistochemistry

In situ-hybridization for *Xenopus* brachyury, chordin and sox2 expression was performed as previously described (Sive et al., 2000) using embryos fixed in MEMPA buffer (0.1 M MOPS (pH 7.4), 2 mM EGTA, 1 mM $MgSO_4$, 3.7% paraformaldehyde). β-Galactosidase activity was visualized using the Red-Gal® substrate (Sive et al., 2000). Anti-phospho-histone H3 staining was performed as described elsewhere (Christen and Slack, 1999) using embryos fixed in Dent's fixative (80% methanol, 20% DMSO) and 1:500 dilution of the anti-phospho-histone H3 antibody.

Results

Steady state Wee1 protein levels are maintained by de novo protein translation

Maternal Wee1 protein levels remain relatively constant from

meiosis II until mid gastrulation. Although no cell-cycle-dependent degradation of Wee1 protein is observed in intact one-cell embryos, degradation of the nuclear fraction of Wee1 has been detected in cell-free extracts (Michael and Newport, 1998; Ayad et al., 2003). Therefore, to determine whether de novo protein translation contributes to the constant level of Wee1 observed in embryos, we examined the consequence of preventing Wee1 translation with antisense morpholino oligonucleotides (MO), the binding of which to sequences surrounding the initiation methionine blocks translation of the target protein (Heasman, 2002). Because MOs are highly sequence specific and the two reported maternal Wee1 sequences differ in six of the 25 nucleotides surrounding the initiation methionine (Mueller et al., 1995; Murakami and Vande Woude, 1998), we generated antisense MOs to both maternal Wee1 sequences and used a mixture of the two Wee1 MOs (MO-Wee1) for all experiments. Notably, there is no sequence homology between the maternal Wee1 isoforms and the zygotic Wee1B/Wee2 in this region. The Wee1 MOs were injected into both cells of a two-cell embryo and endogenous Wee1 protein levels were evaluated. As shown in Fig. 1A, reduction of Wee1 protein levels was first detected at stage 8 with maximal depletion occurring between stages 9-10. The MO-Wee1-induced depletion was not due to the general inhibition of translation, given that Wee1 protein expression could be restored to endogenous levels when in vitro transcribed RNA encoding wild-type (WT) Wee1 was co-injected with the MO-Wee1 (Fig. 1B). Therefore, we conclude that the constant level of Wee1 protein observed from meiosis II until gastrulation represents a steady state balance between degradation and de novo translation.

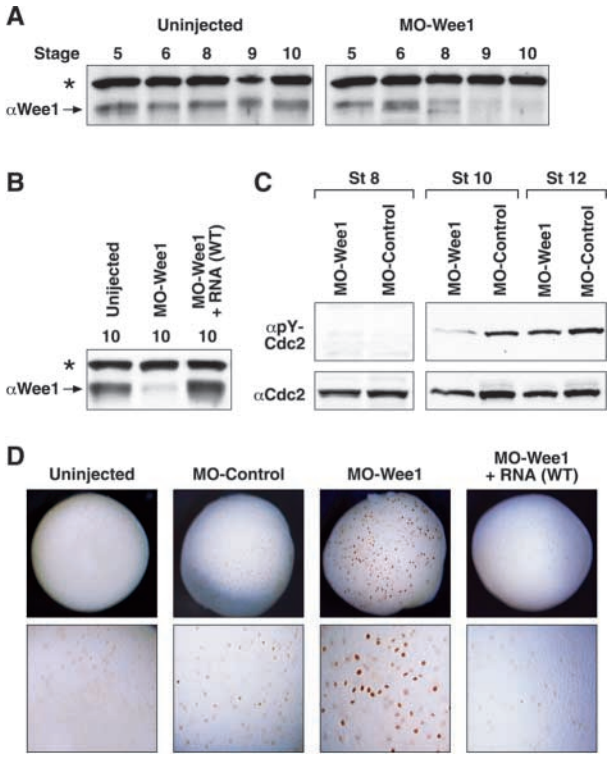

Fig. 1. Wee1-depletion using antisense morpholino oligonucleotides (MO). (A) MO-Wee1 was injected into both cells of a two-cell embryo. Uninjected and MO-Wee1-injected embryos were collected at various stages and lysates examined by immunoblot analysis using anti-Wee1 antibodies (* indicates a nonspecific band). (B) Two-cell embryos were injected with MO-Control, MO-Wee1 or co-injected with MO-Wee1 and WT Wee1 RNA (MO-Wee1+RNA). Embryos were collected at stage 10 and lysates examined as in A. (C) Embryos injected as in B were collected at stage 8, 10 or 12. Lysates were examined by immunoblot analysis using anti-phospho-Cdc2 or anti-Cdc2 antibodies. (D) The mitotic nuclei of injected stage 11 embryos were visualized by whole-mount immunostaining using phospho-histone H3 (αPH3). Mitotic index (*n*=10-12 embryos): MO-control, 8.9% (4739 nuclei); uninjected, 9.9% (1221 nuclei); MO-Wee1, 24.5% (4734 nuclei); MO-Wee1+RNA, 11.4% (2736 nuclei).

Wee1 mediates the tyrosine phosphorylation of Cdc2 in early, but not late, gastrula embryos

To evaluate the contribution of maternal Wee1 to Cdc2 regulation, we examined the effect of Wee1-depletion on the tyrosine phosphorylation state of Cdc2. For this analysis, embryos were collected at stage 10 (when maternal Wee1 is present and prior to zygotic Wee1B/2 translation), at stage 12 (after the degredation of maternal Wee1 and when zygotic Wee1B/2 is present) and also at stage 8 (a stage where minimal MO-induced Wee1 reduction is observed). When the tyrosine phosphorylation state of Cdc2 was examined (Fig. 1C), little inactive tyrosine-phosphorylated Cdc2 was detected in rapidly dividing stage 8 embryos and this level remained unchanged in embryos injected with MO-Wee1. By contrast, significantly higher levels of tyrosine-phosphorylated Cdc2 were observed in stage 10 and stage 12 embryos. As shown in Fig. 1C, depletion of maternal Wee1 reduced the levels of tyrosine-phosphorylated Cdc2 detected in the stage 10 embryos, but had little to no effect on stage 12 embryos. These findings indicate that maternal Wee1 contributes to Cdc2 tyrosine phosphorylation in early gastrula embryos (stage 10), but that other proteins, most likely zygotic Wee1B/Wee2 or Myt1, are required for the tyrosine phosphorylation of Cdc2 in late stage embryos (stage 12).

Wee1 regulates mitotic entry in intact embryos

Tyrosine phosphorylation inhibits the activity of the CyclinB/Cdc2 complex and thereby prevents entry into mitosis. Because Wee1 depletion reduces the level of Cdc2 tyrosine phosphorylation, Wee1-depleted embryos might be expected to contain an increased number of mitotic cells. To determine if this is the case, mitotic nuclei were visualized by immunostaining with an antibody recognizing a mitosis-specific antigen, phospho-histone H3. All nuclei were then visualized with DAPI and a mitotic index was determined. As depicted in Fig. 1D, a marked increase in the number of mitotic nuclei was detected in embryos injected with MO-Wee1, but not in MO-control embryos. This increase could be reversed by co-injection of exogenous WT-Wee1 RNA, suggesting that Wee1 regulates entry into mitosis in intact embryos.

Wee1 is required for *Xenopus* gastrulation

Strikingly, in the course of these studies, we found that Wee1 depletion severely altered the external morphology of gastrulating embryos. Gastrulation is the complex process whereby a hollow ball of cells (blastula) is transformed into a multi-layered embryo (gastrula). Involution begins on the dorsal side of the embryo at the blastopore lip. As gastrulation

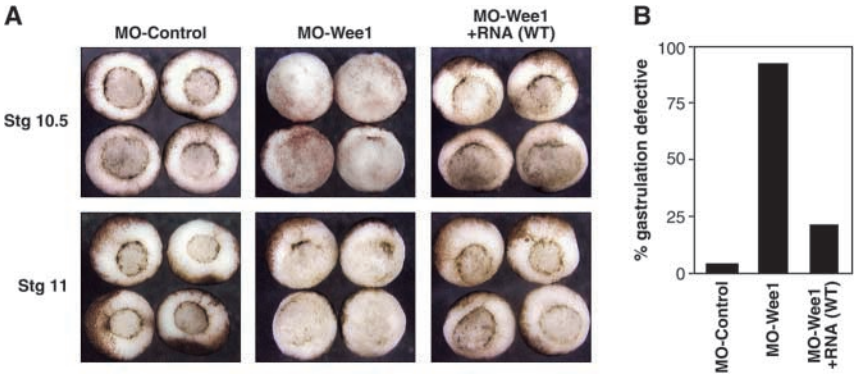

Fig. 2. Wee1 is required for *Xenopus* gastrulation. (A) Embryos injected with MO-Control, MO-Wee1 or MO-Wee1+WT Wee1 RNA were examined for blastopore formation at stage 10.5 and 11. MO-Wee1 disrupts blastopore formation, which is rescued by WT-Wee1 RNA. (B) Percentage of embryos with gastrulation defects: MO-control (*n*=64), MO-Wee1 (*n*=243) and MO-Wee1+WT RNA (*n*=47).

proceeds, the area of involuting cells spreads laterally and ventrally, resulting in the appearance of a circular 'yolk plug' in the vegetal region of the embryo. Because previous work had established that inhibiting cell division has little effect on *Xenopus* gastrulation (Cooke, 1973b; Cooke, 1973a; Gurdon and Fairman, 1986; Symes and Smith, 1987; Grainger and Gurdon, 1989), it seemed plausible that enhanced cell division would have no effect, or would simply advance the onset of gastrulation. Instead, we found that gastrulation was profoundly disrupted when M-phase entry was promoted by Wee1 depletion. At stages 10.5 and 11, when control embryos had a clearly defined blastoporal pigment line and blastoporal groove, MO-Wee1-injected embryos had minimal or no blastopore (Fig. 2). The gastrulation defects induced by Wee1-depletion could be reversed by coinjection of exogenous WT-Wee1 RNA (Fig. 2), indicating that these defects were due to the lack of Wee1 protein.

Wee1 depletion inhibits convergent-extension movement but not mesoderm gene expression in animal cap explants

Xenopus gastrulation requires the precise spatial and temporal induction of zygotic transcription coordinated with a series of complex morphogenetic movements. A subset of these events can be examined in ectodermal explants taken from the animal hemisphere of stage 8-9 embryos (hereon referred to as animal cap explants). Activin treatment of animal cap explants induces mesodermal gene expression and convergent-extension movements similar to those observed in the intact embryo (Smith, 1987; Symes and Smith, 1987; Keller and Danilchik, 1988). Therefore, to further address the role of Wee1 in gastrulation, we examined the effect of Wee1-depletion in animal cap explants. Two-cell embryos were injected with either MO-Wee1 or MO-control, and animal caps were prepared when the embryos reached stage 8.5-9. As depicted in Fig. 3A, untreated control explants remained ball shaped, whereas activin treatment induced the distinct elongation of the tissue. Wee1-depletion had no effect on the morphology of untreated explants, but greatly reduced the tissue elongation observed in activin-treated explants (78% showed reduced elongation *n*=49; Fig. 3A). This inhibition could be partially rescued by restoring Wee1 protein levels via mRNA co-injection.

Zygotic transcription begins at the MBT and lengthening of the cell cycle at this developmental stage has been proposed to contribute to the induction of zygotic transcription (Kimelman et al., 1987). Because Wee1 is a negative regulator of M-phase entry and Wee1 depletion results in increased cell cycle progression, it is possible that the gastrulation defects induced by Wee1 depletion are simply a consequence of suppressed zygotic transcription. Therefore, we examined the effect of Wee1 depletion on the expression of three zygotically transcribed genes: brachyury, goosecoid and chordin. By RT-PCR, no gene expression was observed in the absence of activin treatment; however, each of the genes was expressed in all of the activin-treated explants (Fig. 3B). Collectively, these results suggest that the enhanced cell cycle progression resulting from Wee1-depletion does not inhibit zygotic gene expression, but instead, affects the ability of the tissue to initiate the normal program of convergent-extension movements.

Wee1-depletion disrupts morphogenetic movements in the intact embryo

Convergent-extension is one of several different types of tissue movement involved in gastrulation. To further assess the effect of Wee1-depletion on cell movement and morphogenesis in vivo, we took advantage of the fate-mapping studies performed by Bauer et al. (Bauer et al., 1994). For these experiments, we followed the fate of the B1 dorsal blastomere [for nomenclature see elsewhere (Dale and Slack, 1987; Bauer et al., 1994)] because the progeny of this cell undergo extensive movement during gastrulation. Embryos were injected with MO-Wee1, MO-Wee1 + WT RNA or MO-Control at the two-cell stage. β-Galactosidase RNA was then injected into both B1 blastomeres at the 32-cell stage, and the migration pattern of the B1 progeny was determined at stage 11.5-12 by visualization of the β-gal-positive cells. At stage 11.5, the B1 clone normally forms a narrow column on the dorsal side of the embryo, extending from the edge of the blastopore into the animal hemisphere. As shown in Fig. 3C, this localization pattern was observed in uninjected and control MO-injected embryos. In marked contrast, B1-derived cells were spread broadly across the dorsal equator in Wee1-depleted embryos (Fig. 3C), consistent with the pre-gastrula fate map prior to any cell movements. These cell migration defects were significantly reverted by co-injection of exogenous WT-Wee1 RNA (Fig. 3C).

Next, we assessed the internal morphology of the embryos by bisecting the area of β-gal staining. In control embryos, involution was clearly observed and was greater on the dorsal side. The β-gal positive cells from the B1 clone were dorsally located, extending from the blastopore lip into the animal hemisphere (Fig. 3D). By contrast, no involution was observed in Wee1-depleted embryos (Fig. 3D), consistent with the

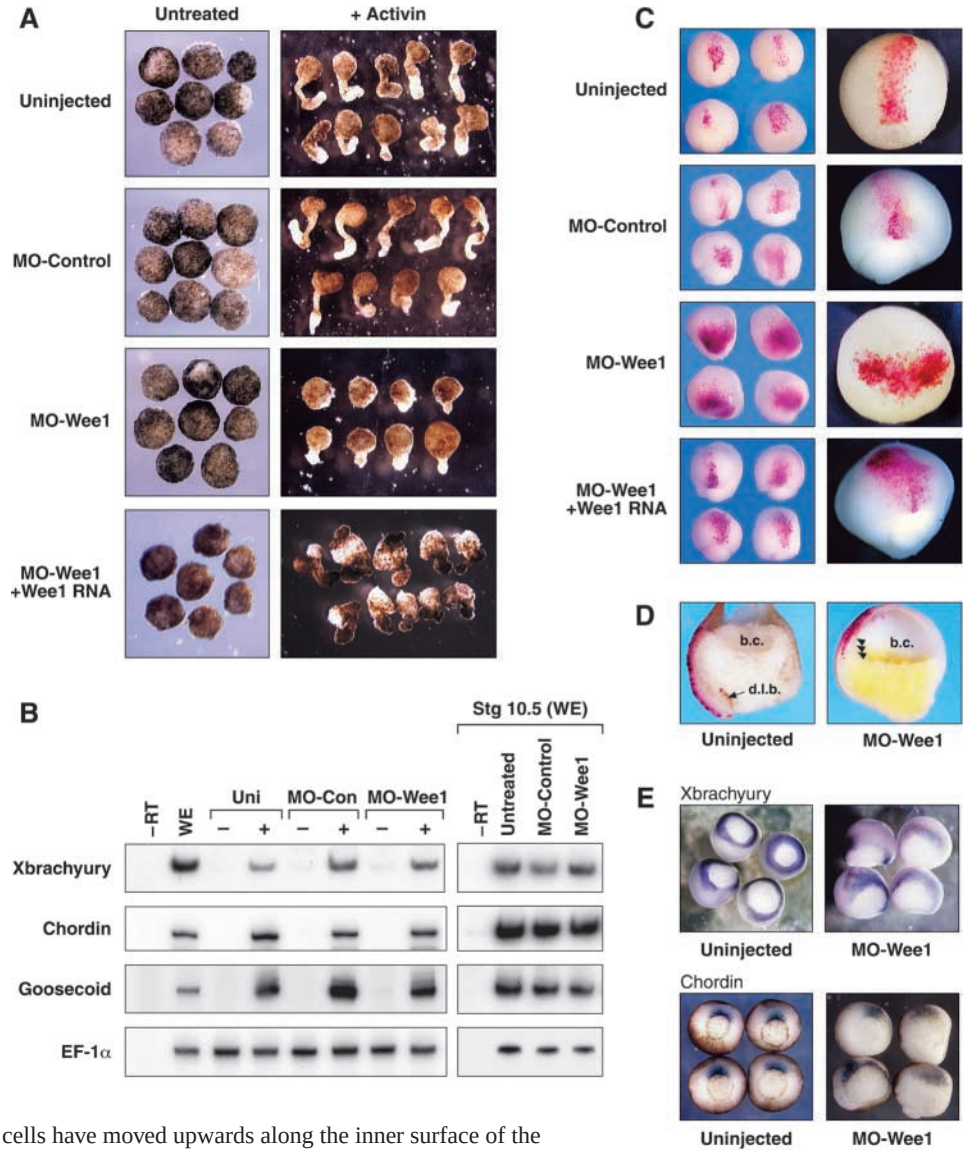

Fig. 3. Wee1 depletion inhibits morphogenesis but not zygotic gene expression. (A) Animal cap explants prepared from uninjected embryos and embryos injected with MO-Control, MO-Wee1 or MO-Wee1+WT Wee1 RNA were left untreated or were treated with activin and cultured until stage 22-23. (B) RNA was isolated from animal cap explants prepared as in A and from stage 10.5 whole embryos (WE) that were either uninjected or had been injected with Control-MO or MO-Wee1 at the two-cell stage. Expression of brachyury, goosecoid and chordin was examined by RT-PCR analysis. cDNA levels were normalized to EF-1α, and a sample lacking reverse transcriptase (–RT) was also included. (C) Two-cell embryos were injected with MO-control, MO-Wee1 or MO-Wee1 + WT RNA. β-Gal RNA was injected into the B1 blastomeres at the 32-cell stage and β-gal activity visualized at stage 11.5-12. The B1 clone forms a narrow midline band extending between the blastopore (bottom) and animal hemisphere (top) in uninjected (*n*=12) and MO-Control injected embryos (*n*=23), while the B1 progeny form a broad band across the dorsal equator in MO-Wee1 embryos (MO-Wee1; *n*=36). this defect is significantly reversed by co-injection of WT Wee1 RNA (MO-Wee1+WT RNA; *n*=16). (D) The embryos shown in C were bisected through the area of β-gal staining. In uninjected embryos, the labeled cells extend from the animal hemisphere (top) to the dorsal blastopore lip (dbl). In Wee1-depleted embryos (MO-Wee1), no epibolic spread towards the vegetal pole (bottom) or involution occurs. However, some of the inner vegetal cells have moved upwards along the inner surface of the blastocoel roof (b.c.; arrow heads). (E) Expression of Xbrachyury (upper two panels, MO-Wee1, *n*=67) and chordin (MO-Wee1, *n*=54, lower two panels) was determined by in situ histochemistry (blue staining).

external morphology (Fig. 2). We also found that epiboly was somewhat impaired. Epiboly is a process that involves the radial intercalation of cells in the blastocoel roof and results in the vegetal migration of cells from the equator (Gerhart and Keller, 1986; Keller and Winklbauer, 1992). As shown in Fig. 3D, the β-gal positive cells in Wee1-depleted embryos did not migrate vegetally, as was observed in the control embryos. However, some interior yolky vegetal cells on the dorsal side of the embryo did migrate upward along the inner surface of the blastocoel roof (arrowheads, Fig. 3D). Taken together, these findings show that Wee1 depletion severely impairs several of the major morphogenetic movements involved in gastrulation.

Wee1-depletion does not prevent zygotic gene expression in vivo

To further verify that the morphological defects were due to impaired tissue movement and not to inhibition of zygotic gene expression, we examined the expression of brachyury, chordin and goosecoid in whole embryos collected at stage 10.5. By RT-PCR analysis, all three zygotic genes were transcribed in the MO-Wee1-injected embryos, consistent with the results observed in the animal cap assays (Fig. 3B). When the expression pattern of brachyury and chordin was examined by in situ hybridization, we found that although both genes were expressed, their expression domain was more diffuse in Wee1-depleted embryos, consistent with a reduction in morphogenetic movements that would normally compress the mesoderm to the rim of the blastopore lip (Fig. 3E). Sox2 expression in the presumptive neural plate was similarly affected (data not shown). Thus, although the transcription of these zygotic genes is induced at the appropriate time, the spatial pattern of expression is somewhat altered.

Wee1 tyrosine phosphorylation in post MBT and gastrula embryos

The above findings indicate that Wee1 suppresses entry into

mitosis by inhibiting Cdc2 and that this function is crucial for the elaborate tissue movements involved in vertebrate gastrulation. The depletion experiments, however, do not define the time at which Wee1 function is required. Therefore, to gain insight into the timing and mechanism of Wee1 function, we examined the level and phosphorylation state of Wee1 and several other cell cycle components during *Xenopus* embryogenesis (Fig. 4). Consistent with previous reports (Kim et al., 1999), Cdc25A was detected in rapidly dividing embryos (stage 7 and 8) but was not observed in post-MBT embryos. Cdc25C levels remained constant from meiosis I (oocyte) through gastrulation (stage 11.5), as did the total levels of Cdc2. However, inactive tyrosine-phosphorylated Cdc2 was significantly greater in stage VI oocytes, in the first mitotic cell cycle (egg 30′) and post-MBT embryos (stages 9,10 and 11.5). As previously reported (Murakami and Vande Woude, 1998), the Wee1 protein was present from meiosis II (egg) until early-gastrulation (stage 10). Tyrosine-phosphorylated Wee1 was detected in the first mitotic cell cycle and strikingly, was also observed after the MBT and during early gastrulation (stage 9 and 10), coincident with the appearance of increased levels of tyrosine-phosphorylated Cdc2.

In the first mitotic cell cycle Wee1 phosphorylates itself on tyrosine residues at amino acid positions 90, 103 and 110, and the phosphorylation of these sites positively regulates the biological activity of Wee1 (Murakami et al., 1999). To address whether the Wee1 residues tyrosine-phosphorylated after the MBT are the same as those phosphorylated in the first mitotic cell cycle, we first generated FLAG-epitope tagged Wee1 constructs encoding WT-Wee1, kinase-dead Wee1 (KD-Wee1) and a Wee1 protein in which Tyr90, Tyr103 and Tyr110 were mutated to phenylalanine (YYY/FFF-Wee1). The FLAG-tagged Wee1 proteins were then expressed in developing embryos and their phosphorylation state examined. Consistent with the tyrosine phosphorylation profile of endogenous Wee1, exogenous FLAG-tagged WT Wee1 was tyrosine phosphorylated after the MBT and at gastrulation (stages 8-10, Fig. 5A). Significantly, no tyrosine-phosphorylated KD- or YYY/FFF-Wee1 was detected at any point during early embryogenesis, even though both mutants were well expressed (Fig. 5A). The finding that KD-Wee1 is not tyrosine phosphorylated demonstrates that the post-MBT tyrosine phosphorylation is most likely to be due to autophosphorylation. Moreover, the lack of YYY/FFF-Wee1 tyrosine phosphorylation indicates that the sites phosphorylated post-MBT are the same as those previously identified and which positively regulate Wee1 activity.

The upregulation of Wee1 activity is required for normal gastrulation

The developmental time course of Wee1 tyrosine phosphorylation suggests that the upregulation of Wee1 activity induced by these phosphorylation events may be important for normal gastrulation. To address this issue, we examined whether the KD- or YYY/FFF-Wee1 proteins could functionally replace the endogenous Wee1 kinase. As shown in Figs 2 and 3, injection of exogenous RNA encoding WT Wee1 largely reverts the defects induced by Wee1-depletion. Therefore, we repeated the MO-Wee1-depletion experiments, co-injecting RNAs encoding either KD- or YYY/FFF-Wee1. Strikingly, neither KD- nor YYY/FFF-Wee1 was able to rescue the gastrulation defects induced by MO-Wee1 (Fig. 5B,C). These results demonstrate that the intrinsic kinase activity as well as the upregulation of Wee1 activity are required for Wee1 function in early gastrula embryos. In addition, these findings confirm that the rescue mediated by coinjection of WT-Wee1 RNA with MO-Wee1 is the result of functional Wee1 protein expression and not due to sequestration of the antisense-MO by exogenous Wee1 RNA.

The KD- and YYY/FFF-Wee1 mutants act as dominant inhibitors of Wee1 function in early gastrula embryos

For various protein kinases, inactive mutants or those that

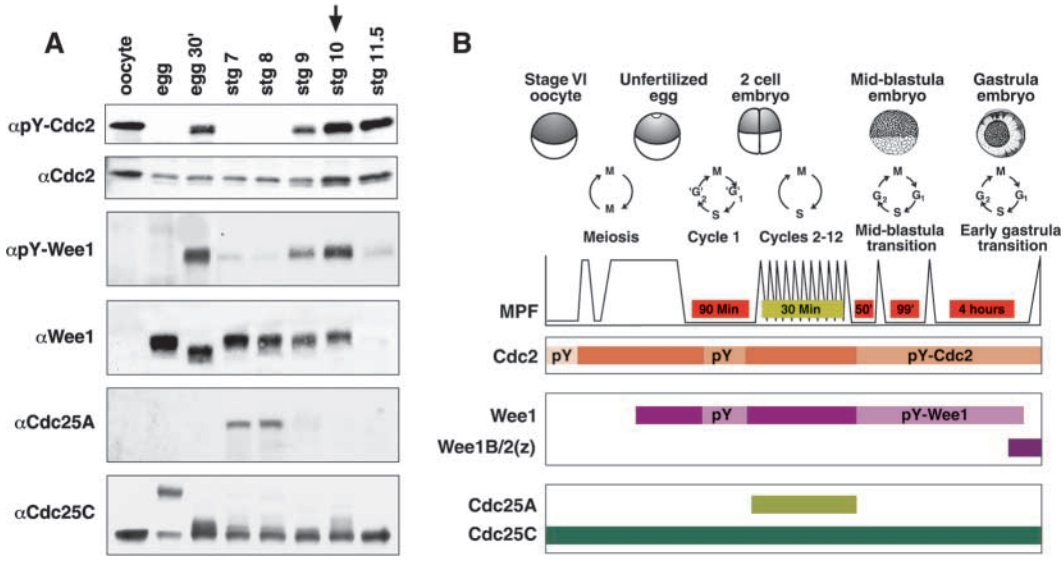

Fig. 4. Wee1 is tyrosine phosphorylated at the MBT and gastrulation. (A) Lysates prepared from stage VI oocytes (oocyte), eggs arrested at metaphase of meiosis II (egg), embryos in the first mitotic cell cycle (egg 30 minutes), cleavage stage embryos (stage 7 and 8) and embryos after the MBT (stage 9), the early gastrula stage (stage 10), and at mid-gastrula stage (stage 11.5) were examined by immunoblot analysis using antibodies recognizing Wee1, Cdc25A, Cdc25C, phospho-Cdc2 and total Cdc2. One oocyte or embryo equivalent was loaded per lane. Tyrosine-phosphorylated Wee1 was detected by probing Wee1 immunoprecipitates with anti-phosphotyrosine antibody. (B) Depicted are developmental expression profiles of members of the Wee1 kinase and Cdc25 phosphatase families and the developmentally regulated tyrosine phosphorylation (pY) of Cdc2 and Wee1.

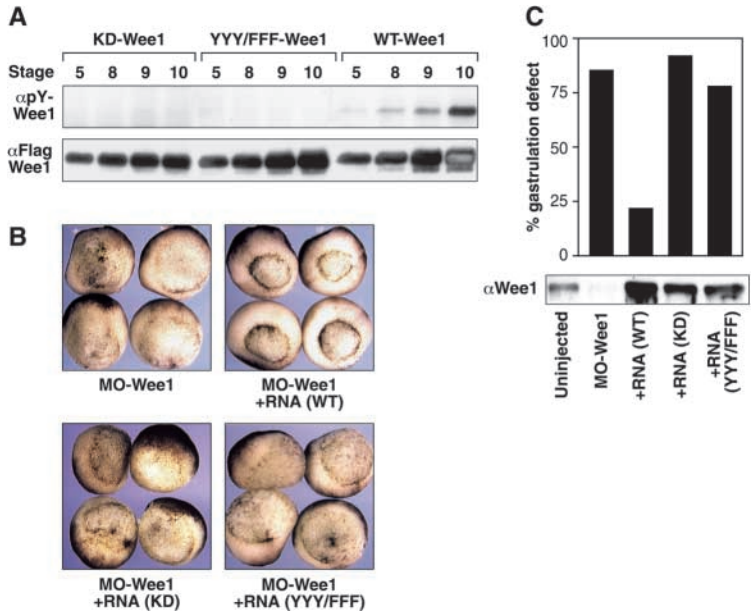

Fig. 5. Upregulation of Wee1 activity by tyrosine autophosphorylation is required for normal gastrulation. (A) Both cells of a two-cell embryo were injected with RNAs encoding KD-Wee1, YYY/FFF-Wee1 or WT-Wee1. Anti-Flag immune complexes isolated from lysates prepared at stages 5, 8, 9 and 10 were examined by immunoblot analysis using anti-phosphotyrosine and anti-FLAG antibodies. (B,C) Embryos injected with MO-Wee1 alone, MO-Wee1+RNA (WT), MO-Wee1+RNA (KD) or MO-Wee1+RNA (YYY/FFF) were scored for gastrulation defects at stage 11. Number of embryos examined: MO-Wee1 (n=17); MO-Wee1+WT Wee1 (n=34); MO-Wee1+KD-Wee1 (n=42); MO-Wee1+YYY/FFF-Wee1 (n=35). Rescue RNAs were injected at 2 ng/embryo. Lysates from the injected embryos were examined by immunoblot analysis using anti-Wee1 antibody.

cannot be activated have been found to function in a dominant inhibitory manner, presumably interfering with the ability of their endogenous counterparts to interact with upstream activators and/or downstream substrates. Therefore, we next examined whether KD- or YYY/FFF-Wee1 would act as dominant inhibitors. For these experiments, two additional Wee1 constructs were generated, one that encodes a stop codon at amino acid 4 (Stop-Wee1) and one that contains a frame-shift mutation resulting in truncation of the protein at amino acid 22 (Shift-Wee1). RNAs encoding the Wee1 constructs were injected into the two dorsal blastomeres of a four-cell embryo, and embryos were subsequently examined at stage 11.5-12. As depicted in Fig. 6, ectopic expression of either KD- or YYY/FFF-Wee1 produced gastrulation defects (Fig. 6A,B). However, in contrast to the MO-Wee1-injected embryos (Fig. 2), only part of the blastopore was disrupted. By co-injecting β-gal RNA to delineate the region of the embryos expressing the exogenous RNA-encoded proteins, we found that the incomplete disruption of the blastopore was due to limited diffusion of the injected RNAs (Fig. 6A). Injection of control RNAs, Stop-Wee1, Shift-Wee1 or β-gal alone had no effect on gastrulation (Fig. 6A), indicating that the defects observed were not simply due to RNA injection. Thus, both KD- and YYY/FFF-Wee1 act in a dominant inhibitory manner to suppress normal gastrulation, further supporting the model that intrinsic kinase activity and tyrosine phosphorylation are required for Wee1 function in the early gastrula embryos.

Cell cycle down regulation is required for normal gastrulation

To verify that the cell cycle regulatory function of Wee1 is crucial for normal gastrulation, we examined the consequence of overexpressing Cdc25C, a phosphatase that activates Cdc2, on *Xenopus* gastrulation. RNA encoding Cdc25C was injected into the two dorsal blastomeres of a four-cell embryo, and embryos were monitored as described above. Strikingly, we found that overexpression of Cdc25C disrupted blastopore formation in the area expressing the exogenous protein (Fig. 7A,B). In addition, when compared with control embryos, those overexpressing Cdc25C had reduced levels of Cdc2 tyrosine phosphorylation (Fig. 7A) and a greater number of mitotic cells (Fig. 7C). Given that the Cdc25C phosphatase and the Wee1 kinase have opposing effects on Cdc2 activity, it would be expected that the defects of Cdc25C overexpression might be reversed by increasing the expression of Wee1. Indeed, as shown in Fig. 7D,E, the gastrulation defects induced

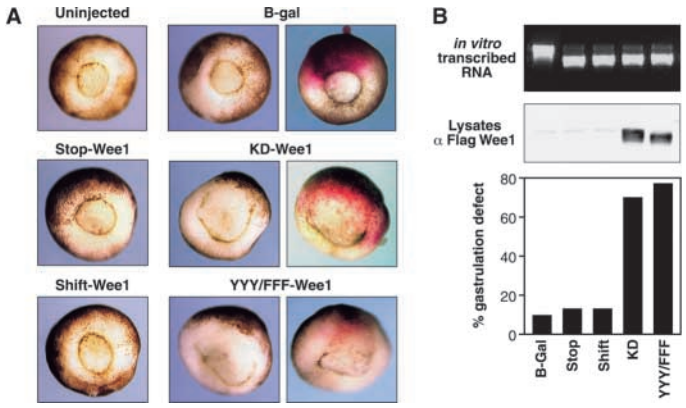

Fig. 6. KD-Wee1 and YYY-FFF-Wee1 act as dominant-inhibitors to disrupt gastrulation. The two dorsal blastomeres of four-cell embryos were injected with 5-6 ng of RNA encoding β-gal, KD-, YYY/FFF-, Shift- or Stop-Wee1 and embryos were examined for blastopore formation at stage 11.5-12. Co-injection of Wee1 and β-gal (100 pg) and the subsequent staining for β-gal activity (red) shows the area of the embryo expressing the exogenous RNAs. (B) RNAs used in A were analyzed by agarose gel electrophoresis and ethidium bromide staining (top panel). Lysates prepared from embryos in A were examined by immunoblot analysis using the FLAG antibody (middle panel). Percentage of embryos with gastrulation defects (bottom panel). Number of embryos examined: β-gal (n=55), Stop- (n=78), Shift- (n=72), KD- (n=82) and YYY/FFF-Wee1 (n=86).

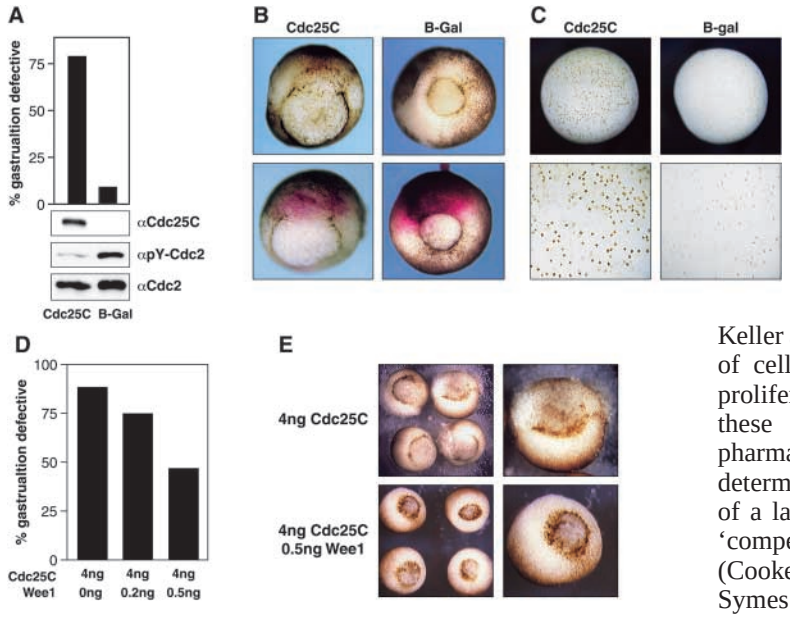

Fig. 7. Cdc25C overexpression disrupts gastrulation. (A,B) The two dorsal blastomeres of four-cell embryos were injected with β-gal RNA (4 ng) or co-injected with β-gal (100 pg) and His-Cdc25C (3 ng) and embryos were scored for gastrulation defects at stage 11.5-12. Number of embryos examined: Cdc25C (*n*=141), β-Gal (*n*=162). (A, lower panels) Stage 10.5-11 embryo lysates were examined by immunoblot analysis using anti-His-epitope, Cdc2 and phospho-Cdc2 antibodies. (C) Following injection as in A, mitotic nuclei of stage 11 embryos were visualized. Mitotic index (*n*=10-12 embryos): β-gal, 8.3% (6928 nuclei), Cdc25C, 28.1% (5550 nuclei). (D,E) Embryos were injected with 4 ng of Cdc25C RNA and either 0, 0.2 or 0.5 ng of Wee1 RNA. Number of embryos examined: Cdc25C + 0 ng Wee1 (*n*=74), Cdc25C + 0.2 ng Wee1 (*n*=100) and Cdc25C + 0.5 ng Wee1 (*n*=86). Note that increased expression of Wee1 counteracts the defects induced by Cdc25C overexpression.

by Cdc25C were rescued by co-injecting increasing amounts of WT-Wee1 RNA. These findings indicate that the balance between the kinases and phosphatases that modulate Cdc2 activity is crucial for normal cell cycle regulation during development and further support the model that normal gastrulation requires the inhibition of cell cycle progression.

Discussion

During *Xenopus* embryogenesis, major developmental events coincide with elevated levels of inactive tyrosine-phosphorylated Cdc2, indicating that regulation of this phosphorylation event may play a crucial role in normal development (Ferrell et al., 1991). One kinase that mediates the tyrosine phosphorylation of Cdc2 is Wee1. In *Xenopus*, the maternal Wee1 protein is expressed from meiosis II until mid gastrulation, at which time expression of zygotic Wee1B/Wee2 begins (Murakami and Vande Woude, 1998; Leise and Mueller, 2002; Okamoto et al., 2002). Maternal Wee1 has been shown to be an important inhibitor of CyclinB/Cdc2 in the first mitotic cell cycle; however, a role for Wee1 at later times in embryogenesis had not been previously established. In this report, we address this issue by using antisense morpholinos to deplete embryos of endogenous Wee1 protein. We show that Wee1 contributes to the regulation of Cdc2 tyrosine phosphorylation and M-phase entry in early gastrula embryos. Moreover, we find that a functional Wee1 protein is required for key morphogenetic movements involved in gastrulation.

Wee1 is required for normal gastrulation

In *Xenopus*, gastrulation is a complex morphogenetic process whereby a simple blastula is transformed into an embryo with three germ layers and a distinct dorsoventral body plan (Gerhart and Keller, 1986; Keller and Winklbauer, 1992). Early studies examining the role of cell division in *Xenopus* gastrulation indicated that cell proliferation was not required for gastrulation. Specifically, these studies showed that inhibiting cell division with pharmacological inhibitors had no effect on gastrulation as determined by the morphology of the embryo, the transcription of a late mesodermal marker (cardiac actin), and the loss of 'competence' to respond to mesoderm-inducing factors (Cooke, 1973b; Cooke, 1973a; Gurdon and Fairman, 1986; Symes and Smith, 1987; Grainger and Gurdon, 1989). In addition, a more recent study has shown that MO depletion of Cyclin E slows cell division and delays embryogenesis, but results in embryos that are morphologically normal (Audic et al., 2001). However, careful spatial analysis of cell division in *Xenopus* embryos has revealed that involuted dorsal mesodermal cells do not divide during gastrulation (Saka and Smith, 2001), suggesting that inhibition of cell proliferation might in fact be required for normal gastrulation. If this were the case, then enhanced cell proliferation should be detrimental to the gastrulation process. Our findings directly support this model. Here, we show that Wee1-depletion, which promotes M-phase entry and cell cycle progression, severely disrupts gastrulation. The role of cell cycle regulation was further substantiated by our findings that inappropriately promoting cell division by overexpressing the Cdc25C phosphatase also disrupted gastrulation and that the Cdc25C-induced defects could be counteracted by increased expression of the Wee1 kinase. Thus, cell cycle progression appears to be incompatible with the processes involved in normal gastrulation.

Wee1 does not contribute to the onset of zygotic transcription

Interestingly, we found that Wee1 is not needed for the induction of zygotic transcription. Between the MBT and gastrulation, the cell cycle is gradually transformed from a minimal 30-minute cycle to a 4 hour cell cycle (Howe et al., 1995). The functional relationship between cell cycle length and the induction of zygotic transcription is derived from experiments where the artificial expansion of the cell cycle prior to the MBT resulted in premature zygotic transcription (Kimelman et al., 1987). Based upon these studies, the acceleration of the cell cycle during/after the MBT might be expected to block or inhibit zygotic transcription. In this report, we find that even though Wee1-depletion does promote M-phase entry, it is not sufficient to prevent the onset of zygotic transcription. Although this finding is somewhat unexpected, it is likely that the expansion of the cell cycle after the MBT is a complex process that involves the programmed degradation of Cyclin E1, Chk1-mediated degradation of Cdc25A, as well as the positive regulation of Wee1 (Howe and Newport, 1996;

Kim et al., 1999; Shimuta et al., 2002) (this report). In addition, the programmed degradation of Cyclin A1, and possibly some post-translational regulation of Cdc25C, may also contribute to the further expansion of the cell cycle during gastrulation (Howe et al., 1995; Rempel et al., 1995; Hartley et al., 1996). Thus, several events may work together as a molecular rheostat to slow the cell cycle from 30 minutes to 4 hours. As a result, blocking only one of these events may not be sufficient to interfere with the onset of zygotic transcription.

Embryonic cell cycle regulation and gastrulation in frogs and flies

Modulation of the cell cycle appears to play an important role in both *Xenopus* and *Drosophila* embryogenesis. Prior to gastrulation, both organisms undergo a burst of rapid cell divisions followed by a gradual expansion of the cell cycle (Newport and Kirschner, 1982; Edgar et al., 1986). Zygotic cell cycle components are synthesized after the MBT and previous studies have indicated that zygotic proteins do play a role in regulating Cdc2 activity during gastrulation (Edgar and O'Farrell, 1989). In *Drosophila*, cell cycle inhibition is observed at the ventral furrow (Foe, 1989), a region somewhat analogous to the *Xenopus* blastopore, and this inhibition is achieved by the removal of a zygotic activator of Cdc2. Specifically, the spatially restricted expression of the Tribbles protein results in the degradation of the String/Cdc25C phosphatase in cells surrounding the ventral furrow (Groβhans and Wieschaus, 2000; Mata et al., 2000; Seher and Leptin, 2000). In *Xenopus*, the zone of non-mitotic cells in the mid-late gastrula is identical to area of zygotic Wee1B/Wee2 RNA expression (Saka and Smith, 2001; Leise and Mueller, 2002), suggesting that zygotic expression of a Cdc2 inhibitor, Wee1B/Wee2, might play an analogous role in frog embryogenesis. Interestingly, the expansion of the cell cycle after the MBT (and during gastrulation) is regulated by zygotic components in *Drosophila*, but is regulated by maternally derived components in *Xenopus* (Newport and Dasso, 1989; Edgar and Datar, 1996). In *Xenopus*, the maternally regulated program of cell cycle expansion has been implicated in the onset of zygotic transcription, cytoplasmic blebbing and pseudopod formation (at the MBT) (Newport and Kirschner, 1982), but has not been previously implicated in the coordinated tissue morphogenesis that takes place during gastrulation. We demonstrate that the maternal Wee1 protein contributes to the cell cycle downregulation that occurs during *Xenopus* gastrulation. Our findings also indicate that the maternally directed program of cell cycle control, rather than simply facilitating the transcription of zygotic components, plays a direct role in morphogenesis.

The requirement of cell cycle regulation for the coordinated cell movements of gastrulation is another shared feature of *Drosophila* and *Xenopus* embryogenesis. In flies, the Tribbles-mediated degradation of Cdc25C permits the invagination of mesodermal cells at the ventral furrow, one of the earliest events of gastrulation (Groβhans and Wieschaus, 2000; Mata et al., 2000; Seher and Leptin, 2000). Similarly, in this study, we found that cell cycle inhibition mediated by Wee1 is important for epiboly, involution and convergent-extension, all of which are major morphogenetic processes that contribute to normal *Xenopus* gastrulation. Thus, although flies and frogs may use different molecular components to regulate the embryonic cell cycle, it appears that in both organisms the inhibition of cell division is essential for the complex morphogenetic movements required for gastrulation.

Wee1 regulation

In this study, we find that Wee1 is upregulated by tyrosine autophosphorylation following the MBT and at gastrulation. This upregulation appears to be required for Wee1 function in early gastrula embryos given that neither kinase-inactive Wee1 or a Wee1 protein containing mutations in the tyrosine phosphorylation sites were able to rescue the defects produced by MO-Wee1-depletion. These findings are consistent with previous observations that upregulation of Wee1 activity by tyrosine autophosphorylation is critical for Wee1 function in the first mitotic cell cycle (Murakami et al., 1999). Taken together, these studies indicate that the maternal Wee1 protein functions at distinct developmental points to coordinate cell cycle progression with events that control the organization of the embryonic body plan. Moreover, we believe this work contributes to a growing body of evidence that cell cycle regulation is likely to be crucial for a wide variety of morphogenetic processes. Wee1 is a primary cell cycle target of the budding morphogenesis checkpoint in *S. cerevisiae* (Sia et al., 1998; Lew, 2000), and in mammalian cells, there is evidence that inhibition of cell proliferation is necessary for cell migration (Nagahara et al., 1998). Collectively, these studies suggest that 'morphogenesis' checkpoints, which coordinate cell shape changes and movement with cell proliferation, will be crucial for normal development and organogenesis, and may also play an important role in the balance between deregulated cell proliferation and metastasis.

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