DNA replication checkpoint control of Wee1 stability by vertebrate Hsl7

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Introduction

Entry into mitosis in all eukaryotic cells is controlled by the Cdc2 (cell division control protein 2)–cyclin B kinase complex. Activation of this complex is restrained in the presence of damaged or incompletely replicated DNA by checkpoint pathways that act to maintain inhibitory phosphorylations of Cdc2 on Thr 14 and Tyr 15 (Ellledge, 1996; Lew and Kornbluth, 1996). These sites are phosphorylated by related kinases, Wee1 and Myt1, and are dephosphorylated by the Cdc25 phosphatase. Accordingly, checkpoint pathways act to inhibit Cdc25 function and ensure the continued Cdc2-suppressive activity of Wee1 (and possibly Myt1). Recently, it has been reported that Wee1 protein stability is controlled by DNA-responsive checkpoint pathways in extracts prepared from Xenopus eggs. Intriguingly, it was found that Wee1 is stabilized during DNA replication (or following DNA damage) and that it is degraded within nuclei at the time of mitotic entry (Michael and Newport, 1998). In Xenopus laevis it has been reported that Wee1 ubiquitination and proteasomal degradation are driven by a Skp1–cullin–F box (SCF) E3 ligase complex containing a novel F-box protein, Tome-1 (trigger of mitotic entry; Ayad et al., 2003). Human Wee1A, however, is reportedly ubiquitinated by an SCF complex containing β-TrCP (β transducin repeat containing protein; Watanabe et al., 2004). This difference may reflect either species differences or the fact that an embryonic form of Wee1 was studied in Xenopus whereas a somatic form of Wee1 was analyzed in human cells. Other vertebrate factors responsible for promoting Wee1 degradation or linking it to DNA-responsive checkpoints have not yet been identified.

In the yeast Saccharomyces cerevisiae, the stability of the Wee1 homologue, Swe1 is under the control of a checkpoint pathway monitoring budding and the status of the actin cytoskeleton (the morphogenesis checkpoint; Sia et al., 1996). This checkpoint is thought to prevent mitotic entry, at least in part, through stabilization of Swe1 (Sia et al., 1998). Looking to this system for candidate Wee1 regulators, we were struck by the requirement for an evolutionarily conserved methyltransferase, Hsl7 (histone synthetic lethal 7), in promoting Swe1 degradation. The morphogenesis checkpoint regulates interaction of Hsl7 with an associated kinase, Hsl1, to prevent Swe1 degradation. Consistent with these observations, overexpression of Hsl7 can override the morphogenesis checkpoint, allowing mitotic entry even when budding is inhibited (McMillan et al., 1999). These data place S. cerevisiae Hsl7 as a central regulator of Swe1 protein stability. In contrast, the Hsl7 homologue (Skb1) in the fission yeast, Schizosaccharomyces pombe, appears to...
have a cell cycle role diametrically opposed to that of *S. cerevisiae* Hsl7. Specifically, Skb1 has been reported to inhibit, rather than promote, mitotic entry through direct binding to the mitotic Cdc2–cyclin complex (Gilbreth et al., 1998). In addition to its activity in cell cycle regulation, Skb1 has been reported to regulate morphogenesis (in association with a PAK family kinase) and to be involved in the hyperosmotic stress response, which stimulates Skb1 methyltransferase activity (Yang et al., 1999; Bao et al., 2001).

Although Hsl7 homologues have been reported in vertebrates, including humans, analyses of their potential role (stimulatory or inhibitory) in controlling mitotic entry has been hampered by a potentially distinct requirement for vertebrate Hsl7 as a component of the methylosome regulating mRNA splicing (Friesen et al., 2001). Therefore, to analyze Hsl7 cell cycle function we turned to the *Xenopus* egg extract, which can undergo multiple cell cycles in vitro without any de novo mRNA transcription, allowing an analysis of cell cycle regulation in a system free of confounding effects on mRNA processing (Friesen et al., 2001). We report here that *Xenopus* Hsl7 controls entry into M phase by controlling the intranuclear stability of Wee1. Moreover, just as overexpression of Hsl7 can override the morphogenesis checkpoint in budding yeast, overproduction of *Xenopus* Hsl7 can short-circuit the DNA replication checkpoint, allowing mitotic entry in the presence of incompletely replicated DNA. These data strongly suggest that the Hsl7–Wee1 cell cycle control module can be used for controlling entry into mitosis in vertebrates, as well as in *S. cerevisiae*, but that this module has been co-opted by vertebrates to respond to a different checkpoint signal than in yeast.

**Results**

**Identification of *Xenopus* Hsl7**

To identify potential *Xenopus* Hsl7 homologues, we searched the Washington University *Xenopus* EST database, and identified five Hsl7-related EST clones of various lengths. After DNA sequencing, we identified a full-length *Xenopus* Hsl7 cDNA (hereafter referred to as xHsl7) bearing 26% identity/44% similarity to yeast Hsl7 and 83% identity/92% similarity to human Hsl7 (known as JBP1 [Janus kinase binding protein 1]/PRMT5; Genbank/EMBL/DDBJ accession no. AY535008) (Fig. 1). We were further encouraged to pursue analysis of this clone as we found that recombinant *S. cerevisiae* Hsl7 protein readily bound *Xenopus* Wee1 in egg extracts (Fig. 2 A). This suggested that the Hsl7–Wee1 interaction might be conserved and that the *Xenopus* protein might also play a role in regulating Wee1 function and mitotic entry.

**Overexpression of xHsl7 accelerates entry into mitosis**

As a first step in evaluating xHsl7 function, we wished to overexpress xHsl7 in cycling extracts of *Xenopus* eggs, which are able to oscillate between S and M phases of the cell cycle. For this purpose, we supplemented translationally competent (though transcriptionally inactive) cycling egg extracts with...
excess mRNA encoding xHsl7, allowing production of xHsl7 to levels approximately twofold that found endogenously (unpublished data). Under these conditions, we found that excess xHsl7 could markedly accelerate entry into mitosis, as shown by both microscopic monitoring of nuclear envelope breakdown and assays of histone H1-directed Cdc2 kinase activity (Fig. 2 B). Interestingly, this acceleration was absent in extracts that had not been supplemented with nuclei, suggesting that the effects of xHsl7 on the cell cycle were nuclear dependent (Fig. 2 C). (Note that the extract shown here is distinct from the one used in Fig. 2 B. In this and subsequent figures, the absolute timing of mitosis differed from extract to extract, but the relative rates of mitotic entry under different treatment conditions remained constant.) The different response of extracts containing or lacking nuclei to xHsl7 addition did not reflect any differences in the intrinsic stability of xHsl7 protein under these conditions, since xHsl7 was equally stable in the presence and absence of nuclei (Fig. 2 D). In addition, an xHsl7 mutant altered at well-conserved residues required for protein methyltransferase activity was as potent as wild type in accelerating mitotic entry, consistent with recent reports that methyltransferase activity is dispensable for Hsl7-mediated cell cycle regulation in S. cerevisiae (unpublished data; Ma, 2000; Theesfeld et al., 2003).

Figure 2. Excess xHsl7 accelerates entry into mitosis in a nuclear-dependent manner. (A) GST-yeast Hsl7 (lane 1) or GST (lane 2) were incubated in Xenopus egg extracts, retained on glutathione Sepharose, and immunoblotted with anti-Wee1 antibodies. (B) xHsl7-encoding mRNA or buffer was incubated in cycling extracts with sperm nuclei [5,000/μl] and an ATP-regenerating system. Aliquots were stored at the indicated times and assayed for their ability to phosphorylate histone H1 in the presence of [γ-32P]ATP. Phosphorylated histone was resolved by SDS-PAGE, subjected to autoradiography, and quantified by phosphoimagery with the zero time point normalized to 1. Square, buffer; circle, FLAG-xHsl7 mRNA addition. Arrows indicate the time of the nuclear envelope break down and the chromosome condensation as monitored by microscope. (C) Same assay as panel B except that sperm nuclei were absent. (D) In vitro–translated [35S] radiolabeled xHsl7 and nucleoplasm [as an internal control] were added to cycling extracts with or without nuclei present. Samples were withdrawn at the indicated times, resolved by SDS-PAGE, and subjected to autoradiography to monitor xHsl7 stability. Mitosis was observed at 90 min by fluorescence microscopy of extracts containing Hoechst-stained nuclei.

Depletion or inhibition of Hsl7 delays the entry into mitosis

Using a commercially available antibody directed against the human Hsl7 homologue, JBP1, we were readily able to detect endogenous xHsl7 (Fig. 3 A). Moreover, this antibody could detect xHsl7 co-immunoprecipitated with endogenous Wee1 (Fig. 3 B). This interaction was further confirmed by retrieval of Wee1 from egg extracts by recombinant GST–xHsl7 linked to glutathione sepharose (Fig. 3 C). These data suggested that xHsl7 could positively regulate mitotic entry, perhaps through an interaction with Wee1. Given these data, we wished to assess the consequences of xHsl7 removal or inactivation on entry into mitosis. Therefore, we used Hsl7 antibodies to immunodeplete xHsl7 from cycling extracts. As shown in Fig. 4 A, extracts depleted with control IgG underwent two rounds of mitosis, as measured by peaks of histone H1-directed kinase activity, whereas extracts depleted of xHsl7 were entirely blocked in their ability to enter mitosis. To examine the role of xHsl7 using another approach, we added anti-Hsl7 antibodies directly to cycling extracts with the goal of interfering with xHsl7 function. Consistent with a role for xHsl7 in promoting mitotic entry, we found that addition of purified anti-Hsl7 antibodies, but not control IgG, to cycling egg extracts was able to significantly delay entry into mitosis as measured by histone H1-directed kinase activity assays and microscopic observation of chromosome condensation/nuclear envelope breakdown (Fig. 4, B and C). Moreover, readdition of recombinant xHsl7 restored the normal timing of mitotic entry, demonstrating that the effects of the anti-Hsl7 antibody were specific (Fig. 4, B and C). These data suggest that xHsl7 is required for efficient entry into M phase in Xenopus.

Wee1 is required for the cell cycle effects of xHsl7

Given the ability of xHsl7 to bind Wee1, we wished to determine whether the effect of xHsl7 overexpression on mitotic entry was Wee1 mediated. Initially, we found that immunodepletion of Wee1 or addition of excess xHsl7 produced similar accelerations in mitotic entry (unpublished data). These data precluded an experiment in egg extracts to determine whether xHsl7 could accelerate mitotic entry in the absence of Wee1, as the kinetics of cyclin B accumulation in the cycling extract make it difficult to observe cell cycle accelerations greater...
than those seen with depletion of Wee1 alone. Consequently, to determine if xHsl7 effects were Wee1 mediated, we took advantage of the fact that G2-arrested stage VI Xenopus oocytes, which contain endogenous xHsl7 at levels similar to that found in egg extracts, lack endogenous Wee1 (Nakajo et al., 2000). Oocytes were injected with mRNA for the overexpression of FLAG-tagged xHsl7 and then treated with progesterone, which stimulates entry into M phase, as monitored by breakdown of the nuclear envelope, or germinal vesicle breakdown (GVBD). However, when we introduced mRNA encoding

Xenopus Wee1 into oocytes, we observed an inhibition of GVBD, likely due to tyrosine phosphorylation of stockpiled Cdc2–cyclin B complexes. This Wee1-induced delay in GVBD could be almost entirely reversed by production of excess xHsl7 (Fig. 5 A), demonstrating that xHsl7 can regulate M phase progression in the oocyte milieu, but only in the presence of Wee1.

To assess the effects of xHsl7 on Cdc2-directed Wee1 kinase activity, we took interphase Xenopus cytosol (lacking membranes and thus devoid of Myt1) as a source of robust Wee1 kinase activity and either immunodepleted endogenous xHsl7 or supplemented the extract with excess recombinant xHsl7 (which, like excess xHsl7 RNA, was able to accelerate mitotic entry in cycling extracts). The treated cytosols were then incubated for 10 min at room temperature with recombinant human cyclin B1 was then added and the reaction was allowed to proceed for 10 min. xWee1 activity was determined by assaying the phosphorylation level of Cdc2 by immunoblotting with anti-phospho Cdc2.
beled in vitro translated Wee1, we noted that xHsl7 overproduction promoted specific loss of the nuclear Wee1, as detected by SDS-PAGE and autoradiography of manually dissected oocyte nuclei (Fig. 6 A; nuclear envelope breakdown was prevented by addition of the Cdc2 inhibitor roscovitine). Although Wee1 was also lost from the cytoplasm during these experiments, this loss was not stimulated by xHsl7 overproduction. Moreover, since Wee1 is rapidly transported into oocyte nuclei, we suspect that much of the loss of cytoplasmic Wee1 reflects nuclear import (unpublished data). As in S. cerevisiae, xHsl7-stimulated loss of Wee1 was abrogated by disruption of the well-conserved residues responsible for methyltransferase activity (Fig. 6 B), nor was it inhibited by leptomycin B treatment to stop Crm1-mediated nuclear export (Fig. 6 C; note the increase in nuclear Wee1 inhibited by leptomycin B treatment to stop Crm1-mediated nuclear export (Fig. 6 C; note the increase in nuclear Wee1 in control injections due to inhibition of nuclear export. *Xenopus* Hsl7 injection counteracts even the added burden of imported Wee1 that cannot be exported). These data are consistent with data reported previously suggesting intranuclear Wee1 degradation, particularly as nuclear export of Swe1 depends on the Crm1 homologue, Expo1 (Lew, D.J., personal communication).

Given our data suggesting that xHsl7 promoted Wee1 degradation, we wished to determine if the observed ability of xHsl7 to reverse the Weel1-mediated delay in oocyte maturation could potentially be accounted for by accelerated proteasomal degradation of Wee1. To determine if this was the case, we treated oocytes with the proteasome inhibitor MG132 and repeated the experiment shown in Fig. 5 A. We found not only that the inhibitory effect of Weel1 on oocyte maturation was more pronounced in the presence of MG132 (consistent with a role for the proteasome in controlling Weel1 function), but that xHsl7 was unable to counteract the effects of Weel1 in the absence of proteasome activity (Fig. 6 D). Taken together with the observed effects of xHsl7 on nuclear Weel1 levels, these data suggest that xHsl7 antagonizes Weel1 by promoting its proteasomal degradation.

One important feature of xHsl7-stimulated nuclear Weel1 degradation in oocytes was its dependence upon progesterone treatment, suggesting that M phase–promoting factors were required for Weel1 degradation (such factors must be distinct from Cdc2–cyclin B, as these experiments were performed in the presence of roscovitine; unpublished data). Interestingly, Weel1 exhibited a noticeable electrophoretic shift within the nucleus, but not cytoplasmic, fraction before degradation, which may reflect phosphorylation by kinases active at G2/M (Fig. 6 A). It is also of interest to note that xHsl7 and Wee1 were unable to interact in *Xenopus* egg extracts lacking nuclei, consistent with an intranuclear locus of xHsl7 action (Fig. 6 E); we have also found that xHsl7 is efficiently transported into nuclei (unpublished data). These data suggest either that xHsl7 and Weel1 do not achieve sufficient concentration in the cytoplasm for interaction or that additional intranuclear factors are required for xHsl7–Weel1 binding. Consistent with these data, xHsl7 and Weel1 did not interact in mitotic extracts of *Xenopus* eggs where there is no nuclear compartmentalization (Fig. 6 F).
In replication checkpoint
Hsl7 overexpression overrides the DNA replication checkpoint. Using similar logic, it was attractive to speculate that overexpression of Hsl7 will override the morphogenesis checkpoint; Lee et al., 2001), recent reports indicate that Wee1 is also regulated at the level of protein stability. In particular, it has recently been demonstrated that DNA-responsive checkpoints can act to stabilize Wee1, at least in part, by inhibiting xHsl7–Wee1 interactions.

**Discussion**

As an inhibitor of Cdc2–cyclin B activation, the Wee1 kinase is critical for determining the timing of entry into mitosis. Although the catalytic activity of Wee1 can be controlled by various upstream factors (e.g., 14–3–3 binding, Chk1 phosphorylation; Lee et al., 2001), recent reports indicate that Wee1 is also regulated at the level of protein stability. In particular, it has been demonstrated that DNA-responsive checkpoints can act to stabilize the Wee1 protein (Michael and Newport, 1998), thereby ensuring the continued suppression of Cdc2–cyclin B until DNA replication is completed or DNA damage is repaired. In this study we have shown that the Xenopus Hsl7 protein acts upstream of Wee1 to control its intranuclear stability.
Accordingly, inactivation of xHsl7 was able to slow mitotic entry, although its overexpression accelerated the onset of M phase, even in the presence of unreplicated DNA. These data position Xenopus Hsl7 as a novel vertebrate regulator of mitotic entry and suggest that xHsl7 may play an important role in transducing DNA-responsive checkpoint signals.

**Hsl7 and the control of nuclear Wee1 stability**

The ability of xHsl7 to accelerate mitotic entry in egg extracts only in the presence of nuclei is consistent with previous reports suggesting that Wee1 degradation is an intranuclearly controlled event. Indeed, we also found that overexpression of xHsl7 in oocytes had no effect on M phase entry unless Wee1 was present and this effect was apparently exerted at the level of nuclear Wee1 stability. There are a number of possible explanations for this nuclear requirement. First, it may be that xHsl7 and Wee1 only achieve sufficient concentration for binding when both are present within nuclei. Consistent with this idea, we observed a strong interaction between these proteins only in the presence of nuclei. It is also possible that a specific nuclear structure serves to promote their colocalization and interaction. For example, modulation of xHsl7–Wee1 interactions in response to the DNA replication checkpoint may reflect a direct effect of replication structures on xHsl7–Wee1 binding. It is also possible that the effect of nuclei reflects a requirement for specific nuclearly localized cofactors in Wee1 degradation. For example, we note that both Wee1 and xHsl7 appear to be phosphorylated just before the disappearance of Wee1. It may be that nuclear kinases contribute to Wee1 degradation.

Our data suggest that Pxl1 can facilitate Wee1 degradation by promoting xHsl7–Wee1 interactions. Moreover, the reported down-regulation of polo-like kinases by DNA-responsive checkpoints could potentially lead to stabilization of Wee1 by preventing efficient xHsl7–Wee1 binding. Recently, it was reported that Pkl1 can stimulate interactions between human Wee1 and β-TrCP through phosphorylation of Wee1 (Watanebe et al., 2004). One intriguing possibility is that the Pkl1 stimulation of xHsl7–Wee1 interactions leads to enhanced interactions of Wee1 with SCF E3 ubiquitin ligases. Although this issue merits further investigation, we have observed only weak (potentially nonspecific) interactions between Xenopus Wee1 and its two reported F-box interactors (β-TrCP and Tomel) and these interactions do not appear to be markedly altered by either xHsl7 overexpression or depletion. With regard to β-TrCP interactions, it is worth noting that the Pkl1 phosphorylation site on the somatic human Wee1A (S53) that has been implicated in Wee1 degradation is not conserved in Xenopus Wee1 (Watanebe et al., 2004). Thus, further evaluation of Pkl1 involvement in promoting xHsl7–Wee1 binding and Wee1 degradation will require identification of sites phosphorylated by Pkl1 on Xenopus Wee1. As preliminary in vitro kinase assays using purified Pkl1 and Wee1 proteins suggest that there are likely to be multiple sites of Pkl1 phosphorylation on Xenopus Wee1 (unpublished data), it may require extensive mutagenesis to identify sites relevant for promoting Wee1 degradation (note the presence of at least 20 Cdc5 sites on S. cerevisiae Swe1; Sakchaiisri et al., 2004).

With regard to the role of phosphorylation in promoting xHsl7–Wee1 interactions, it is also of interest that one of the key regulators of Swe1 stability in yeast is an Hsl7-interacting kinase, Hsl1 (McMillan et al., 1999; Cid et al., 2001). A true vertebrate Hsl1 has not yet been identified, in part because Hsl1 is a member of a large class of protein kinases, most of which do not function in cell cycle control, and the conservation between Hsl1-like proteins in different species is too low to allow definitive identification of a vertebrate ortholog by sequence analysis. It may soon be possible, however, to identify such a protein through interaction with our Xenopus Hsl7 clone.

**Multiple roles for Hsl7**

Hsl7 homologues have been reported to have different roles in different species (Gilbreth et al., 1998; McMillan et al., 1999; POLLACK et al., 1999; Shulewitz et al., 1999; Yang et al., 1999; Ma, 2000; Bao et al., 2001; Friesen et al., 2001; Meister et al., 2001). For example, unlike in S. cerevisiae where Hsl7 has been shown to regulate Swe1 stability (McMillan et al., 1999), the S. pombe Hsl7 has been reported to inhibit Cdc2 through direct binding (Gilbreth et al., 1998). We have been unable to detect any such direct interactions between xHsl7 and Cdc2 in Xenopus (unpublished data). Because the vertebrate cell cycle shares with S. pombe the use of Cdc2 tyrosine phosphorylation to control both the timing of unperturbed mitotic entry and cell cycle delay in response to DNA checkpoints (Rhind and Russell, 1998; Norbury et al., 1991), it is perhaps surprising that xHsl7 function is more akin to that of its S. cerevisiae homologue, where DNA responsive checkpoints do not promote a specific G2 arrest. With regard to vertebrate Hsl7, we note that it has been reported that the human homologue, JBP1, can complement an S. cerevisiae Hsl7 mutation (Lee et al., 2000). Moreover, at the start of this work we found that S. cerevisiae Hsl7 can interact physically with Xenopus Wee1.

In both S. cerevisiae and in our own experiments in Xenopus, it appears that residues critical for Hsl7 methyltransferase activity are dispensable for the cell cycle effects of Hsl7 (Theesfeld et al., 2003). These data suggest that Hsl7 functions in at least two modes—as a cell cycle regulator (perhaps through direct binding of its targets or recruitment of other factors to its targets) and as a methyltransferase involved in distinct biological processes. In this regard, it has been reported that the S. pombe Hsl7 homologue is a mediator of hyperosmotic stress whose methyltransferase activity is stimulated under stressor conditions and in human cells, JBP1 can interact with Jak family kinases, presumably to control transcription (Pollack et al., 1999; Bao et al., 2001). In addition, the human 20S methylsomesome that methylates Sm proteins before assembly into SnRNP core particles contains Hsl7 as a catalytic subunit (Friesen et al., 2001; Meister et al., 2001). Our data showing that xHsl7 can affect cell cycle progression in the transcriptionally inactive Xenopus egg extract further strengthens the idea that the effects of xHsl7 on RNA transcription/processing are very likely to be distinct from its cell cycle effects. Additionally, xHsl7 methyltransferase activity is likely to be more important for the former than the latter.
Hsl7–Wee1 as a checkpoint control module
In budding yeast, degradation of Swe1 involves the hierarchical association of Hsl1, then Hsl7, then Swe1 to the mother/bud neck (Shulewitz et al., 1999; Longtine et al., 2000). When budding is prevented, the morphogenesis checkpoint is activated, leading to inactivation of the Hsl1–Hsl7–Swe1 cell cycle control module and consequent stabilization of Swe1 (McMillan et al., 1999). This is accomplished, at least in part, by impairing proper recruitment of Hsl7 to the septin cortex. Although these specifics of Swe1 degradation are obviously not directly applicable to Wee1 degradation in animal cells that do not reproduce by budding, our data indicate that Hsl7 can also be used to control Wee1 stability in vertebrates. In effect, these data demonstrate that cell cycle control “modules” can be co-opted for use by different checkpoint signaling pathways. In S. cerevisiae, Hsl7 does, indeed, regulate Swe1 stability, but this regulatory module is under the control of pathways responding to perturbations in morphogenesis. In vertebrate cells, control of Wee1 stability is important for the operation of DNA-responsive checkpoints that operate to control the state of Cdc2 tyrosine phosphorylation.

Materials and methods
Cloning of Xenopus Hsl7 cDNA
Using the protein sequence of the human Hsl7 homologue, JB1, in a BLAST search against the Washington University Xenopus EST database we identified 5 EST clones homologous to JB1. After sequencing, it was determined that the EST clone with GenBank/EMBL/DDBJ accession number BF613396 contained the full-length Xenopus homologue of JB1, xHsl7.

Plasmid construction and RNA synthesis
GST-xHsl7 was constructed by inserting the Xenopus EST clone into the BglII and HindIII sites of pGEX-KG. Following in vivo transcription of the riboprobe, the GST-xHsl7 RNA was amplified using PCE using FLAG-xHsl7/BglII (5′-TTG CGG CCG CAA TCA CAG GCC AAT TG-3′) and xHsl7/NotI (5′-TTG CGG CCG CAA TCA CAG GCC AAT TG-3′). The amplified fragment was digested with BglII and NotI and subcloned into pSP64T, yielding pSP64T-FLAG-xHsl7. The metytransferase mutant of xHsl7 (Arg364 to Ala) was made using the Quick Change Site-Directed Mutagenesis Kit (Stratagene). The primers used were 5′-GGG CTC GGA GCA GGC CCG GGG CCC CTT-3′ and 5′-AAG GGG TCC CGC GCC TGC TCC GAG CAC-3′. To prepare pSP64T/HA-Wee1, Wee1 was PCR amplified using xWee1/BglII (5′-AGG GTG AGA TCT ATG AGA AGA GCC-3′) and xWee1/NotI (5′-TTG CGG CCG CAA TTA ATA CCC TCC GC-3′) and subcloned into pEBB-HA vector. Then, HA-Wee1 was amplified again by PCR using HA/BglII (5′-CTT GGA TCC ATG TGT CTC AGC CAT-3′) and xWee1/NotI digested with BamHII and NotI, and subcloned into pEBB-HA vector. The PCR fragment was digested with BglII and NotI, and subcloned into pEBB-HA vector. The HaWee1 mRNA was synthesized using a mCAP RNA capping kit.

Co-immunoprecipitation of xHsl7 and Wee1
For co-immunoprecipitation of Wee1 and xHsl7 in interphase extracts, 10 μg anti-Wee1 or control rabbit IgG was bound to 20 μl of protein A-Sepharose 4B beads and incubated with interphase egg extract for 1 h at 4°C. Beads were then washed three times with ELB, eluted with SDS-PAGE sample buffer, resolved by SDS-PAGE, and blotted with anti-Wee1 (Zymed Laboratories). Plx1 and bound proteins were eluted with SDS-PAGE sample buffer, resolved by SDS-PAGE and blotted with anti-Wee1 and treated with 1 M Na3VO4. Kinase assays were initiated buffer. Samples were incubated for 10 min at 4°C and proceeded for 10 min before SDS-PAGE followed by anti-Plx1 immunoblotting.

Histone H1 kinase assay
2 μl of cycling extracts was added to 28 μl of H1 kinase reaction mix (final concentrations, 10 mM Hepes KOH [pH 7.2], 5 mM MgCl2, 50 mM NaCl, 83 μM ATP, 4.2 mM DTT, 5 μM of histone H1) and 2 μCi [γ-32P]ATP. The reactions were incubated at room temperature for 10 min and resolved by 12.5% SDS-PAGE. The bands corresponding to histone H1 were quantified with a phosphorimager (Molecular Dynamics).

Oocyte injection and Wee1 degradation assay
Stage VI oocytes were prepared as described previously (Swenson et al., 1989). 40 nl of mRNAs (40 ng) encoding β-galactosidase or FLAG-xHsl7 were injected into oocytes. Injected oocytes were incubated in 50 μM roscovitine. After 12 h, they were injected with 40 nl of [32P]labeled in vitro–translated Wee1 and treated with 1 μM progesterone and roscovitine in modified Barth’s + Ca buffer. Five oocytes were separated into nuclear and cytoplasmic fractions by manual dissection under mineral oil. Nuclear material was resuspended in SDS-PAGE sample buffer. Cytoplasmic fractions were suspended in buffer (20 mM Hepes KOH, pH 7.5, 20 mM β-glycerophosphate, 15 mM MgCl2, 20 mM EGTA, 1 mM PMSF, and 5 g/l of aprotinin/leupeptin) and spun for 5 min at 13,000 g to remove insoluble material. The fractions were separated on 10% SDS-PAGE and the remaining Wee1 in these fractions was quantified by phosphorimager.

Calc2 Tyr15 phosphorylation
To measure the effect of Hsl7 on Calc2 tyrosine 15 phosphorylation rates, 100 μl aliquots of egg were either depleted with anti-JBP1 sera or with preimmune sera. In addition, 100 μl aliquots of cytosol were supplemented with 10 μl of XB buffer or 10 μl of recombinant xHsl7 in XB buffer. Samples were incubated for 10 min at 4°C with an ATP-regenerating system with or without 0.5 mM Na3VO4. Kinase assays were initiated by addition of 1 μl of cyclin B1A13 and proceeded for 10 min before SDS-PAGE sample buffer addition. 2 μl of each sample was then resolved by SDS-PAGE and blotted with anti-phospho–Calc2 (Cell Signaling Technology).

Microscopy
DNA was stained with Hoechst 33258 and nuclear morphology was examined using a Zeiss Axioskop with a 40× Plan-Neofluar air objective with an NA of 0.75. Images were captured using a Pentamax cooled charge-coupled device camera (Princeton Instruments), interfaced with MetaMorph software (Universal Imaging Corp.) and levels were adjusted using Adobe Photoshop 7.0.

Histone H1 was quantified with a phosphorimager (Molecular Dynamics).
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