Cell

Substrate Competition as a Source of Ultrasensitivity in the Inactivation of Wee1

Sun Young Kim[1] and James E. Ferrell, Jr.[1,*]
[1] Department of Chemical and Systems Biology, Stanford University School of Medicine, 269 West Campus Drive, Center for Clinical Sciences Research, Room 3155, Stanford, CA 94305-5174, USA
*Correspondence: james.ferrell@stanford.edu
DOI 10.1016/j.cell.2007.01.039

SUMMARY

The mitotic regulators Wee1 and Cdk1 can inactivate each other through inhibitory phosphorylations. This double-negative feedback loop is part of a bistable trigger that makes the transition into mitosis abrupt and decisive. To generate a bistable response, some component of a double-negative feedback loop must exhibit an ultrasensitive response to its upstream regulator. Here, we experimentally demonstrate that Wee1 exhibits a highly ultrasensitive response to Cdk1. Several mechanisms can, in principle, give rise to ultrasensitivity, including zero-order effects, multisite phosphorylation, and competition mechanisms. We found that the ultrasensitivity in the inactivation of Wee1 arises mainly through two competition mechanisms: competition between two sets of phosphorylation sites in Wee1 and between Wee1 and other high-affinity Cdk1 targets. Based on these findings, we were able to reconstitute a highly ultrasensitive Wee1 response with purified components. Competition provides a simple way of generating the equivalent of a highly cooperative allosteric response.

INTRODUCTION

The mitotic regulator cyclin B-Cdk1 is controlled by a system of two double-negative feedback loops and a positive feedback loop. Active Cdk1 brings about the inactivation of the nuclear kinase Wee1 and the cytoplasmic, membrane-associated kinase Myt1, which, when active, can inactivate Cdk1 through phosphorylation of Thr 14 and/or Tyr 15 (Fattaey and Booher, 1997; Liu et al., 1997; McGowan and Russell, 1993, 1995; Mueller et al., 1995a, 1995b; Parker and Piwnica-Worms, 1992). In addition, active Cdk1 brings about the activation of Cdc25, which can then dephosphorylate the sites phosphorylated by Wee1 and Myt1 (Hoffmann et al., 1993; Kumagai and Dunphy, 1992; Solomon et al., 1990).

Under the proper circumstances, positive and double-negative feedback loops can exhibit bistability. This means that the system can adopt either of two alternative steady states in response to a constant stimulus and can toggle between these states in response to small changes in stimulus (Cross et al., 2002; Ferrell and Xiong, 2001; Gardner et al., 2000; Laurent and Kellershohn, 1999; Novak and Tyson, 1993; Thron, 1996; Tyson et al., 1996). Recent experimental work has demonstrated that the Cdk1/Wee1/Myt1/Cdc25 system is, in fact, bistable (Pomerening et al., 2003; Sha et al., 2003) and can toggle between a stable interphase state, with Cdk1 and Cdc25 inactive and Wee1 and Myt1 active, and a stable mitotic state with Cdk1 and Cdc25 active and Wee1 and Myt1 inactive.

A bistable signaling system must include a positive feedback loop, a double-negative feedback loop, or the equivalent (Thomas, 1981), although sometimes this feedback loop may be difficult to appreciate (Markevich et al., 2004). However, the presence of positive or double-negative feedback does not guarantee that a system will be bistable; the shapes of the steady-state stimulus/response curves for the individual legs of the loop are important as well. It is also important that some component of the loop exhibit a sigmoidal, ultrasensitive steady-state response—that is, a nonlinear response resembling that of a cooperative enzyme (Ferrell, 1996; Ferrell and Xiong, 2001; Goldbeter and Koshland, 1981, 1982; Huang and Ferrell, 1996)—rather than a hyperbolic, Michaelian response (Figure 1A). For the particular case of a two-component positive or double-negative feedback, it is easy to show that simple Michaelian response functions do not support bistability (see the Supplemental Data available with this article online). This is also true for an arbitrarily large positive feedback loop, provided that the components of the loop constitute a strongly monotone system (Angeli et al., 2004). Given that the Cdk1/Wee1/Myt1/Cdc25 system is bistable (Pomerening et al., 2003; Sha et al., 2003), it seemed plausible that some component of the system would exhibit a highly ultrasensitive steady-state response to its upstream regulator.

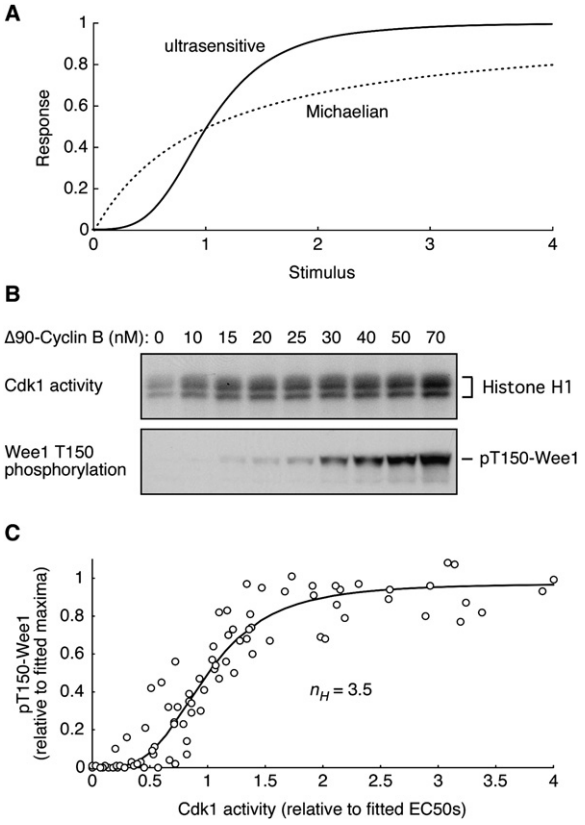

Figure 1. Ultrasensitive Phosphorylation of Wee1 at Thr 150 in *Xenopus* Egg Extracts

(A) Michaelian and ultrasensitive steady-state responses.

(B) Steady-state Cdk1 activity and Wee1 Thr 150 phosphorylation as a function of cyclin concentration. Data were taken from one representative experiment.

(C) Steady-state Wee1 inactivation versus steady-state Cdk1 activity. Data were taken from 13 independent experiments. Because different extracts and cyclin preparations yielded different *EC*50 values, we pooled the data after fitting each individual experiment to the Hill equation and then normalizing it to its fitted maximum and *EC*50 values. The apparent Hill coefficient of 3.5 was obtained by fitting all of the normalized data to the Hill equation.

Ultrasensitivity is not only important for allowing positive feedback loops to generate bistable responses, but also for effective signal propagation down cascades. With Michaelian steady-state responses, an *n*-fold change in stimulus always yields a less than *n*-fold change in response. After several levels in a signaling cascade, the loss in signal contrast can be severe. Ultrasensitivity can help restore the original contrast and can also amplify it, converting graded inputs into more abrupt and switch-like outputs (Brown et al., 1997; Ferrell, 1996, 1997; Goldbeter and Koshland, 1981, 1982; Hooshangi et al., 2005). In addition, ultrasensitivity is required for generating oscillations in negative feedback loops of certain lengths (Walter, 1970). Thus, ultrasensitivity may be important in a wide range of signaling contexts.

Several examples of ultrasensitive responses have been documented experimentally. The earliest were the phosphorylation of phosphorylase and isocitrate dehydrogenase in vitro (LaPorte and Koshland, 1983; Meinke et al., 1986), where the observed sigmoidal responses could be largely attributed to zero-order ultrasensitivity, a phenomenon that occurs when the kinase and/or phosphatase that regulate the steady-state level of substrate phosphorylation are operating near saturation (Goldbeter and Koshland, 1981). More recent examples have included the activation of MEK and p42 MAPK by Mos in *Xenopus* oocyte extracts (Huang and Ferrell, 1996), AMP kinase activation in INS-1 cells (Hardie et al., 1999), and JNK activation in several cell types (Bagowski et al., 2003). Thus, ultrasensitivity is a recurring motif in cell signaling.

A variety of plausible mechanisms have been proposed to account for the ultrasensitivity observed in these systems. For example, the ultrasensitive response of MEK to Mos may be generated by competition between CK2β and MEK for access to Mos (Chen et al., 1997), and the response of p42 MAPK to MEK may be generated by nonprocessive multisite phosphorylation (Burack and Sturgill, 1997; Ferrell and Bhatt, 1997). However, these postulated mechanisms remain largely untested.

Here, we have examined whether the inactivation of Wee1 by Cdk1 in *Xenopus* egg extracts is Michaelian or ultrasensitive. We found that the inactivation of Wee1, as assessed by the steady-state phosphorylation of one critical residue (Thr 150), is highly ultrasensitive, with an apparent Hill coefficient of 3.5. We then tested several plausible mechanisms to account for the observed ultrasensitivity. We found that some of the ultrasensitivity is intrinsic to the core Wee1/cyclin B-Cdk1/Wee1 phosphatase system, with the main mechanism for intrinsic ultrasensitivity being competition between two sets of phosphorylation sites in Wee1 for access to Cdk1. In addition, much of the observed ultrasensitivity is extrinsic to the core system. The main mechanism of extrinsic ultrasensitivity appears to be competition between Wee1 and other high-affinity substrates for access to Cdk1. As proof of principle for the idea of competition as a source of ultrasensitivity, we have reconstituted the highly ultrasensitive, switch-like inhibition of Wee1 by Cdk1 in vitro with purified recombinant components.

RESULTS

We began by measuring the steady-state response of Wee1 to various constant levels of Cdk1 activity in *Xenopus* egg extracts. We prepared interphase cycloheximide-treated egg extracts in which cyclins had been degraded and Cdk1 was inactive. To these extracts, we added various concentrations of purified, bacterially expressed, nondegradable cyclin (sea urchin Δ90-cyclin B) and S_f9-expressed Cdk1AF (*Xenopus* Cdk1 with Thr 14 and Tyr 15 changed to Ala and Phe, respectively, which prevents inactivation of the Cdk1AF by Wee1 and Myt1) and

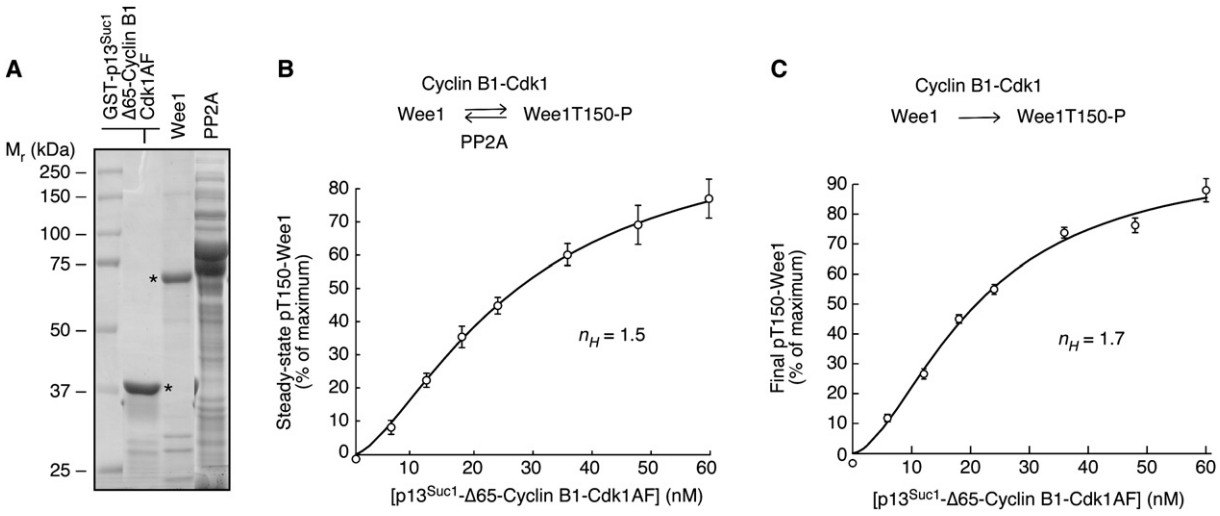

Figure 2. Intrinsic Ultrasensitivity in the Wee1 Response

(A) Coomassie-stained gel of recombinant GST-p13^{Suc1}, Δ65-cyclin B1, Cdk1AF, Wee1, and PP2A proteins. The asterisks denote the comigrating GST-p13^{Suc1}, Δ65-cyclin B1 and Cdk1AF proteins, and the Wee1 protein.

(B) In vitro steady-state response of Wee1 to p13^{Suc1}-Δ65-cyclin B1-Cdk1AF in a reconstituted system. Recombinant Wee1 (60 nM) was incubated with various concentrations of p13^{Suc1}-Δ65-cyclin B1-Cdk1AF complexes in the presence of PP2A for 1 hr. Wee1 Thr 150 phosphorylation was assessed by pT150-Wee1 immunoblotting and densitometry. Data points represent means ± standard errors (n = 6). The apparent Hill coefficient (1.5) was estimated by fitting the data to the Hill equation.

(C) Wee1 Thr 150 phosphorylation after 1 hr incubation with various concentrations of p13^{Suc1}-Δ65-cyclin B1-Cdk1AF. Data points represent means ± standard errors (n = 4).

assessed the steady-state level of Cdk1 activity by histone H1 kinase assays (Figure 1B). Time-course experiments (data not shown) established that by 60 min, Cdk1 activity had reached an apparent steady state. We also included sperm chromatin (500/μL) in the extracts to monitor mitosis and to ensure that the Cdk1 activities achieved were physiologically relevant. We typically obtained nuclear envelope breakdown at a cyclin concentration of 30–40 nM, consistent with previous studies (Pomerening et al., 2003; Sha et al., 2003; Solomon et al., 1990).

As a surrogate for the steady-state activity of the endogenous Wee1 protein, we assessed the phosphorylation of Thr 150 by quantitative immunoblotting with phosphospecific antibodies (Figure 1B). This assay was chosen because Thr 150 phosphorylation correlates well with Wee1 activity and because it has proven to be more reproducible than direct activity assays (Kim et al., 2005). However, since the inactivation of Wee1 depends upon the phosphorylation of two residues, Thr 104 and Thr 150 (Kim et al., 2005), pT150 immunoblotting might underestimate the ultrasensitivity in the Wee1 inactivation response. Care was taken to ensure that the quantified pT150 and H1 phosphorylation signals were linear with respect to the amount of protein loaded per lane (data not shown).

We carried out Cdk1 and Wee1 assays on 13 independent extract preparations, and plotted Wee1 T150 phosphorylation as a function of Cdk1 activity to isolate the response of Wee1 to Cdk1 from any nonlinearity in the response of Cdk1 to cyclin. As shown in Figure 1C, the relationship between Cdk1 activity and Wee1 T150 phosphorylation was steeply sigmoidal and was well-approximated by a Hill function with a Hill coefficient of 3.5. Thus, the steady-state response of Wee1 to Cdk1 in *Xenopus* egg extracts is highly ultrasensitive.

Intrinsic Ultrasensitivity

Several potential mechanisms for ultrasensitivity, including zero-order ultrasensitivity and multisite phosphorylation, would be intrinsic to the core system of Wee1, the Wee1 SP/TP kinase (thought to be mainly cyclin B1-Cdk1), and the Wee1 phosphatase (as yet unidentified). If so, it should be possible to reconstitute an ultrasensitive response with physiological concentrations of purified recombinant proteins in vitro.

Accordingly, we examined the steady-state response of purified Wee1 to purified Cdk1 complexes in the presence of a commercially available phosphatase, PP2A. The Cdk1 complexes consisted of recombinant p13^{Suc1}, Δ65-cyclin B1, and Cdk1AF. Suc1/Cks1 proteins have been shown to be important for the high-affinity interaction of Cdk1 with certain substrates, including Wee1 (Patra and Dunphy, 1996; Patra et al., 1999), and we found that to be the case here as well. The purified Wee1 preparation and the p13^{Suc1}- Δ65-cyclin B1- Cdk1AF preparation ran as single Coomassie-stained bands on SDS polyacrylamide gels (GST-p13^{Suc1}, Δ65-cyclin B1, and Cdk1AF all comigrate at 37 kDa) (Figure 2A). The PP2A preparation showed numerous Coomassie-stained bands, none of which ran with the expected molecular weights of the PP2A A- or C-subunits (Figure 2A). We

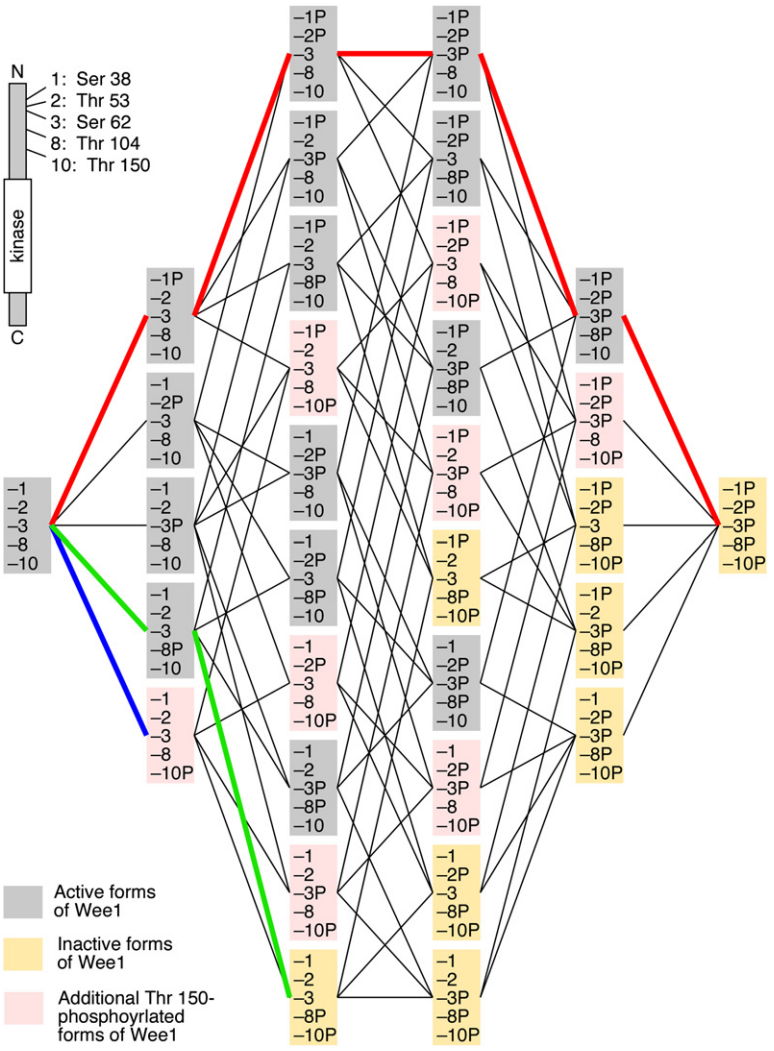

Figure 3. The Wee1 Phosphorylation/Dephosphorylation Network

Five SP/TP Cdk phosphorylation sites have been identified in *Xenopus* Wee1A (Kim et al., 2005). There are 32 possible phosphorylated forms of Wee1 and 80 phosphorylation and dephosphorylation reactions that interconvert them. The phosphorylation of Thr 104 and Thr 150 (sites 8 and 10) is required for inactivation of Wee1, but the other sites (sites 1, 2, and 3) tend to be phosphorylated first. The red, green, and blue edges represent particular paths to Thr 150 phosphorylation as discussed in the text.

empirically chose a concentration of PP2A that would allow ~30 nM $p13^{Suc1}$- Δ65-cyclin B1-Cdk1AF to produce a steady-state level of Wee1-T150 phosphorylation of about 0.5 mol phosphate/mol Wee1.

The reconstituted system produced an ultrasensitive response, with an apparent Hill coefficient of 1.5 (Figure 2B). This indicates that some of the ultrasensitivity in the response of Wee1 to Cdk1 in extracts was intrinsic to the Wee1/Cdk1/phosphatase system. However the ultrasensitivity observed here was significantly less than that of the full extract system (n_H = 3.5), suggesting that much of the ultrasensitivity in the extract was extrinsic—that is, due to factors outside the core Wee1/Cdk1/phosphatase system.

If the ultrasensitivity in the reconstituted Wee1/Cdk1/phosphatase system was due mainly to the mechanism of Wee1 phosphorylation rather than dephosphorylation, then a plot of the cumulative extent of Wee1 phosphorylation at some fixed time for the two-component system of Wee1 and Cdk1 should be similar in shape to the steady-state stimulus/response curve for the three-component Wee1/Cdk1/PP2A system. This was in fact the case (Figures 2B and 2C), allowing us to further simplify the system and to avoid using the least well-defined of the three components, the partially purified PP2A, in many of our further studies. Note that the Cdk1 preparations used in Figures 2B and 2C were different, and the fact that the two curves have similar *EC*50's despite the absence of PP2A in one (Figure 2C) is coincidental.

The Wee1 Phosphorylation Network

The mitotic inactivation of Wee1 involves the phosphorylation of at least five sites (Kim et al., 2005). Two of the sites, Thr 104 and Thr 150 (referred to in Figure 3 as sites 8 and 10, because they are the eighth and tenth SP/TP sequences from the N terminus), are well-conserved and are essential for the inactivation of Wee1. The other three sites, Ser 38, Thr 53, and Ser 62 (sites 1, 2, and 3; Figure 3) are poorly conserved and are dispensable for Wee1 inactivation (Kim et al., 2005). Recent deletion studies suggest

the possibility of three other sites as well (Ser 74, Thr 86, Thr 101) (unpublished data). However, for simplicity we will focus on the five best-established sites.

With five phosphorylation sites there are $2^5 = 32$ potential phosphostates for Wee1 (Figure 3). Eight phosphostates are phosphorylated at both Thr 104 and Thr 150, and so are expected to be inactive (Figure 3, yellow shading). An additional eight phosphostates are phosphorylated at Thr 150 but not Thr 104 (Figure 3, pink shading), and so would be recognized by the pThr 150 antibodies used here as a surrogate for Wee1 inactivation. The 32 phosphostates are connected by 80 phosphorylation/dephosphorylation pathways (Figure 3). At present there is no evidence for an obligatory order of Wee1 phosphorylation, although sites 1, 2 and 3 tend to be phosphorylated more quickly in *Xenopus* egg extracts than Thr 150 (Kim et al., 2005). A phosphorylation network as complicated as that shown in Figure 3 offers myriad possible mechanisms for the generation of ultrasensitivity.

Zero-Order Ultrasensitivity

We first examined whether zero-order ultrasensitivity might account for the intrinsic ultrasensitivity seen in Figure 2B. When opposing phosphatases and kinases are operating close to saturation, the steady-state level of substrate phosphorylation as a function of kinase concentration becomes steeply sigmoidal (Goldbeter and Koshland, 1981) (see also Supplemental Data).

To assess the potential for zero-order ultrasensitivity in the Wee1 response, we measured the apparent K_m values for the phosphorylation and dephosphorylation of Thr 150 in *Xenopus* egg extracts. We prepared a cycloheximide-treated interphase extract and added sufficient Cdk1AF (200 nM) and nondegradable Δ90-cyclin B (100 nM) to drive the extract into M-phase. After 30 min of incubation the extracts were in M-phase, as assessed by DAPI staining of added sperm chromatin (data not shown). We then added various concentrations of purified recombinant Wee1 to the extract for 2 min and assessed the initial rate of Thr 150 phosphorylation by pT150-Wee1 immunoblotting. As shown in Figure 4A, the rate of Thr 150 phosphorylation increased almost linearly with Wee1 concentration up to the highest concentration used, 120 nM. By nonlinear curve fitting we estimated a K_m value of 566 nM. To obtain the apparent K_m for dephosphorylation, we incubated various concentrations of recombinant Wee1 with M-phase extract for 1 hr to maximally phosphorylate the Wee1, added 50 mM EDTA to rapidly stop protein phosphorylation, and at 0 and 25 s assessed the level of Wee1 Thr 150 phosphorylation. Nonlinear curve fitting yielded an apparent K_m of 97 nM (Figure 4B). Plugging these K_m values into the Goldbeter-Koshland equation (see Supplemental Data) yielded a curve with an apparent Hill coefficient of 1.1, substantially less than the observed value of 1.5. Thus, zero-order ultrasensitivity does not appear to account for much of the observed intrinsic ultrasensitivity.

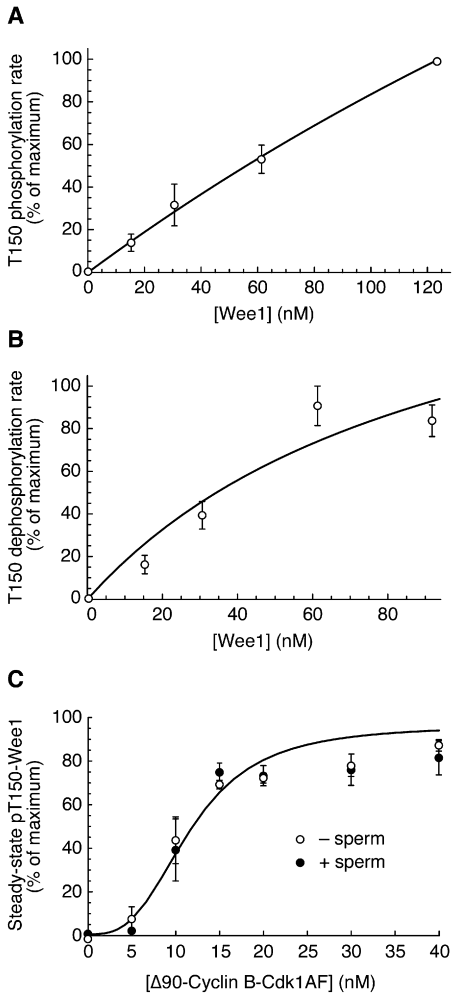

Figure 4. The Role of Zero-Order Ultrasensitivity in the Wee1 Response

(A) Saturation of Wee1 Thr 150 phosphorylation. Various concentrations of recombinant Wee1 proteins were incubated in Δ90-cyclin B-treated extracts for 2 min. The phosphorylation of Wee1 at Thr 150 at 2 min was assessed by pT150-Wee1 immunoblotting and densitometry, and was used to estimate initial velocities. Data points represent means ± standard errors (n = 4). The curve represents the best fit to the Michaelis-Menten equation.

(B) Saturation of Wee1 Thr 150 dephosphorylation. Various concentrations of recombinant Wee1 protein were fully phosphorylated by incubation with M-phase egg extract for 1 hr and then extract was treated with 50 mM EDTA for 25 s. The decrease of phosphorylation between 0 and 25 s was assessed by pT150-Wee1 immunoblotting and densitometry and used to estimate initial velocities. Data points represent means ± standard errors (n = 5). The curve represents the best fit to the Michaelis-Menten equation.

(C) Steady-state response of Wee1 to Cdk1 in the presence and absence of sperm chromatin (1000/μl). Interphase extracts were treated with Δ90-cyclin B and Cdk1AF for 2 hr. The phosphorylation of endogenous Wee1 at Thr 150 was assessed as in (B). Data points represent means ± standard errors (n = 5).

One remaining possibility is suggested by the fact that Wee1 is a nuclear protein. If its phosphorylation and dephosphorylation take place mainly in the nucleus, then

the concentration of Wee1 seen by its kinase and phosphatase could be much higher than 10-20 nM, allowing the converting enzymes to operate close to saturation (Ferrell, 1998). If so, then some of the ultrasensitivity seen in extracts (Figure 1C) should require the presence of nuclei. However, as shown in Figure 4C, there was no apparent difference in the response of Wee1 to Δ90-cyclin B-Cdk1AF in extracts in the presence and absence of added sperm chromatin. Thus, there is no evidence that zero-order ultrasensitivity contributes substantially to the Wee1 response.

Dual Phosphorylation of Thr 104 and Thr 150

The inactivation of Wee1 depends upon the phosphorylation of two sites, Thr 104 and Thr 150 (Kim et al., 2005). If the Thr 104 phosphorylation generally precedes the Thr 150 phosphorylation (Figure 3, green path; Figure 5A), the two-step inactivation of Wee1 might generate enough ultrasensitivity to account for the intrinsic ultrasensitivity (see Supplemental Data for further discussion).

To test this hypothesis, we compared the response of wild-type Wee1 to Wee1 with one of the inactivating phosphorylation sites, Thr 104, mutated to glutamic acid (Wee1-T104E). As shown in Figure 5B, WT-Wee1 and Wee1-T104E were indistinguishable in their responses to $p13^{Suc1}$-Δ65-cyclin B1-Cdk1AF. Thus, the requirement for dual phosphorylation for the inactivation of Wee1 does not contribute significantly to Wee1's intrinsic ultrasensitivity.

A Role for Wee1's Inessential Phosphorylation Sites

We considered two other plausible mechanisms for the intrinsic ultransensitivity of the Wee1 response. The first was suggested by the observation that the three inessential phosphorylation sites in the N terminus of Wee1 (Ser 38, Thr 53, and Ser 62) tend to be phosphorylated prior to Thr 150 (Kim et al., 2005). Thus, long paths to the phosphorylation of Thr 150 (Figure 3, red path) may predominate if all phosphorylation sites are present, even though shorter paths (Figure 3, blue and green paths) can be taken if the inessential sites are missing. These long paths could generate multisite ultrasensitivity, as discussed further in the Supplemental Data, through competition between the essential and inessential sites (Figures 5D and 5E). The second mechanism was based on the fact that Wee1 is not only a substrate of Cdk1 but also a regulator of Cdk1. If a complex between the catalytic domain of Wee1 and the phosphorylation site of Cdk1 accumulates to an appreciable extent, then Wee1 could act as a stoichiometric inhibitor of Cdk1AF. This inhibition could generate the threshold in the response of Wee1 to Cdk1AF.

These two mechanisms were tested by examining whether mutation of the N-terminal phosphorylation sites in Wee1 affected the intrinsic ultrasensitivity in the in vitro system. If the first mechanism (N-terminal phosphorylation sites as decoys) predominated, mutating these sites should eliminate the ultrasensitivity. If the second mechanism (stoichiometric inhibiton of Cdk1AF by the catalytic domain of Wee1) predominated, mutating these sites should have no effect. Accordingly, we generated a quintuple-site mutant of Wee1 (with Ser 38, Thr 53, Ser 62, Thr 101, and Thr 104 all mutated to glutamic acid; designated Wee1-12378E) and compared its response to Cdk1 to that of wild-type Wee1. As shown in Figure 5C, the response of Wee1-12378E was nearly hyperbolic (n_H = 1.1), whereas the response of wild-type Wee1 was slightly ultrasensitive (n_H = 1.4). Thus, the intrinsic ultrasensitivity in Wee1 Thr 150 phosphorylation depends upon the presence of these poorly conserved N-terminal sites. Although inessential for the inactivation of Wee1, they are nevertheless required for the generation of an ultrasensitive response.

If the N-terminal sites in one Wee1 molecule can compete with Thr 150 in a second Wee1 molecule, then adding recombinant Wee1-T150A to the core system of recombinant Wee1, PP2A, and $p13^{Suc1}$-Δ65-cyclin B1-Cdk1AF should increase the observed ultrasensitivity. As shown in Figure 5F, this was in fact the case: adding Wee1-T150A shifted the steady-state response curve to the right and increased the ultrasensitivity from 1.5 to 2.1. This shows that competition between Wee1 molecules for access to Cdk1 can generate ultrasensitivity.

Extrinsic Ultrasensitivity: Intermolecular Competition and Dilution Effects

Much of the ultrasensitivity seen in extracts (Figure 1C) is not accounted for by intrinsic mechanisms (Figure 2), and thus must be due to some protein or proteins extrinsic to the core Wee1/Cdk1/phosphatase system. One attractive hypothesis is competition between Wee1 and other Cdk1 substrates, with Wee1 becoming phosphorylated after these preferred substrates are saturated with Cdk1. A preferred substrate can act much like a stoichiometric inhibitor and can therefore produce a threshold and ultrasensitivity (Supplemental Data). Recent experimental work has established that the budding yeast Cdk1 protein can phosphorylate hundreds of cellular proteins with a range of affinities and efficiencies (Loog and Morgan, 2005; Ubersax et al., 2003). If, as seems likely, vertebrate Cdk1 also interacts with a great number of targets, it seems plausible that some of the *Xenopus* Cdk1 substrates could compete effectively with Wee1 for access to Cdk1.

To explore this idea, we modeled a simple intermolecular competition mechanism between Wee1 and a second substrate, denoted Sub2, for access to active Cdk1 (Figure 6A). If we chose rate constants such that Sub2 was preferred over Wee1 and a substantial concentration of Cdk1-Sub2 complex was formed (a_1 = 1, a_2 = 10; Figures 6A and 6B, blue and red lines), the result was a prominent threshold in the Wee1 stimulus/response curve. Thus, intermolecular competition could contribute to Wee1's observed ultrasensitivity.

One strong prediction of the intermolecular competition model is that the size of the threshold and the shape of the stimulus/response curve should be sensitive functions of the concentration of the competitor (Figure 6B), with the response becoming less ultrasensitive as the competitor

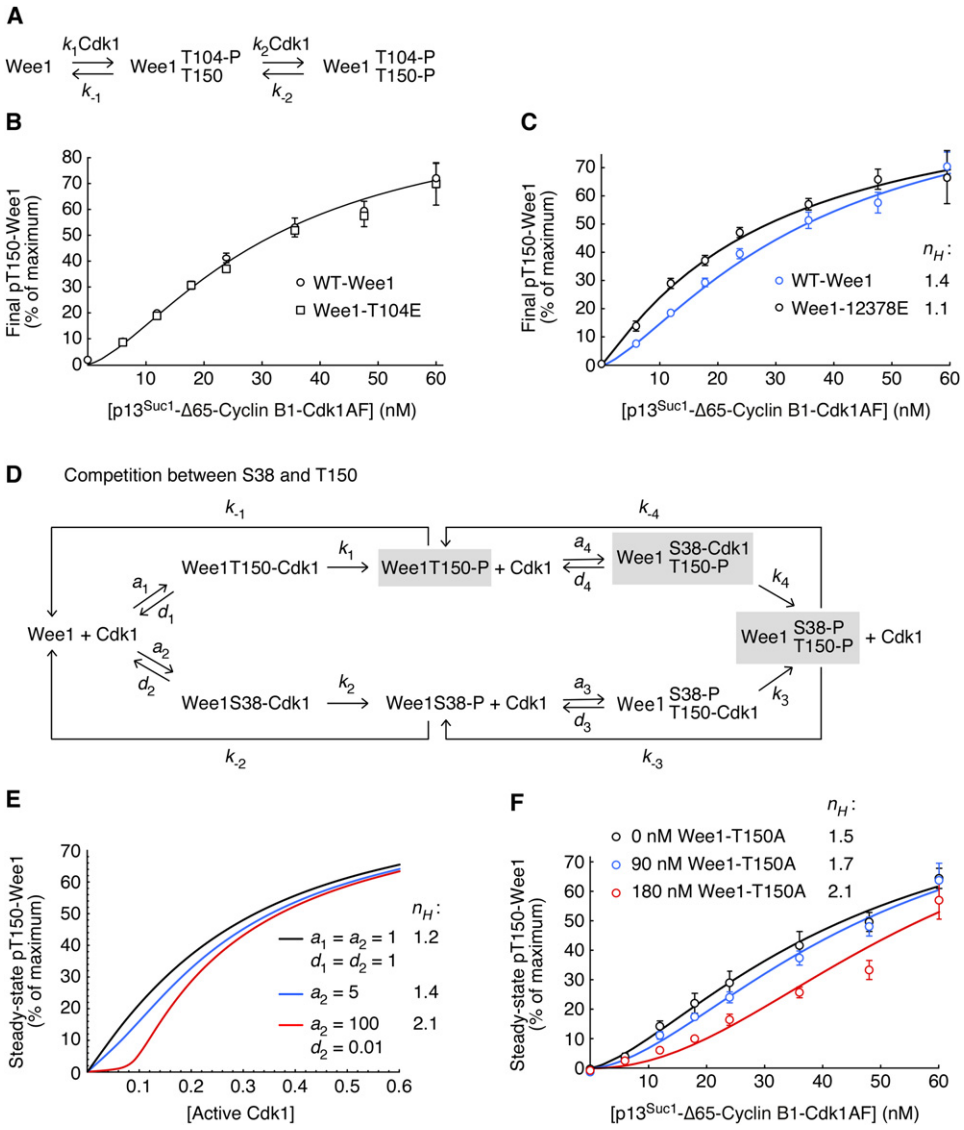

Figure 5. The Role of Multisite Phosphorylation in the Wee1 Response

(A) Inactivation of Wee1 through the sequential phosphorylation of Thr 104 and Thr 150. If nonprocessive, this type of mechanism could yield an ultrasensitive response with an apparent Hill coefficient between 1 and 2 (Equation 13 in the Supplemental Data).

(B) Mutating Thr 104 does not affect Wee1 Thr 150 phosphorylation. WT-Wee1 and Wee1-T104E were incubated for 1 hr with various concentrations of $p13^{Suc1}$-Δ65-cyclin B1-Cdk1AF, and pT150-Wee1 was assessed by immunoblotting. Data points represent means ± standard errors (n = 3).

(C) The role of the inessential N-terminal sites. Wild-type Wee1 and Wee1-12378E (60 nM) were incubated with various concentrations of $p13^{Suc1}$-Δ65-cyclin B1-Cdk1AF complex for 1 hr. pT150-Wee1 was assessed by immunoblotting. Data points represent means ± standard errors (n = 6).

(D) A kinetic scheme for the competition between one inessential decoy site (Ser 38) and one essential site (Thr 150) for Cdk1.

(E) Theoretical steady-state pT150 Wee1 responses in the face of competition between essential and inessential sites within Wee1. For the black curve, all of the elementary rate constants (*a*'s, *d*'s, and *k*'s) in panel (D) were assumed to be equal to 1, except $k_{-1} = k_{-2} = k_{-3} = k_{-4} = 0.1$. For the red and blue curves, d_2 and/or a_2 were changed as specified in the panel. For other parameter choices, other behaviors are possible, including bistability (Markevich et al., 2004).

(F) Intermolecular competition between Wee1 molecules for access to Cdk1. Recombinant Wee1 (60 nM) was incubated with various concentrations of $p13^{Suc1}$-Δ65-cyclin B1-Cdk1AF complexes in the presence of PP2A for 1 hr, as in Figure 2B, in the absence or presence of purified recombinant Wee1-T150A. Data points represent means ± standard errors (n = 3).

decreases in concentration. A model that combines competition between Wee1 sites (between Ser 38 and Thr 150) and between Wee1 and a second substrate (Figure 6C) makes similar predictions (Figure 6D).

We therefore set out to determine whether the ultrasensitivity of the Wee1 response was decreased in diluted extracts. We prepared extracts at three concentrations: undiluted (1×), diluted to 30%-strength (0.3×), or diluted

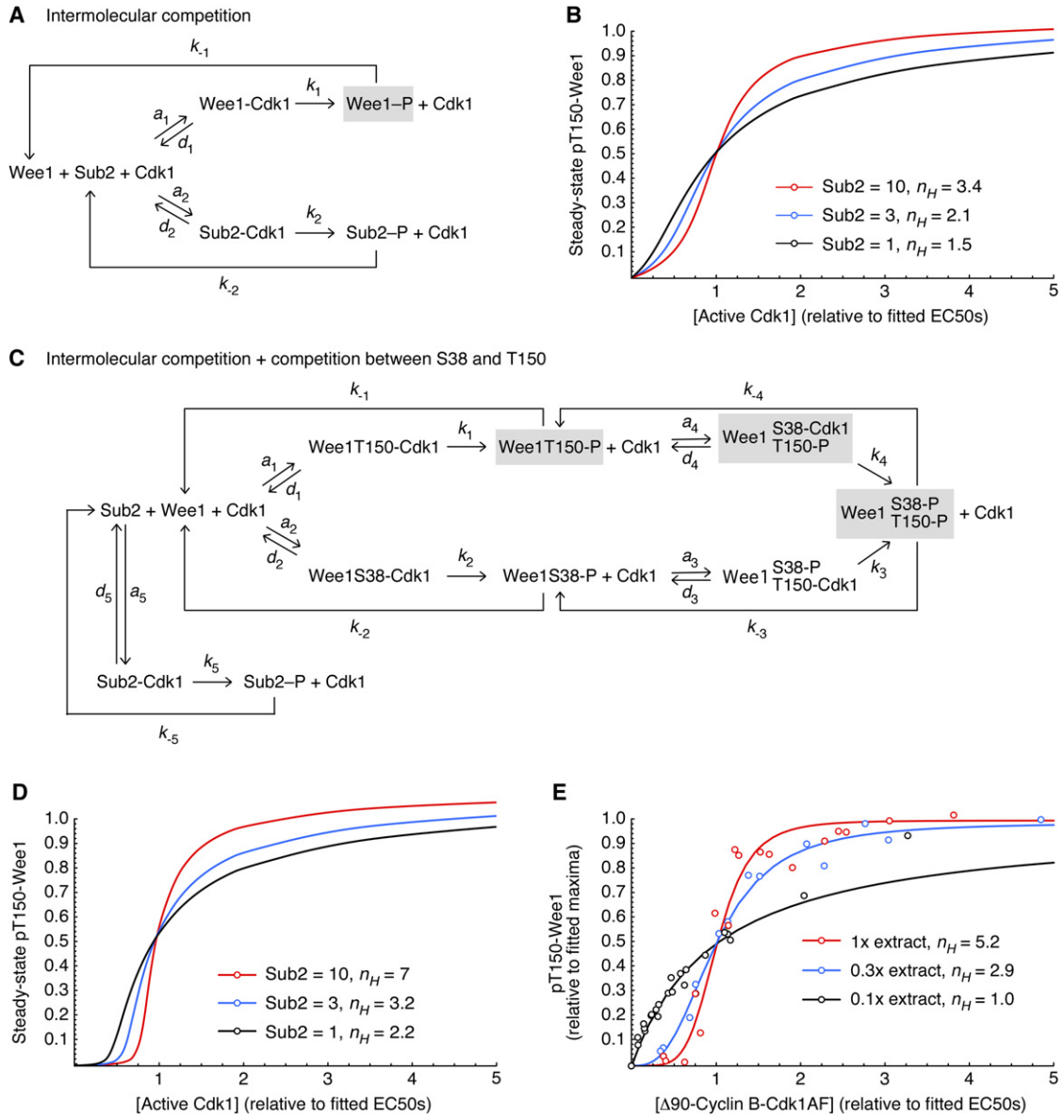

Figure 6. Extrinsic Ultrasensitivity: Intermolecular Competition

(A) Kinetic scheme for intermolecular competition between Wee1 and a second Cdk1 substrate, Sub2.

(B) Theoretical stimulus/response curves for various assumed concentrations of Sub2. For each of the curves shown, it was assumed that $a_1 = d_1 = d_2 = k_1 = k_2 = [\text{Wee1}_{\text{tot}}] = 1$; $a_2 = 10$; $k_{-1} = k_{-2} = 0.1$. To emphasize the relative shapes of the three curves, we have taken each *EC*50 to be equal to 1.

(C) Kinetic scheme for combined competition between Ser 38 and Thr 150 and intermolecular competition between Wee1 and Sub2, for access to active Cdk1.

(D) Theoretical stimulus/response curves for various assumed concentrations of Sub2. For each of the curves shown, it was assumed that all of the *a*'s, *d*'s, *k*'s, and $[\text{Wee1}_{\text{tot}}] = 1$, except $a_2 = a_5 = 100$, $d_2 = d_5 = 0.01$, and $k_{-1} = k_{-2} = k_{-3} = k_{-4} = k_{-5} = 0.1$. To emphasize the relative shapes of the three curves, we have taken each *EC*50 to be equal to 1.

(E) Steady-state response of Wee1 to Cdk1 in diluted extracts. Data were taken from three independent experiments, and each extract was normalized to its fitted maximum and *EC*50 values, again to emphasize the shapes of the curves.

to 10%-strength (0.1×). We restored the concentration of Wee1 in the extracts to 20 nM by adding recombinant Wee1, incubated the extracts with various concentrations of Δ90-cyclin B/Cdk1AF for 90 min at room temperature, and then assessed the steady-state level of Wee1 Thr 150 phosphorylation by immunoblotting. The combined results of three experiments are shown in Figure 6E. The Wee1 response in the 1× extracts was highly ultrasensitive, with an apparent Hill coefficient of 5.2 (Figure 6E, red curve). The Wee1 response in the 0.3× extract was

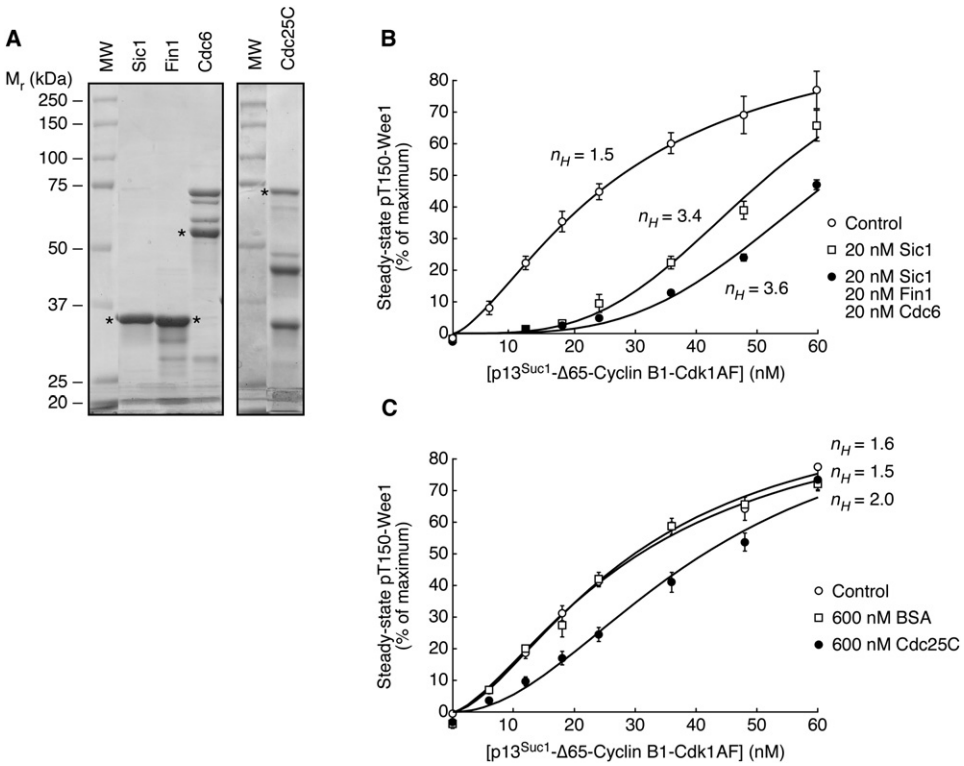

Figure 7. Reconstitution of a Highly Ultrasensitive Response In Vitro

(A) Coomassie-stained gel of recombinant yeast Sic1, yeast Fin1, yeast Cdc6, and *Xenopus* Cdc25C. Asterisks denote the Sic1, Fin1, Cdc6, and Cdc25C bands.

(B) Ultrasensitivity in vitro. Recombinant Wee1 (60 nM) was incubated for 1 hr with PP2A and various concentrations of p13^{Suc1}-Δ65-cyclin B1-Cdk1AF in the presence of no competitor substrate (○); 20 nM Sic1 (□); or 20 nM Sic1, 20 nM Cdc6, 20 nM Fin1 (●). Data are from five independent experiments and are plotted as means ± standard errors. Curves were obtained by nonlinear least squares regression to the Hill equation.

(C) Ultrasensitivity in vitro. Recombinant Wee1 (60 nM) was incubated for 1 hr with PP2A and various concentrations of p13^{Suc1}-Δ65-cyclin B1-Cdk1AF in the presence of no competitor substrate (○); 600 nM bovine serum albumin (BSA) (□); or 600 nM Cdc25C (●). Data are from three independent experiments and are plotted as means ± standard errors. Curves were obtained by nonlinear least squares regression to the Hill equation.

more graded, with an apparent Hill coefficient of 2.9 (Figure 6E, blue curve). In the 0.1× extract the response was indistinguishable from a Michaelian response (Figure 6E, black curve). This supports the hypothesis that much of Wee1's extrinsic ultrasensitivity arises through intermolecular competition.

Reconstitution of a Highly Ultrasensitive Response In Vitro

If the intermolecular competition hypothesis is correct, it should be possible to reconstitute a highly ultrasensitive response in vitro by adding an appropriate competitor to the Wee1/Cdk1 system. Loog and Morgan have identified a number of yeast proteins that are high-affinity substrates for yeast Cdk1-cyclin complexes (Loog and Morgan, 2005). We expressed three of these potential competitors in *E. coli* (Cdc6, Fin1, and Sic1), purified them (Figure 7A), and examined their effects individually and together on the Wee1 stimulus/response curve in vitro.

As shown in Figure 7B, adding 20 nM Sic1 shifted the stimulus/response curve to the right and made the response more ultrasensitive. Adding Cdc6 and Fin1 together with Sic1 (Figure 7B) shifted the curve further to the right and slightly increased the apparent Hill coefficient. The extent of ultrasensitivity achieved with Sic1 alone (n_H = 3.4) or with all three competitors (n_H = 3.6) in vitro was comparable to that seen in the extract system (n_H = 3.5). Similar results were obtained for the cumulative response of Wee1 in the absence of added PP2A in vitro (data not shown). We also carried out reconstitution experiments using recombinant bacterially expressed *Xenopus* Cdc25C (Figure 7A). The N terminus of Cdc25C contains 13 SP/TP motifs, any of which might bind Cdk1. At a concentration of 600 nM (cf. an estimated endogenous concentration of 140 nM [Kumagai and Dunphy, 1992]), Cdc25C shifted the Wee1 stimulus/response curve to the right and made the response more ultrasensitive (n_H = 2.0) (Figure 7C). Taken together, these findings experimentally

confirm that intermolecular competition can generate a highly ultrasensitive response.

DISCUSSION

A simple phosphorylation-dephosphorylation cycle, with neither the kinase nor the phosphatase saturated by substrate, will exhibit a graded, Michaelian response to stimuli. However, a number of mechanisms, including cooperativity, enzyme saturation, multisite phosphorylation, and stoichiometric inhibitors, can produce a qualitatively different type of response—a sigmoidal, ultrasensitive response. Ultrasensitivity is an important building block for several complex systems-level behaviors, including bistability.

Here, we have shown that the response of Wee1 to Cdk1 in *Xenopus* egg extracts is highly ultrasensitive (Figure 1C). The steady-state stimulus/response curve is fairly flat at low and high stimulus levels, but switches from off to on over a narrow 3- to 4-fold range of Cdk1 concentrations. In general a response like this can be useful for filtering noise and amplifying sensitivity. In the case of the Cdk1/Wee1 system, we suspect that Wee1's ultrasensitivity also permits the generation of a bistable response, which helps make interphase and M-phase discrete states (Pomerening et al., 2003; Sha et al., 2003) and helps keep the Cdk1/APC oscillator from approaching a stable steady state with an intermediate level of Cdk1 activity (Pomerening et al., 2005).

Intrinsic Ultrasensitivity from Competition between Wee1 Phosphorylation Sites

A small amount of Wee1's ultrasensitivity can be observed in a simple reconstituted system consisting of purified recombinant Wee1, PP2A, and $p13^{Suc1}$- Δ65-cyclin B1-Cdk1AF (Figure 2). This intrinsic ultrasensitivity appears not to represent a simple zero-order effect; the Wee1 phosphatase and the Wee1 kinase are not sufficiently saturated by Wee1 to produce the experimentally observed intrinsic ultrasensitivity (Figure 4). The intrinsic ultrasensitivity requires the presence of several poorly conserved N-terminal phosphorylation sites (Figure 5C), which suggests that these sites act as decoys, competing with Thr 150 (and possibly Thr 104) for access to active Cdk1. At least some of this competition is probably intermolecular, since the addition of Wee1-T150A to the in vitro system increases the ultrasensitivity of the wild-type Wee1 response (Figure 5F).

These findings provide a rationalization for a puzzling aspect of Wee1 evolution. The N-terminal regulatory region of Wee1 is highly divergent across species. Nevertheless, all Wee1 species sequenced to date contain multiple N-terminal SP/TP phosphorylation sites, although the number and exact location of the sites varies considerably. For example, *Xenopus* embryonic Wee1A contains 10 SP/TP sites; human somatic Wee1A contains 15; *Drosophila* Dwee1 contains 8; *S. pombe* Wee1 contains 24; and *S. cerevisiae* Swe1 contains 13. If the function of many or most of these sites is simply to soak up the first increments of active Cdk1 presented to Wee1, as opposed to mediating some complicated allosteric rearrangement, it could explain why the evolutionary constraints on the sites appear to be so weak.

These findings also help to rationalize recent observations on the regulation of Swe1 by phosphorylation (Harvey et al., 2005). Wee1 proteins undergo multiple N-terminal phosphorylations just before cells enter mitosis (McGowan and Russell, 1995; Mueller et al., 1995a; Parker et al., 1995; Tang et al., 1993; Watanabe et al., 1995), and usually the SP/TP phosphorylation of Wee1 correlates with its inactivation. Indeed, mutating all of the SP/TP sites in *Xenopus* Wee1A renders it constitutively active (Kim et al., 2005). However, Harvey and colleagues found that when some sites in Swe1 are mutated to nonphosphorylatable residues, the resulting Wee1 allele has low activity, not high activity (Harvey et al., 2005). It seems plausible that these sites might be decoy sites rather than inactivation sites, and that mutating them frees Cdk1 to phosphorylate mitotic substrates.

Note that two other important substrates and regulators of Cdk1, Myt1 and Cdc25, also have multiple poorly conserved SP/TP residues. In the case of Myt1, many of the SP/TP residues reside in a C-terminal regulatory domain that has been implicated in Cdk1 binding (Wells et al., 1999). Thus, it seems plausible that this type of intrinsic competition is a recurring theme in Cdk1-substrate interactions.

Extrinsic Ultrasensitivity and Intermolecular Competition

Although some of Wee1's observed ultrasensitivity appears to be intrinsic to the core Wee1/Cdk1/phosphatase system, much of it is not (Figure 1C versus Figure 2B). Our working hypothesis is that this extrinsic ultrasensitivity is mainly due to competition between Wee1 and other Cdk1 substrates for access to Cdk1. Several observations support this hypothesis. First, modeling studies show that such a mechanism could plausibly account for the observed extrinsic ultrasensitivity (Figures 6C–6D). Second, comprehensive studies of Cdk1 substrates in yeast indicate that Cdk1 has hundreds of substrates (Ptacek et al., 2005; Ubersax et al., 2003), many containing multiple SP/TP sites that might be expected to tie Cdk1 up more effectively than single sites could (Klein et al., 2003). Third, a key prediction of the intermolecular competition model, that the steady-state stimulus/response curve should become less ultrasensitive as the concentration of the extract is lowered, was observed experimentally (Figure 6E). Fourth, a highly ultrasensitive response could be reconstituted in vitro by adding Sic1 or Cdc25C to the core Wee1/Cdk1/phosphatase system (Figures 7B and 7C). Taken together, this evidence supports the hypothesis that intermolecular competition accounts for the extrinsic ultrasensitivity observed in the response of Wee1 in extracts. It was somewhat surprising that Sic1 produced a substantial threshold at a concentration of 20 nM (Figure 7B), when

its apparent K_m for Cdk1 was found to be approximately 150 nM (data not shown). This suggests that the effective K_d for the Sic1-Cdk1 complex under steady-state phosphorylation conditions might be substantially lower than the apparent K_m, which was derived from initial velocity experiments. For example, some partially phosphorylated form of Sic1 might bind Cdk1 more strongly than nonphosphorylated Sic1 does.

Sic1 is unusual in that it is not only a Cdk substrate, but also a stoichiometric Cdk inhibitor. We suspect that these aspects of Sic1 function are two sides of the same coin—that its ability to inhibit the phosphorylation of other Cdk1 substrates simply reflects its ability to form long-lived complexes with Cdk1 while it itself is being phosphorylated (Nash et al., 2001; Verma et al., 1997). This idea is supported by structural studies of the binding of p27 Kip1, another protein that acts as both a substrate and stoichiometric inhibitor of Cdks, to cyclin A-Cdk2 complexes (Russo et al., 1996).

A definitive test of the intermolecular competition model awaits the identification of the relevant competitors. One possibility is Xic1, a Cdk1 substrate and inhibitor related to p21 Cip1 and p27 Kip1 (Shou and Dunphy, 1996; Su et al., 1995). However, Xic1 is a poor inhibitor of cyclin B/Cdk1 (Shou and Dunphy, 1996; Su et al., 1995) and is too low in abundance to be an effective stoichiometric competitor (Shou and Dunphy, 1996). In addition, purified recombinant Xic1 (10 nM) had no apparent affect on the phosphorylation of Wee1 (60 nM) by cyclin B1-Cdk1 (0-60 nM) in vitro (data not shown), indicating that at physiological concentrations (2 nM), Xic1 is unlikely to contribute to the ultrasensitivity of Wee1.

Intermolecular competition should impose an ultrasensitive response on not only Wee1, but also any Cdk1 substrate with similar or weaker binding to Cdk1. This might explain why a number of proteins phosphorylated downstream of Cdk1, including Wee1, Cdc25, and p42 MAPK, all exhibit similarly switch-like responses to more graded changes in Cdk1 activity in extracts (Pomerening et al., 2005).

Intermolecular competition has the potential to be a true systems-level mechanism for generating an ultrasensitive response. It is possible that scores or hundreds of substrates contribute to the ultrasensitive response of Wee1 to Cdk1, and to the bistability of the Cdk1/Cdc25/Wee1/Myt1 system. Mutations in any of these substrates might have an effect on the function of Wee1, albeit a subtle one if a great number of nonmutated substrates would continue to contribute normally to the ultrasensitivity.

Competition, Ultrasensitivity, and Temporal Thresholds

It seems likely that competition is important beyond its role in generating ultrasensitive steady-state responses. Georgi and coworkers have shown that there is a discrete temporal ordering in the phosphorylation of Cdk1 substrates, with Wee1 and Cdc25 phosphorylated early, and the APC component Cdc27 phosphorylated late (Georgi et al., 2002). Likewise, competition between APC substrates appears to be critical for determining the order of polyubiquitination and proteolysis of various APC substrates (Rape et al., 2006). In the same way that competition can introduce a concentration threshold into a steady-state response, it can introduce a temporal threshold into a dynamic response. The importance of generating both sharp temporal thresholds and sharp concentration thresholds is readily apparent; competition provides a simple general mechanism for generating both.

EXPERIMENTAL PROCEDURES

Steady-State Wee1 Inactivation with *Xenopus* Egg Extracts

Interphase egg extracts were prepared in the presence of cycloheximide (100 μg/ml) as previously described (Murray, 1991; Smythe and Newport, 1991). Extracts were supplemented with nondegradable sea urchin Δ90-cyclin B (0 - 100 nM) and noninhibitable Cdk1AF (200 nM). Demembranated sperm chromatin was routinely added (500/μl) to monitor progression into mitosis. Samples of egg extract were taken after 2 hr and frozen on dry ice for subsequent histone H1 kinase assay and phospho-Thr150 Wee1 blotting. Samples equivalent to 0.2 μl extract were used for histone H1 kinase assay and the resulting ^{32}P-labeled histone was detected and quantified by SDS gel electrophoresis and blotting followed by phosphoimaging. For phospho-Thr150 Wee1 blotting, 1 μl of extract sample was subjected to SDS polyacrylamide gel electrophoresis, blotted, and probed with affinity-purified pT150-Wee1 antibody (1:1000). The phosphorylated band was quantified by Photoshop or GelDoc (Bio-Rad, Hercules, CA). Care was taken to ensure that the band intensity was linear with respect to protein loading.

Expression of Recombinant Proteins in Insect Cells

N-6xHis/Flag-tagged *Xenopus* Wee1A (the product of the embryonic Wee1 gene), N-6xHis/Flag-tagged *Xenopus* Cdk1AF, and C-6xHis/Flag-tagged nondegradable *Xenopus* cyclin B1 (Δ65-cyclin B1) were expressed in S_f9 cells and affinity-purified using anti-Flag M2 agarose (Sigma-Aldrich, St. Louis, MO) as described previously (Pomerening et al., 2005). Wee1-T104E and Wee1-12378E mutants were constructed by QuickChange mutagenesis (Stratagene, La Jolla, CA). Primer sequences are provided in the Supplemental Data.

p13^{Suc1}-Δ65-cyclin B1-Cdk1AF complexes were prepared by a modification of a published procedure (Patra and Dunphy, 1996). GST- and Flag-tagged p13^{Suc1} from bacterial culture (200 ml) was bound to glutathione-Sepharose (0.4 ml, Amersham, Piscataway, NJ). Δ65-cyclin B1 and Cdk1AF (50 ml each) were individually expressed in S_f9 cells, combined, lysed with 8 ml of lysis buffer (50 mM Tris-Cl, 150 mM NaCl, 1 mM EDTA, 10 mM β-mercaptoethanol, 0.5 mM ATP, 10 mM $MgCl_2$, 0.1% Nonidet P-40, 10 μg/ml leupeptin, 10 μg/ml chymostatin, and 10 μg/ml pepstatin [pH 7.4]). After incubation for 30 min at room temperature, S_f9 cell lysates were added to p13^{Suc1}-bound glutathione resin and agitated for 2 hr at 4°C. The resin was washed three times with KB buffer (20 mM HEPES, 150 mM NaCl, 5 mM $MgCl_2$, 10% sucrose, 0.1% Nonidet P-40 [pH 7.7]) and once with KB buffer without detergent. p13^{Suc1}-Δ65-cyclin B1-Cdk1AF complexes were eluted with 50 mM Tris-Cl (pH 8.0) containing 10 mM glutathione.

Preparation of Recombinant Proteins in Bacteria

Constructs for bacterial expression of *S. cerevisiae* Sic1, Cdc6, and Fin1, in the pET28a vector, were obtained from D. O. Morgan (UCSF, CA). Proteins were expressed in bacteria with 0.4 mM IPTG induction for 3 hr at 29°C, and purified using nickel-NTA Superflow as described in the 6xHis-tagged protein manual (Qiagen, Valencia, CA). In the case of Fin1, the cell pellet was lysed in 8 M urea, which was gradually

removed from the protein preparation by washing the column with buffers containing decreasing concentrations of urea. Protein concentrations were determined from Coomassie-stained SDS-PAGE gels using bovine serum albumin as a standard.

Phosphorylation and Dephosphorylation of Wee1 with Egg Extracts

For phosphorylation reactions, cycloheximide-treated (100 μg/ml) interphase extracts were prepared and incubated with Cdk1AF (200 nM) and nondegradable Δ90-cyclin B (100 nM) for 30 min. Various concentrations of recombinant Wee1 protein was then added to the extract, incubated for two minutes, and reaction was terminated by adding SDS sample buffer. Wee1 phosphorylation was analyzed by pT150-Wee1 immunoblotting as described above.

For dephosphorylation reactions, recombinant Wee1 proteins were incubated with cycloheximide-treated extracts preincubated with Cdk1AF (200 nM) for 1 hr to produce maximal phosphorylation. Dephosphorylation initiated by the addition of 50 mM EDTA, was allowed to proceed for 25 s, and was stopped by the addition of SDS sample buffer. Dephosphorylation was estimated from the change of pT150-Wee1 signal between 0 and 25 s detected by pT150-Wee1 immunoblotting.

In Vitro Reconstituted Wee1 Inactivation

Recombinant Wee1 (60 nM) was incubated with various concentrations of $p13^{Suc1}$-Δ65-cyclin B1-Cdk1AF complex and PP2A (Upstate, Temecula, CA) in the presence or absence of Sic1, Fin1, and Cdc6 (20 nM each). The kinase reaction was carried out at 30°C for 1 hr in kinase buffer (15 μl) containing 50 mM Tris-Cl (pH 7.5), 10 mM $MgCl_2$, 0.3 mM ATP, 2 mM dithiothreitol, 0.01% Brij35, and 1 mM EGTA.

Supplemental Data

Supplemental Data include Supplemental Experimental Procedures, Supplemental References, and three figures and can be found with this article online at http://www.cell.com/cgi/content/full/128/6/1133/DC1/.

ACKNOWLEDGMENTS

We thank Bill Dunphy and Peter Jackson for providing Kix1/Xic1 constructs and antibody, Mike Tyers for providing Sic1 antibodies, Joe Pomerening for providing Cdk1AF and Δ65-cyclin B1 baculoviruses and Cdc25C protein, Dave Byron for the $p13^{Suc1}$ construct, Jianbo Yue for Δ90-cyclin B protein and frog sperm, Zach Serber and Jeff Ubersax for help with protein preparation, Dave Morgan, Mart Loog, Doug Kellogg, Sally Kornbluth, Seth Margolis, and Xiaoxia Lin for helpful discussions, and members of the Ferrell lab for comments on the manuscript. This work was supported by a grant from the National Institutes of Health (GM46383).

Received: October 5, 2006
Revised: November 28, 2006
Accepted: January 31, 2007
Published: March 22, 2007

REFERENCES

Angeli, D., Ferrell, J.E., Jr., and Sontag, E. (2004). Detection of multistability, bifurcations, and hysteresis in a large class of biological positive-feedback systems. Proc. Natl. Acad. Sci. USA *101*, 1822–1827.

Bagowski, C.P., Besser, J., Frey, C.R., and Ferrell, J.E., Jr. (2003). The JNK cascade as a biochemical switch in mammalian cells. Ultrasensitive and all-or-none responses. Curr. Biol. *13*, 315–320.

Brown, G.C., Hoek, J.B., and Kholodenko, B.N. (1997). Why do protein kinase cascades have more than one level? Trends Biochem. Sci. *22*, 288–289.

Burack, W.R., and Sturgill, T.W. (1997). The activating dual phosphorylation of MAPK by MEK is nonprocessive. Biochemistry *36*, 5929–5933.

Chen, M., Li, D., Krebs, E.G., and Cooper, J.A. (1997). The casein kinase II beta subunit binds to Mos and inhibits Mos activity. Mol. Cell. Biol. *17*, 1904–1912.

Cross, F.R., Archambault, V., Miller, M., and Klovstad, M. (2002). Testing a mathematical model of the yeast cell cycle. Mol. Biol. Cell *13*, 52–70.

Fattaey, A., and Booher, R.N. (1997). Myt1: a Wee1-type kinase that phosphorylates Cdc2 on residue Thr14. Prog. Cell Cycle Res. *3*, 233–240.

Ferrell, J.E., Jr. (1996). Tripping the switch fantastic: how a protein kinase cascade can convert graded inputs into switch-like outputs. Trends Biochem. Sci. *21*, 460–466.

Ferrell, J.E., Jr. (1997). How responses get more switch-like as you move down a protein kinase cascade. Trends Biochem. Sci. *22*, 288–289.

Ferrell, J.E., Jr. (1998). How regulated protein translocation can produce switch-like responses. Trends Biochem. Sci. *23*, 461–465.

Ferrell, J.E., Jr., and Bhatt, R.R. (1997). Mechanistic studies of the dual phosphorylation of mitogen-activated protein kinase. J. Biol. Chem. *272*, 19008–19016.

Ferrell, J.E., Jr., and Xiong, W. (2001). Bistability in cell signaling: how to make continuous processes discontinuous, and reversible processes irreversible. Chaos *11*, 227–236.

Gardner, T.S., Cantor, C.R., and Collins, J.J. (2000). Construction of a genetic toggle switch in Escherichia coli. Nature *403*, 339–342.

Georgi, A.B., Stukenberg, P.T., and Kirschner, M.W. (2002). Timing of events in mitosis. Curr. Biol. *12*, 105–114.

Goldbeter, A., and Koshland, D.E., Jr. (1981). An amplified sensitivity arising from covalent modification in biological systems. Proc. Natl. Acad. Sci. USA *78*, 6840–6844.

Goldbeter, A., and Koshland, D.E., Jr. (1982). Sensitivity amplification in biochemical systems. Q. Rev. Biophys. *15*, 555–591.

Hardie, D.G., Salt, I.P., Hawley, S.A., and Davies, S.P. (1999). AMP-activated protein kinase: an ultrasensitive system for monitoring cellular energy charge. Biochem. J. *338*, 717–722.

Harvey, S.L., Charlet, A., Haas, W., Gygi, S.P., and Kellogg, D.R. (2005). Cdk1-dependent regulation of the mitotic inhibitor Wee1. Cell *122*, 407–420.

Hoffmann, I., Clarke, P.R., Marcote, M.J., Karsenti, E., and Draetta, G. (1993). Phosphorylation and activation of human cdc25-C by cdc2–cyclin B and its involvement in the self-amplification of MPF at mitosis. EMBO J. *12*, 53–63.

Hooshangi, S., Thiberge, S., and Weiss, R. (2005). Ultrasensitivity and noise propagation in a synthetic transcriptional cascade. Proc. Natl. Acad. Sci. USA *102*, 3581–3586.

Huang, C.-Y.F., and Ferrell, J.E., Jr. (1996). Ultrasensitivity in the mitogen-activated protein kinase cascade. Proc. Natl. Acad. Sci. USA *93*, 10078–10083.

Kim, S.Y., Song, E.J., Lee, K.J., and Ferrell, J.E., Jr. (2005). Multisite M-phase phosphorylation of Xenopus Wee1A. Mol. Cell. Biol. *25*, 10580–10590.

Klein, P., Pawson, T., and Tyers, M. (2003). Mathematical modeling suggests cooperative interactions between a disordered polyvalent ligand and a single receptor site. Curr. Biol. *13*, 1669–1678.

Kumagai, A., and Dunphy, W.G. (1992). Regulation of the cdc25 protein during the cell cycle in Xenopus extracts. Cell *70*, 139–151.

LaPorte, D.C., and Koshland, D.E., Jr. (1983). Phosphorylation of isocitrate dehydrogenase as a demonstration of enhanced sensitivity in covalent regulation. Nature *305*, 286–290.

Laurent, M., and Kellershohn, N. (1999). Multistability: a major means of differentiation and evolution in biological systems. Trends Biochem. Sci. *24*, 418–422.

Liu, F., Stanton, J.J., Wu, Z., and Piwnica-Worms, H. (1997). The human Myt1 kinase preferentially phosphorylates Cdc2 on threonine 14 and localizes to the endoplasmic reticulum and Golgi complex. Mol. Cell. Biol. *17*, 571–583.

Loog, M., and Morgan, D.O. (2005). Cyclin specificity in the phosphorylation of cyclin-dependent kinase substrates. Nature *434*, 104–108.

Markevich, N.I., Hoek, J.B., and Kholodenko, B.N. (2004). Signaling switches and bistability arising from multisite phosphorylation in protein kinase cascades. J. Cell Biol. *164*, 353–359.

McGowan, C.H., and Russell, P. (1993). Human Wee1 kinase inhibits cell division by phosphorylating p34cdc2 exclusively on Tyr15. EMBO J. *12*, 75–85.

McGowan, C.H., and Russell, P. (1995). Cell cycle regulation of human WEE1. EMBO J. *14*, 2166–2175.

Meinke, M.H., Bishop, J.S., and Edstrom, R.D. (1986). Zero-order ultrasensitivity in the regulation of glycogen phosphorylase. Proc. Natl. Acad. Sci. USA *83*, 2865–2868.

Mueller, P.R., Coleman, T.R., and Dunphy, W.G. (1995a). Cell cycle regulation of a Xenopus Wee1-like kinase. Mol. Biol. Cell *6*, 119–134.

Mueller, P.R., Coleman, T.R., Kumagai, A., and Dunphy, W.G. (1995b). Myt1: a membrane-associated inhibitory kinase that phosphorylates Cdc2 on both threonine-14 and tyrosine-15. Science *270*, 86–90.

Murray, A.W. (1991). Cell cycle extracts. Methods Cell Biol. *36*, 581–605.

Nash, P., Tang, X., Orlicky, S., Chen, Q., Gertler, F.B., Mendenhall, M.D., Sicheri, F., Pawson, T., and Tyers, M. (2001). Multi-site phosphorylation of a CDK inhibitor sets a threshold for the onset of S-phase. Nature *414*, 514–521.

Novak, B., and Tyson, J.J. (1993). Numerical analysis of a comprehensive model of M-phase control in Xenopus oocyte extracts and intact embryos. J. Cell Sci. *106*, 1153–1168.

Parker, L.L., and Piwnica-Worms, H. (1992). Inactivation of the p34cdc2-cyclin B complex by the human WEE1 tyrosine kinase. Science *257*, 1955–1957.

Parker, L.L., Sylvestre, P.J., Byrnes, M.J., Liu, F., and Piwnica-Worms, H. (1995). Identification of a 95-kDa WEE1-like tyrosine kinase in HeLa cells. Proc. Natl. Acad. Sci. USA *92*, 9638–9642.

Patra, D., and Dunphy, W.G. (1996). Xe-p9, a Xenopus Suc1/Cks homolog, has multiple essential roles in cell cycle control. Genes Dev. *10*, 1503–1515.

Patra, D., Wang, S.X., Kumagai, A., and Dunphy, W.G. (1999). The xenopus Suc1/Cks protein promotes the phosphorylation of G(2)/M regulators. J. Biol. Chem. *274*, 36839–36842.

Pomerening, J.R., Kim, S.Y., and Ferrell, J.E., Jr. (2005). Systems-level dissection of the cell-cycle oscillator: bypassing positive feedback produces damped oscillations. Cell *122*, 565–578.

Pomerening, J.R., Sontag, E.D., and Ferrell, J.E., Jr. (2003). Building a cell cycle oscillator: hysteresis and bistability in the activation of Cdc2. Nat. Cell Biol. *5*, 346–351.

Ptacek, J., Devgan, G., Michaud, G., Zhu, H., Zhu, X., Fasolo, J., Guo, H., Jona, G., Breitkreutz, A., Sopko, R., et al. (2005). Global analysis of protein phosphorylation in yeast. Nature *438*, 679–684.

Rape, M., Reddy, S.K., and Kirschner, M.W. (2006). The processivity of multiubiquitination by the APC determines the order of substrate degradation. Cell *124*, 89–103.

Russo, A.A., Jeffrey, P.D., Patten, A.K., Massague, J., and Pavletich, N.P. (1996). Crystal structure of the p27Kip1 cyclin-dependent-kinase inhibitor bound to the cyclin A-Cdk2 complex. Nature *382*, 325–331.

Sha, W., Moore, J., Chen, K., Lassaletta, A.D., Yi, C.S., Tyson, J.J., and Sible, J.C. (2003). Hysteresis drives cell-cycle transitions in Xenopus laevis egg extracts. Proc. Natl. Acad. Sci. USA *100*, 975–980.

Shou, W., and Dunphy, W.G. (1996). Cell cycle control by Xenopus p28Kix1, a developmentally regulated inhibitor of cyclin-dependent kinases. Mol. Biol. Cell *7*, 457–469.

Smythe, C., and Newport, J.W. (1991). Systems for the study of nuclear assembly, DNA replication, and nuclear breakdown in Xenopus laevis egg extracts. Methods Cell Biol. *35*, 449–468.

Solomon, M.J., Glotzer, M., Lee, T.H., Philippe, M., and Kirschner, M.W. (1990). Cyclin activation of $p34^{cdc2}$. Cell *63*, 1013–1024.

Su, J.Y., Rempel, R.E., Erikson, E., and Maller, J.L. (1995). Cloning and characterization of the Xenopus cyclin-dependent kinase inhibitor p27XIC1. Proc. Natl. Acad. Sci. USA *92*, 10187–10191.

Tang, Z., Coleman, T.R., and Dunphy, W.G. (1993). Two distinct mechanisms for negative regulation of the Wee1 protein kinase. EMBO J. *12*, 3427–3436.

Thomas, R. (1981). Quantum Noise. In Springer Series in Synergetics, C.W. Gardiner, ed. (Berlin: Springer), pp. 180–193.

Thron, C.D. (1996). A model for a bistable biochemical trigger of mitosis. Biophys. Chem. *57*, 239–251.

Tyson, J.J., Novak, B., Odell, G.M., Chen, K., and Thron, C.D. (1996). Chemical kinetic theory: understanding cell-cycle regulation. Trends Biochem. Sci. *21*, 89–96.

Ubersax, J.A., Woodbury, E.L., Quang, P.N., Paraz, M., Blethrow, J.D., Shah, K., Shokat, K.M., and Morgan, D.O. (2003). Targets of the cyclin-dependent kinase Cdk1. Nature *425*, 859–864.

Verma, R., Annan, R.S., Huddleston, M.J., Carr, S.A., Reynard, G., and Deshaies, R.J. (1997). Phosphorylation of Sic1p by G1 Cdk required for its degradation and entry into S phase. Science *278*, 455–460.

Walter, C.F. (1970). The occurrence and the significance of limit cycle behavior in controlled biochemical systems. J. Theor. Biol. *27*, 259–272.

Watanabe, N., Broome, M., and Hunter, T. (1995). Regulation of the human WEE1Hu CDK tyrosine 15-kinase during the cell cycle. EMBO J. *14*, 1878–1891.

Wells, N.J., Watanabe, N., Tokusumi, T., Jiang, W., Verdecia, M.A., and Hunter, T. (1999). The C-terminal domain of the Cdc2 inhibitory kinase Myt1 interacts with Cdc2 complexes and is required for inhibition of G(2)/M progression. J. Cell Sci. *112*, 3361–3371.