A mechanism for how Cdr1/Nim1 kinase promotes mitotic entry by inhibiting Wee1

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Abstract

To enter into mitosis, cells must shut off the cell cycle inhibitor Wee1. SAD family protein kinases regulate Wee1 signaling in yeast and humans. In *S. pombe*, two SAD kinases (Cdr1/Nim1 and Cdr2) act as upstream inhibitors of Wee1. Previous studies found that *S. pombe* Cdr1/Nim1 directly phosphorylates and inhibits Wee1 *in vitro*, but different results were obtained for budding yeast and human SAD kinases. Without a full understanding of Cdr1 action on Wee1, it has been difficult to assess the *in vivo* relevance and conservation of this mechanism. Here, we show that both Cdr1 and Cdr2 promote Wee1 phosphorylation in cells, but only Cdr1 inhibits Wee1 kinase activity. Inhibition occurs when Cdr1 phosphorylates a cluster of serine residues linking α-helices G and H of the Wee1 kinase domain. This region is highly divergent among different Wee1 proteins, consistent with distinct regulatory mechanisms. A *wee(4A)* mutant that cannot be phosphorylated by Cdr1 delays mitotic entry and causes elongated cells. By disrupting and re-targeting Cdr1 localization, we show that Cdr1 inhibition of Wee1 occurs in cells at cortical nodes formed by Cdr2. Based on our results, we propose a two-step model for inhibition of Wee1 by Cdr1 and Cdr2 at nodes.

Introduction

Eukaryotic cells enter into mitosis due to regulated activation of the ubiquitous cyclin-dependent kinase Cdk1. During interphase, Cdk1 is kept inactive by the protein kinase Wee1,

which phosphorylates a conserved tyrosine (Cdk1-Y15) to inhibit Cdk1 activity (Nurse, 1975; Gould and Nurse, 1989; Featherstone and Russell, 1991; Lundgren *et al.*, 1991; Parker *et al.*, 1992; Coleman *et al.*, 1993). As cells enter into mitosis, this inhibitory phosphorylation is removed by the phosphatase Cdc25 to activate Cdk1 (Russell and Nurse, 1986; Dunphy and Kumagai, 1991; Gautier *et al.*, 1991; Kumagai and Dunphy, 1991; Strausfeld *et al.*, 1991). To enter into mitosis, cells require mechanisms to inhibit Wee1, thereby relieving the "brake" on Cdk1. However, many of the upstream mechanisms that regulate Wee1 are not well defined. The fission yeast *S. pombe* has served as a long-standing model system for studying this conserved module that controls cell division. These rod-shaped cells enter into mitosis and divide at a reproducible size due to the activities of Wee1, Cdc25, and other Cdk1 regulators. Decades of work have identified key factors that act upstream of Cdk1, but it has remained a challenge to place these factors into defined pathways and to understand their biochemical mechanisms.

Genetic screens in fission yeast defined two SAD-family protein kinases, Cdr1/Nim1 and Cdr2, as upstream inhibitors of Wee1 (Russell and Nurse, 1987; Young and Fantes, 1987; Breeding *et al.*, 1998; Kanoh and Russell, 1998). Both *cdr1* and *cdr2* mutants divide at a larger size than wild type cells due to uninhibited Wee1 (Russell and Nurse, 1987; Young and Fantes, 1987; Breeding *et al.*, 1998; Kanoh and Russell, 1998). Further, the cell size defects of *cdr1* and *cdr2* mutants are non-additive (Feilotter *et al.*, 1991; Martin and Berthelot-Grosjean, 2009; Moseley *et al.*, 2009), suggesting redundant or related inhibitory mechanisms. Wee1 becomes increasingly phosphorylated as cells grow during G2 (Lucena *et al.*, 2017), and this phosphorylation is reduced in *cdr1*Δ and *cdr2*Δ mutants (Allard *et al.*, 2018), consistent with increasing inhibition by Cdr1-Cdr2. Cdr2 appears to act primarily through localization of Wee1. Cdr2 forms cortical node structures in the cell middle, and recruits both Cdr1 and Wee1 to these sites (Morrell *et al.*, 2004; Martin and Berthelot-Grosjean, 2009; Moseley *et al.*, 2009; Allard *et al.*, 2018). Cdr1/Nim1 appears to be the key protein that directly regulates Wee1 activity. *In vitro*, Cdr1 has been shown to phosphorylate and inhibit Wee1 kinase activity (Coleman *et al.*, 1993; Parker *et al.*, 1993; Wu and Russell, 1993). However, key questions have remained open regarding this mechanism. The *in vivo* relevance of Cdr1 phosphorylating Wee1 has not been tested because direct phosphorylation sites have been unknown. In addition, different results have been obtained for regulation of Wee1 by SAD family kinases in budding yeast and humans (Kellogg, 2003; Lu *et al.*, 2004; Sakchaisri *et al.*, 2004; Keaton and Lew, 2006). In this study, we identify the Wee1 sites that are phosphorylated by Cdr1, and show that this phosphorylation is required for Wee1 regulation in cells. By disrupting and then re-targeting Cdr1 localization, we propose that Cdr1 phosphorylates and inhibits Wee1 at cortical nodes formed by Cdr2. Our combined results lead to a mechanistic model for Wee1 regulation by SAD kinases in fission yeast.

Results and Discussion

Cdr1 and Cdr2 act as upstream inhibitors of Wee1 (Figure 1A). To test their mechanisms of action on Wee1, we overexpressed either Cdr1, Cdr2, or the empty vector in *cdr1*Δ*cdr2*Δ cells, and then monitored the phosphorylation and activity of Wee1 (Figure 1B). Wee1 phosphorylation was monitored by SDS-PAGE band shift (Lucena *et al.*, 2017; Allard *et al.*, 2018), and Wee1 activity was assessed by monitoring Cdk1-pY15 levels. Cdr1 overexpression induced hyperphosphorylation of Wee1 and concomitant loss of Cdk1-pY15, indicating inhibition of Wee1 kinase activity, consistent with previous work (Coleman *et al.*, 1993; Parker

et al., 1993; Wu and Russell, 1993). In contrast, Cdr2 overexpression induced hyperphosphorylation of Wee1 but no change in Cdk1-pY15. Thus, both Cdr1 and Cdr2 induce phosphorylation of Wee1, but the regulatory output of these two kinases is distinct. Consistent with these biochemical results, only over-expression of Cdr1 but not of Cdr2 resulted in reduced cell size in *cdr1*Δ*cdr2*Δ cells (Figure 1C). We also found that phosphorylation of endogenous Wee1 in fission yeast cells was reduced in the catalytically inactive mutant *cdr1(K41A)* (Figure 1D), consistent with previous results using *cdr1*Δ cells (Allard *et al.*, 2018). Given the role of Cdr1 in regulating Wee1 kinase activity, along with open questions regarding its underlying mechanism, we investigated this pathway further.

Previous studies found that Cdr1 can directly phosphorylate and inhibit Wee1 kinase activity *in vitro* (Coleman *et al.*, 1993; Parker *et al.*, 1993; Wu and Russell, 1993). However, in budding yeast, the Cdr1-like kinase Hsl1 does not phosphorylate or inhibit Wee1-like kinase Swe1 (Kellogg, 2003; Sakchaisri *et al.*, 2004; Keaton and Lew, 2006). These conflicting results indicate that a more detailed analysis of the mechanism for Cdr1-Wee1 regulation is needed. Consistent with previous work (Coleman *et al.*, 1993; Parker *et al.*, 1993; Wu and Russell, 1993), co-expression of Wee1 and active Cdr1 in Sf9 insect cells caused a shift in the SDS-PAGE migration of Wee1 (Figure 1E). This shift was due to phosphorylation and was not induced by co-expression with the catalytically inactive mutant *cdr1(K41A)* (Figures 1E-G). Further, the shift was not due to Wee1 auto-phosphorylation because we observed a similar result using the inactive mutant *wee1(K596L)* (Figure 1F). As a more direct test, we performed *in vitro* kinase assays with purified proteins (Figure S1A-E). Using recombinant Cdr1 expressed and purified from bacteria, we found that Cdr1 directly phosphorylated Wee1, but Cdr1(K41A) did not (Figure 1H). We performed two-step *in vitro* kinase assays to test the effects of this phosphorylation on Wee1 activity. Wee1 that was phosphorylated by Cdr1 did not phosphorylate its substrate Cdk1-Y15, whereas Wee1 retained activity after incubation with Cdr1(K41A) (Figure 1I). Taken together, our results show that Cdr1 phosphorylates Wee1 in fission yeast cells, insect cells, and *in vitro*. Our findings confirm and extend past work showing that Cdr1 directly phosphorylates Wee1, and this modification inhibits Wee1 kinase activity (Coleman *et al.*, 1993; Parker *et al.*, 1993; Wu and Russell, 1993).

Next, we sought to identify the sites on Wee1 that are targeted by Cdr1 for inhibitory phosphorylation. We used LC-MS/MS to map Wee1 phosphorylation sites from three independent experiments. First, we purified Wee1 from insect cells following its expression either alone or in combination with Cdr1. We identified sites that were phosphorylated specifically upon co-expression with Cdr1. Second, we performed a similar experiment with the inactive mutant Wee1(K596L) to ensure that identified sites were not due to auto-phosphorylation. Third, we performed *in vitro* kinase assays by mixing purified Wee1 or inactive Wee1(K596L) with either active Cdr1 or inactive Cdr1(K41A), and then mapped Wee1 phosphorylation sites specifically induced by active Cdr1. A number of phosphorylation sites were identified in multiple experiments throughout the Wee1 sequence (Figure S2B). We focused primarily on sites within the kinase domain because Cdr1 regulates Wee1 kinase activity and phosphorylates the kinase domain alone (Figure 2A, S2A).

To pinpoint which of these phosphorylation sites mediate inhibition of Wee1 by Cdr1 in cells, we generated a panel of mutants in which different phosphorylated residues were changed to alanine, thereby preventing phosphorylation (Figure S2C). We reasoned that a non-phosphorylatable Wee1 mutant would be hyperactive, leading to an elongated cell length at division similar to *cdr1*Δ cells. These mutant constructs were integrated into the genome and

were expressed by the *wee1* promoter as the sole copy in these cells. By analyzing combinations of point mutations, we determined that some mutations (*e.g.* S21A and S822A) had no effect on cell size, while others (*e.g.* S781A) caused a *wee* phenotype indicative of loss-of-function. Importantly, we generated one mutant that mimicked the *cdr1*Δ phenotype. We named this mutant *wee1(4A)* because it prevents phosphorylation at 4 sites: S771, S787, S794, and S798.

The phosphorylation sites mutated in *wee1(4A)* are clustered within the C-lobe of the kinase domain and have interesting regulatory potential. Using sequence alignments and structural modeling, this cluster falls mostly within a loop that connects α-helices G and H of the Wee1 kinase domain (Figure 2B-C) (Squire *et al.*, 2005). This loop is extended in *S. pombe* Wee1 as compared to human Wee1, so the sites are not obviously present in the human polypeptide sequence. Interestingly, this loop is dramatically extended and asparagine-rich in the *S. cerevisiae* Swe1 sequence, and therefore the conserved sites may not be subject to a related regulatory mechanism (Figure 2C). Several eukaryotic protein kinases have extended loops connecting α-G and α-H within the GHI subdomain (Hanks and Hunter, 1995; Scheeff and Bourne, 2005). This subdomain acts as a substrate docking site and connects to the activation segment to regulate catalytic activity (Deminoff *et al.*, 2009; Taylor *et al.*, 2012). Thus, post-translational modifications in this region have the potential to regulate kinase activity.

We performed a series of *in vivo* experiments to test key predictions for the *wee1(4A)* mutant. First, if Cdr1 functions by phosphorylating these residues, then cellular phosphorylation of Wee1 should be reduced in the *wee1(4A)* mutant. Consistent with this model, wee1(4A) phosphorylation was reduced in cells when compared to wild type Wee1, and its phosphorylation was not altered by *cdr1*Δ or *cdr2*Δ mutations (Figure 3A). Second, if both *cdr1*Δ and *wee1(4A)* mutants are elongated at division due to the same Cdr1-Wee1 pathway, then combining these two mutations should not generate additive or synthetic defects. Indeed, *cdr1*Δ *wee1(4A)* cells divided at the same size as *cdr1*Δ cells (Figure 3B), showing that *wee1(4A)* and *cdr1*Δ mutant phenotypes were non-additive. Third, *wee1(4A)* mutations should be additive or synthetic in combination with mutations in *cdc25*, which operates through a different signaling pathway. Past work has shown that *cdr1* mutations are synthetically lethal with *cdc25* mutant alleles (Young and Fantes, 1987). We found that both *wee1(4A)* and *cdr1*Δ were synthetically lethal with *cdc25-dD* (Figure 3C). These combined experiments show that *wee1(4A)* prevents signal transduction through the Cdr2-Cdr1-Wee1 pathway.

Next, we addressed several potential mechanisms that could explain the phenotype of *wee1(4A)* cells. Increased cell size at division for *wee1(4A)* could be caused by changes to Wee1 expression, localization, and/or regulation of kinase activity by Cdr1. We confirmed that wee1(4A) protein level does not increase and still localizes to cortical nodes (Figure 3A,D), which are the sites where Wee1 colocalizes with Cdr1 and Cdr2 (Martin and Berthelot-Grosjean, 2009; Moseley *et al.*, 2009; Allard *et al.*, 2018). However, Cdr1 was unable to induce hyperphosphorylation of the wee1(4A) kinase domain in insect cells (Figure 3E). Using two-step *in vitro* kinase assays, we found that Cdr1 readily inhibits Wee1 but not wee1(4A) (Figure 3F). Unlike wild type Wee1, the wee1(4A) mutant can still phosphorylate Cdk1-pY15 after treatment with Cdr1. These results indicate that the primary defect of the wee1(4A) mutant is loss of inhibition by Cdr1, although we note that the 4A mutation does not completely abolish Wee1 phosphorylation and regulation by Cdr1 *in vitro*. Taken together, our results show that phosphorylation of these four residues by Cdr1 inhibits Wee1 activity both *in vitro* and in cells.

Wee1 has also been shown to be phosphorylated and inhibited by Cdk1 in budding yeast, humans, and other systems (Tang *et al.*, 1993; Mueller *et al.*, 1995; Harvey *et al.*, 2005). This

feedback sharpens the ultrasensitive nature of Cdk1 activation for mitotic entry (Pomerening *et al.*, 2003; Sha *et al.*, 2003; Kim and Ferrell, 2007), but whether it also occurs in fission yeast has not been tested. We considered the possibility that Cdr1 might "prime" or otherwise regulate Wee1 phosphorylation by Cdk1. To test this idea, we used the analog-sensitive cdk1-asM17 allele (Aoi *et al.*, 2014), which can use bio-orthoganol 6-Bn-ATPγS to thiophosphorylate direct substrates (Allen *et al.*, 2007; Hertz *et al.*, 2010). First, we confirmed that *S. pombe* cdk1-asM17 directly thio-phosphorylates Wee1 and Wee1(K596L) (Figure S3A). Thus, Cdk1 phosphorylates Wee1 in fission yeast, similar to other systems. Next, we found that Wee1 phosphorylation by Cdr1 did not affect the level of thio-phosphorylation by cdk1-asM17 (Figure S3B). We conclude that Cdk1 feedback on Wee1 is mechanistically independent of Cdr1.

Finally, we examined the spatial control of this inhibitory mechanism in cells. Cdr1 and Wee1 both localize to cortical nodes in the cell middle (Martin and Berthelot-Grosjean, 2009; Moseley *et al.*, 2009; Allard *et al.*, 2018). These nodes are formed by Cdr2 oligomers that are required for Cdr1 and Wee1 recruitment (Martin and Berthelot-Grosjean, 2009; Moseley *et al.*, 2009; Allard *et al.*, 2018). We previously identified a mutant *cdr1(Δ460-482)* that fails to localize to nodes and results in elongated cell size at division like *cdr1*Δ (Figure 4A) (Opalko and Moseley, 2017). We tested the effects of artificially recruiting mEGFP-cdr1(Δ460-482) back to nodes using cdr2-GBP-mCherry, which contains the GFP-binding peptide (GBP). In this system, mEGFP-cdr1(Δ460-482) colocalized with cdr2-GBP-mCherry at nodes (Figure 4B). More importantly, recruitment back to nodes had strong effects on both cell size and Wee1 phosphorylation (Figure 4C-D). In the *cdr1(Δ460-482)* mutant, Wee1 was not hyperphosphorylated and cells divided at an increased size, similar to *cdr1*Δ. However, recruitment of mEGFP-cdr1(Δ460-482) back to nodes by cdr2-GBP-mCherry caused Wee1 to be even more hyperphosphorylated than in wild type cells. A similar effect was seen using full-length mEGFP-cdr1. Along with enhanced Wee1 hyperphosphorylation, these cells divided at a smaller size than wild type cells. These results show that Cdr1 localization to nodes is a limiting factor for regulation of Wee1 and cell size at division. Further, they demonstrate that cdr1(Δ460-482) retains full activity but can only phosphorylate Wee1 at nodes, strongly supporting a model where inhibitory phosphorylation of Wee1 occurs at nodes.

Our results, combined with past work, suggest a two-step mechanism for regulation of a Wee1 molecule at nodes (Figure 4E). Wee1 localizes to nodes in transient bursts that last between 5-15 seconds (Allard *et al.*, 2018). This localization requires Wee1's non-catalytic amino terminus, which can be phosphorylated by Cdr2 (Kanoh and Russell, 1998; Allard *et al.*, 2018). In kinase-dead *cdr2* mutants, Wee1 localizes to nodes in extremely short bursts (< 2 seconds) (Allard *et al.*, 2018). As the first step towards inhibition, we propose that Cdr2 phosphorylates the Wee1 amino terminus to slow the off-rate for Wee1 release, thereby "trapping" the modified molecule in the node. In the second step, Cdr1 within the node can then phosphorylate the Wee1 kinase domain to inhibit the catalytic activity of this molecule through the mechanism described in this study. These two steps are consistent with a large number of past results, and show how these two related kinases control distinct aspects of a shared mechanism to inhibit Wee1. An open question going forward is why this reaction needs to occur at nodes. It is possible that nodes simply increase the local concentration of Cdr1 and Wee1. Indeed, both Cdr1 and Wee1 are expressed at low levels, which could preclude an efficient interaction in the cytoplasm. This possibility is supported by the finding that Cdr1 overexpression bypasses the need for Cdr2, leading to Wee1 hyperphosphorylation, inhibition of Wee1, and reduced cell size (Figure 1B-C). An alternative possibility is that the structure of a

node promotes efficient phosphorylation of Wee1, for example by promoting a more active conformation of Cdr1. These and other possibilities are not mutually exclusive, and additional work on this system may reveal general principles for the emerging theme of signal transduction in cortical clusters.

In conclusion, we have answered long-standing questions regarding how Cdr1/Nim1 inhibits Wee1 in fission yeast cells. Cdr1 inhibits the kinase activity of Wee1 by directly phosphorylating a cluster of residues connecting α-helices G and H. The sequence of this region is evolutionarily divergent, likely explaining why Cdr1-like kinases may not act directly on Wee1 in some other species. However, the functional role of this structural region within kinase domains suggests that other kinases containing insertions within the GHI subdomain may be regulated by mechanisms similar to the one we describe here. Phosphorylation of these residues in Wee1 explains the cell size defects of *cdr1*Δ mutants, and likely represent the functional output of the well-studied Pom1-Cdr2-Cdr1-Wee1 pathway (Martin and Berthelot-Grosjean, 2009; Moseley *et al.*, 2009; Pan *et al.*, 2014; Allard *et al.*, 2018, 2019; Gerganova *et al.*, 2019). Efficient signal transduction in this pathway requires organization within oligomeric clustered structures at the cell cortex. Given the critical role of these structures in relaying cell size information to the core cell cycle machinery, additional insight into the underlying mechanisms will have implications for cell size control. Molecular clusters are a feature of many other signal transduction pathways, so the Cdr2/Cdr1/Wee1 network will also serve as a model for broader mechanistic studies of signal transduction.

Materials and Methods

Yeast strains and growth

Standard *S. pombe* media and methods were used (Moreno *et al.*, 1991). Strains and plasmids used in this study are listed in supplementary table S1. Sf9 constructs were cloned via restriction digest into Fastbac vector (Thermo Fisher). Mutations in the Wee1 or Cdr1 gene were made by Gibson assembly (QuantaBio), or by site-directed mutagenesis by Quikchange (Stratagene). Wee1 was expressed in a pJK148 plasmid containing wildtype Wee1 along with 1000bp each of the Wee1 promoter and terminator. To introduce *wee1* mutations into cells, we deleted one copy of *wee1+* from a wild type diploid strain (JM5334) using PCR-based homologous recombination to generate the heterozygous diploid strain JM5337. Mutant *wee1* alleles in pJK148-based plasmids were then transformed into the *leu1* locus. Diploid cells were then sporulated, and the resulting spores were separated via tetrad dissection. Colonies that grew on leu- plates and were G418 resistant were verified by colony PCR and by western blot analysis.

To test for synthetic lethality with *cdc25* mutants, we used a *cdc25-degron-DAmP::hygro* strain. *wee1+* (JM5578), *wee1(4A)* (JM5709), and *cdr1*Δ*::kanMX6* (JM483) were crossed to *cdc25-degron DAMP::hygro* (JM5886/JM5887). A full plate of tetrads was analyzed (9-10 tetrads). Crosses with the *wee1(4A)* yielded a synthetic lethality with *cdc25-degron-DAmP(dD)* both with and without a wildtype copy of Wee1 at the endogenous locus. When *cdc25-dD* was crossed to a *cdr1*Δ with the *wee1*Δ*::kanMX6 leu1(Pwee1-wee1-Twee1)* background, *cdr1*Δ cells were also synthetically lethal with *cdc25-degron DAMP* both with and without a wildtype copy

of Wee1 at the endogenous locus. To look specifically at the genetic interaction between Cdr1 and Cdc25 with one copy of wildtype Wee1 present, a *cdr1Δ::kanMX6* strain in a wildtype background was crossed to *cdc25-dD*. For mEGFP-Cdr1(Δ460-482), Cdr1 was mutated in a pJK148 vector containing 970 bp of Cdr1 promoter, mEGFP-Cdr1 and 300 bp of Cdr1 terminator. Each vector was then integrated into the *leu1* locus in *cdr1Δ::kanMX6 leu1-32*.

For Cdr1 or Cdr2 overexpression, pREP3x plasmids were transformed into *cdr1::kanMX6 cdr2Δ::ura4 ULA-* (JM2070) cells and grown in EMM-leu +thiamine. Cells were then washed vigorously and resuspended in EMM-leu to induce expression of Cdr1 or Cdr2, starting at time point 0.

Sf9 cells Co-expression and Wee1 purification

Sf9 cells were grown in Grace's media supplemented (Gibco) with 10% heat inactivated FBS, 10µg/mL Gentamicin and 0.25 µg/mL Amphotericin B, at 27˚C. Constructs were expressed in Sf9 cells using Bac-to-Bac Baculovirus expression system (Thermo Fisher). Wee1 constructs were co-expressed with Cdr1/Cdr1(K41A) for 3 days. Cells were resuspended in SDS-PAGE sample buffer (65mM Tris pH 6.8, 3% SDS, 10% glycerol, 10% 2-mercaptoethanol, 50mM sodium fluoride, 50mM β–gylcerophosphate, 1mM sodium orthovanadate, complete EDTA-free protease inhibitor tablet (Pierce)), boiled for 5 mins, and the resulting lysates were separated by SDS-PAGE.

For purification of Wee1 from Sf9 cells, 14His-3C-MBP-Wee1 was expressed in Sf9 cells for 3 days. Cells were then harvested and resuspended in lysis buffer (50mM Tris pH 7.5, 150mM NaCl, 75mM sodium fluoride, 75mM β–gylcerophosphate, 1mM PMSF, 10mM imidazole pH 8, complete EDTA-free protease inhibitor tablet (Pierce)). Cells were lysed by French press. Triton X-100 was added to a final concentration of 1%, and glycerol was added to a concentration of 0.5%. Lysates were clarified by centrifuging at 15,000rpm for 15 minutes at 4˚C. Lysates were then added to TALON ® (TaKaRa) resin and incubated for 1 hour at 4˚C. The resin was then washed with wash buffer (50mM Tris pH 7.5, 500mM NaCl, 75mM sodium fluoride, 75mM β–gylcerophosphate, 20mM imidazole pH 8). Wee1 was then eluted using 500mM imidazole pH 8. For purification and mass spec, Wee1 was purified using the MBP tag due to co-expression with 10His-Cdr1. The approach was similar as above, except for the use of an amylose resin (NEB). Wee1 was then resuspended in elution buffer (1% SDS, 15% glycerol, 50mM Tris pH 8.7, 150mM NaCl) and boiled for 5 minutes. The sample was reduced and alkylated prior to separation by SDS-PAGE.

Lambda Phosphatase

For lambda phosphatase treatment, FLAG-Wee1 was co-expressed with Cdr1-MBP-14His for 3 days. Cells were harvested and lysed in lysis buffer (20mM HEPES pH 7.4, 1mM EDTA, 300mM NaCl, 0.2% Triton X-100, complete EDTA-free protease inhibitor tablet (Pierce), 75mM sodium fluoride, 75mM β–gylcerophosphate, 1mM sodium orthovanadate, 1mM PMSF) by repeated freeze thaw cycles. Lysates were centrifuged at 15,000 rpm at 4˚C for 15 minutes. Clarified lysates were incubated with FLAG M2 magnetic beads (Sigma) for 1 hour. Beads were then vigorously washed in lysis buffer lacking phosphatase inhibitors. Samples were split and treated with 800U of lambda phosphatase (NEB) or mock treated at 30˚C for 1 hour.

***In vitro* kinase assay and Western Blots**

GST-Cdr1(1-354) and GST-Cdr1(1-354)(K41A) were expressed in BL21 *E. coli* at 16˚C. For these assays, Cdr1 was always freshly purified on the same day that it would be used. Cells were lysed in lysis buffer (1xPBS, 300mM NaCl, 1mM DTT, 75mM sodium fluoride, 75mM β–gylcerophosphate, 1mM PMSF) by French press. Triton X-100 was added to a final concentration of 1% and glycerol to a concentration of 0.5%. Lysates were then centrifuged at 15,000 rpm for 20 minutes at 4˚C. Clarified lysates were then incubated on gluthathione-agarose (Sigma) for 1 hour at 4˚C. Agarose resin was then washed with wash buffer (1xPBS, 500mM NaCl, 1mM DTT, 75mM sodium fluoride, 75mM β–gylcerophosphate) followed by 50mM Tris pH 7.5, 150mM NaCl. GST-Cdr1 was maintained on glutathione-agarose resin for *in vitro* kinase reactions. Briefly, 0.3µg of purified 14His-3C-MBP-Wee1 was incubated with approximately 5-10µg of GST-Cdr1 in kinase buffer (50mM Tris pH 7.5, 10mM $MgCl_2$ 1mM DTT, 2mM ATP, 3µM okadaic acid, 20mM glutathione pH 8) shaking, for 1 hour. To test Wee1 ability to phosphorylate Cdk1, 0.1µg Wee1 was incubated with Cdr1 for 10 minutes. Then reactions were spun down and soluble Wee1 was then added to Cdk1-Cdc13 complexes for 15 minutes. Cdk1-Cdc13 was immunoprecipitated from the fission yeast strain, *cdc13-FLAG wee1-50 mik1*Δ*::ura4*+ after growth at the non-permissive temperature for 3 hours. Cdc13-FLAG was then immunoprecipitated as described above.

For thiophosphate ester *in vitro* kinase assay, following *in vitro* kinase assay with Cdr1 as described above, ATP-γS (Axxora BLG-B072-05) was added to each reaction at a final concentration of 50 µM. The reaction was allowed to proceed for 15 minutes. The reaction was then quenched with 20mM EDTA and a final concentration of 2.5mM p-nitrobenzyl mesylate (Abcam Biochemicals) was added and incubated for 2 hours at room temperature. Phosphorylation was then probed using thiophosphate ester antibody RabMAb (ab92570) according to manufacturer's protocol.

For western blots, whole cell extracts of *S. pombe* were made by flash freezing 2 O.D. of cells. Cells were then lysed in 100µl sample buffer (65mM Tris pH 6.8, 3% SDS, 10% glycerol, 10% 2-mercaptoethanol, 50mM sodium fluoride, 50mM β–gylcerophosphate, 1mM sodium orthovanadate, complete EDTA-free protease inhibitor tablet (Pierce)) in a Mini-beadbeater-16 for 2 minutes. Blots were probed with anti-FLAG M2 (Sigma), anti-GST (Covance), anti-Cdr1 (Opalko and Moseley, 2017), anti-Wee1 (Allard *et al.*, 2018), anti-cdc2 (Santa Cruz Biotechnologies sc-53217), anti-pY15 (Cell Signaling #9111L), anti-His (Santa Cruz Biotechnologies sc- 8036), and anti-thiophosphate ester (Abcam ab92570).

Mass spectrometry

Gel bands from Sf9 coexpression of Wee1 and Cdr1 and *in vitro* kinase assays were excised and destained overnight at 37˚C using 50mM ammonium bicarbonate/50% acetonitrile. The destained gel bands were digested in 50mM ammonium bicarbonate using either trypsin or GluC/LysC at 37°C for 16 hours or Proteinase K at 37°C for 1 hour. Following digestion, peptides were extracted using 5% formic acid/50% acetonitrile and dried. Peptides were analyzed on a Q-Exactive Plus mass spectrometer (Thermo Fisher Scientific) equipped with an Easy-nLC 1000 (Thermo Fisher Scientific). Raw data were searched using COMET in high-resolution mode (Eng *et al.*, 2013) against *S.pombe* sequence database, with appropriate enzyme specificity, and carbamidomethylcysteine as static modification. Oxidized methionine and phosphorylated serine, threonine, and tyrosine were searched as variable modifications. Quantification of LC-MS/MS spectra was performed using MassChroQ (Valot *et al.*, 2011) with retention time alignment for smart quantification. Peak areas of Wee1 peptides were normalized

to total amount of Wee1 in the sample. Probability of phosphorylation site localization was determined by phosphoRS (Taus *et al.*, 2011).

Microscopy

Microscopy was performed at room temperature with a DeltaVision Imaging System (Applied Precision), equipped with an Olympus IX-71 inverted wide-field microscope, a Photometrics CoolSNAP HQ2 camera, and Insight solid-state illumination unit. All images are single focal plane images with a 5µm scale bar. Blankophor was used to identify septating cells for cell size measurements. ImageJ was used to measure cell length.

Swiss-Model and Sequence Alignment

Swiss-Model was used to thread the kinase domain sequence of *S. pombe* Wee1 into the crystal structure of human Wee1 kinase domain (Guex *et al.*, 2009). PyMOL Molecular Graphics system, Version 2.0 Schrödinger, LLC. was used to visualize the model. For sequence alignments, clustal omega was used to align the Wee1 amino acid sequences from human, and *S. pombe* Wee1 and *S. cerevisiae* Swe1 (Madeira *et al.*, 2019).

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Figure legends

Figure 1: Cdr1 phosphorylates and inhibits Wee1. (A) Schematic of Cdr1, Cdr2, Wee1 pathway. (B) Whole-cell extracts (WCE) were separated by SDS-PAGE and western blotted against endogenous Wee1. Cdk1-pY15 levels were monitored for Wee1 activity, asterisks mark background bands. (C) DIC images of *cdr1*Δ*cdr2*Δ cells with the indicated over-expression plasmids. Scale bar is 5 μm. (D) WCE were separated by SDS-PAGE and blotted against endogenous Wee1. Cdk1 is used as a loading control, asterisk denotes background band. (E) Cdr1 phosphorylates Wee1 in Sf9 cells. Wee1 was co-expressed with Cdr1 or Cdr1(K41A) in Sf9 cells. (F) Co-expression of Wee1(K596L) with Cdr1/Cdr1(K41A) in Sf9 cells. (G) Cdr1-dependent band shift is due to phosphorylation of Wee1. Wee1 was expressed alone or co-expressed with Cdr1, immunoprecipated, and treated with λ-phosphatase. (H) Cdr1 phosphorylates Wee1 directly *in vitro.* GST-Cdr1(1-354) was expressed and purified from bacteria, and mixed with ATP and purified 14His-MBP-Wee1. (I) Cdr1-dependent phosphorylation of Wee1 inhibits Wee1 kinase activity. Wee1 was phosphorylated by Cdr1 as in (H), and then incubated with Cdk1-Cdc13 immunoprecipitated from *S. pombe*. Cdk1-pY15 was used to monitor Wee1 kinase activity.

Figure 2: Identification of Cdr1-dependent phosphorylation sites on Wee1. (A) Cdr1 phosphorylates the Wee1 kinase domain. 10His-Cdr1 or 10His-Cdr1(K41A) was co-expressed in Sf9 cells with either FLAG-Wee1(545-877) or FLAG-Wee1(545-877)(K596L). (B) Model of *S. pombe* kinase domain threaded into human Wee1 from Swiss-Model. Green region indicates the N-terminal lobe; blue highlights the C-terminal lobe. Phosphorylated residues in the extended loop are marked in red. (C) Sequence alignment of Wee1 from human, *S. pombe*, and *S. cerevisiae.* Red serines are phosphorylated by Cdr1. Black amino acids are conserved.

Figure 3: Phenotypes of phosphorylation-resistant *wee1(4A)* mutant. (A) WCE samples were separated by SDS-PAGE, and blotted to detect Wee1. Cdk1 is used as a loading control, asterisk marks background band. (B) Cell size measurements of the indicated strains. Values are mean ± SD for n>50 each strain. (C) *wee1(4A)* is synthetically lethal with *cdc25-degron DAMP,* similar to a *cdr1*Δ. Tetrad analysis for the indicated crosses, black boxes indicate double mutants. (D) Wee1 localization to nucleus and cortical nodes is not affected in the Wee1(4A) mutant. Middle focal plane images. Scale bar is 5 μm. Boxes are enlarged images of nodes denoted on the image by a yellow arrow. (E) *4A* mutation prevents Cdr1-dependent phosphorylation of the Wee1 kinase domain. Co-expression of wildtype or Wee1(4A) with Cdr1/Cdr1(K41A). (F) Wee1(4A) has impaired Cdr1 inhibition. Wee1 kinase activity was tested as in Fig 1I.

Figure 4: Wee1 inhibition occurs at nodes. (A) Middle focal plane images of mEGFP-tagged Cdr1 or Cdr1(Δ460-482), co-expressed with Cdr2-mRuby as a node marker. Scale bar, 5 µm. (B) Colocalization of mEGFP-cdr1(460-482Δ) Cdr2-GBP-mCherry. Middle focal plane images. Scale bar is 5 µm. (C) Tethering Cdr1(460-482Δ) to nodes rescues size defect. Cell size measurements of indicated strains. Line marks mean; error bars mark SD, n>50 for each strain. (D) Cdr1 localization dictates Wee1 phosphorylation state. WCE of indicated strains were separated by SDS-PAGE. Note upper, hyperphosphorylated band for Wee1 upon node targeting of Cdr1. Cdk1 was used as a loading control, asterisk marks background band. (E) Model for two-step inhibition of a Wee1 molecule at a node.

Supplemental Figure S1: Cdr1 phosphorylates and inhibits Wee1. (A) Western blot for the indicated Cdr1 constructs in Figure 1E. (B) Western blot for the indicated Cdr1 constructs in Figure 1F. (C) Input of Wee1 and Cdr1 from Sf9 cells prior to immunoprecipitation used for Figure 1G. (D) Coomassie-stained SDS-PAGE gel of purified Wee1 constructs used for *in vitro* kinase assays. (E) Coomassie-stained SDS-PAGE gel of purified GST-Cdr1(1-354) and GST-Cdr1(1-354)(K41A) used for *in vitro* kinase assays.

Supplemental Figure S2: Identification of Cdr1-dependent phosphorylation sites on Wee1. (A) Western blot for Cdr1 expression in the samples analyzed in Figure 2A. (B) Schematic of Wee1 with phosphorylated residues. Black bars indicate residues that were phosphorylated in all 3 co-expression data sets. Red bars are sites that were present in the co-expression data sets as well as the *in vitro* kinase assay data set. (C) Cell size measurements of serine to alanine mutations. n>25.

Supplemental Figure S3: Cdk1 phosphorylation of Wee1 is independent of Cdr1. (A) Cdk1 phosphorylates Wee1 *in vitro*. Left panel: Wildtype Cdk1 or Cdk1-asM17 was immunoprecipitated from fission yeast cells, and incubated with purified 14His-MBP-Wee1. These in vitro kinase assays used bio-orthoganol 6-Bn-ATPγS, which can only be utilized by Cdk1-asM17 to thiophosphorylate direct substrates. Note that wildtype Cdk1 cannot utilize ATP-γS. Right panel: Cdk1-asM17 does not phosphorylate 14His-MBP. (*) denotes a background band. (B) Cdk1-asM17 thio-phosphorylates both Wee1 and Wee1(K596L), and this activity is not affected by prior phosphorylation of Wee1 constructs by Cdr1. regardless of Wee1 kinase activity.

Supplemental Table S1. Strains and plasmids used in this study.

Supplemental Table S2. Mass spectrometry data generated in this study.

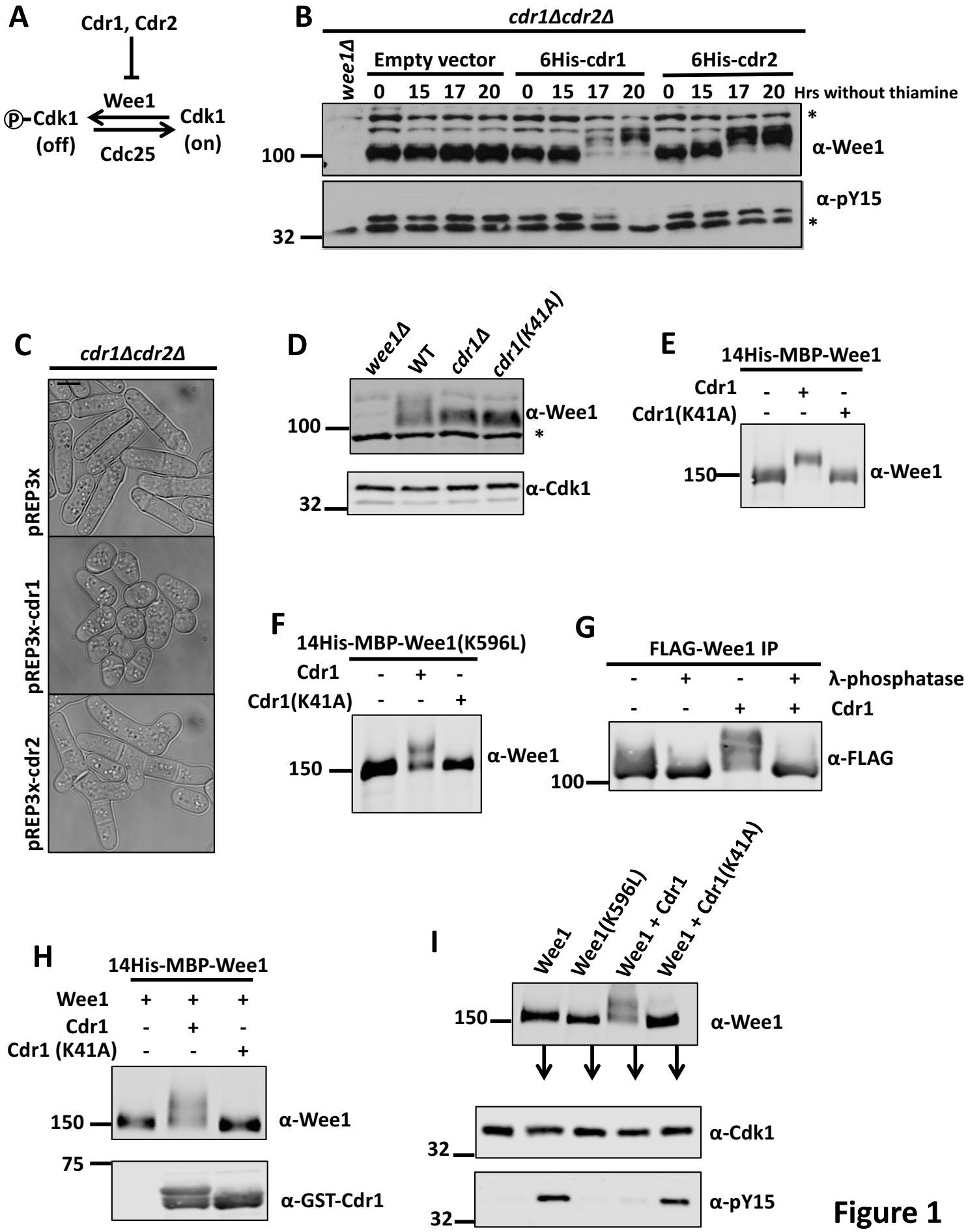
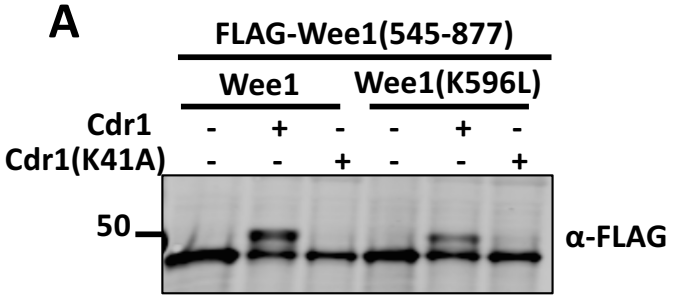
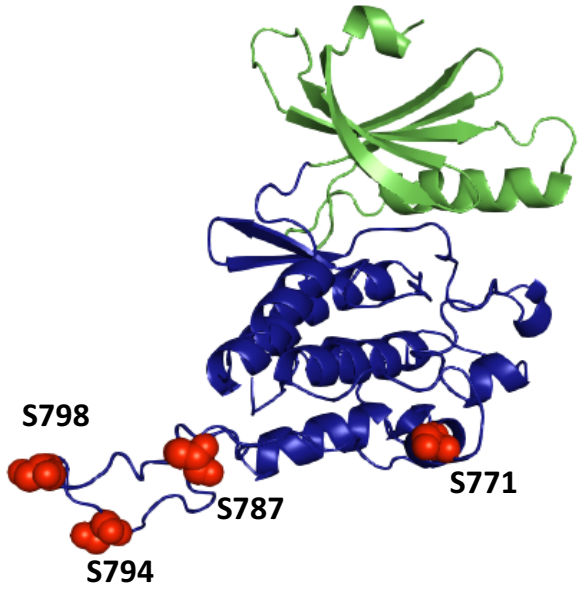

A
Cdr1, Cdr2
Wee1
Ⓟ-Cdk1 (off)
Cdk1 (on)
Cdc25
B
cdr1Δcdr2Δ
wee1Δ
Empty vector
6His-cdr1
6His-cdr2
0 15 17 20 0 15 17 20 0 15 17 20 Hrs without thiamine
100
α-Wee1
α-pY15
32
C
cdr1Δcdr2Δ
pREP3x
pREP3x-cdr1
pREP3x-cdr2
D
wee1Δ
WT
cdr1Δ
cdr1(K41A)
100
α-Wee1
32
α-Cdk1
E
14His-MBP-Wee1
Cdr1 - + -
Cdr1(K41A) - - +
150
α-Wee1
F
14His-MBP-Wee1(K596L)
Cdr1 - + -
Cdr1(K41A) - - +
150
α-Wee1
G
FLAG-Wee1 IP
- + - + λ-phosphatase
- - + + Cdr1
100
α-FLAG
H
14His-MBP-Wee1
Wee1 + + +
Cdr1 - + -
Cdr1 (K41A) - - +
150
α-Wee1
75
α-GST-Cdr1
I
Wee1
Wee1(K596L)
Wee1 + Cdr1
Wee1 + Cdr1(K41A)
150
α-Wee1
32
α-Cdk1
32
α-pY15

Figure 1

A



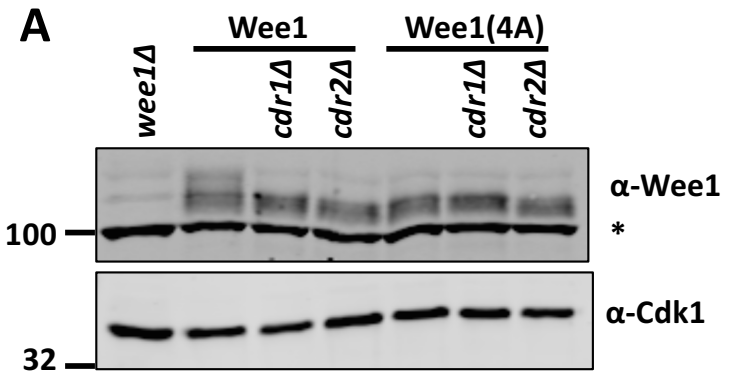
B



C

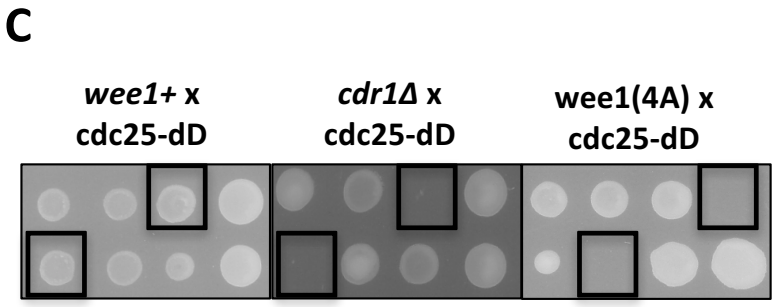
```
                                   α-G
Wee1_Human:          515  PLPRNGDQWHEIRQGRLPRIPQV----------------------
Wee1_S. pombe:       764  VLPDNGQSWQKLRSGDLSDAPRLSSTDNGSSLTSSS---------
Swe1_ S. cerevisiae: 651  VLPDNGNAWHKLRSGDLSDAGRLSSTDIHSESLFSDITKVDTNDL

Wee1_Human:               ---------------------------------------------
Wee1_S. pombe:            ---------------------------------------------
Swe1_ S. cerevisiae:      FDFERDNISGNSNNAGTSTVHNNSNINNPNMNNGNDNNNVNTAAT

                                                        α-H
Wee1_Human:          532  ----------------------LSQEFTELLKVMIHP
Wee1_S. pombe:       800  ------------RETPANSIIGQGGLDRVVEWMLSP
Swe1_ S. cerevisiae: 788  KNRLILHKSSKIPAWVPKFLIDGESLERIVRWMIEP
```

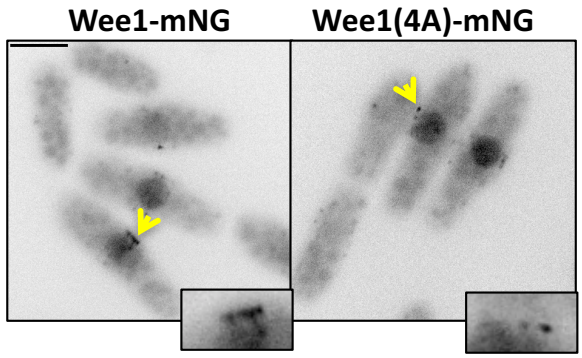
Figure 2

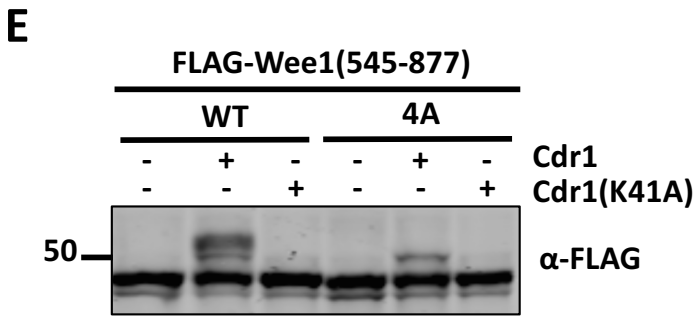


B

Strain	Cell Size
Wildtype	13.3 ± 0.9
wee1(4A)	17.3 ± 1.7
cdr1Δ	16.7 ± 1.1
wee1(4A) cdr1Δ	16.5 ± 1.0







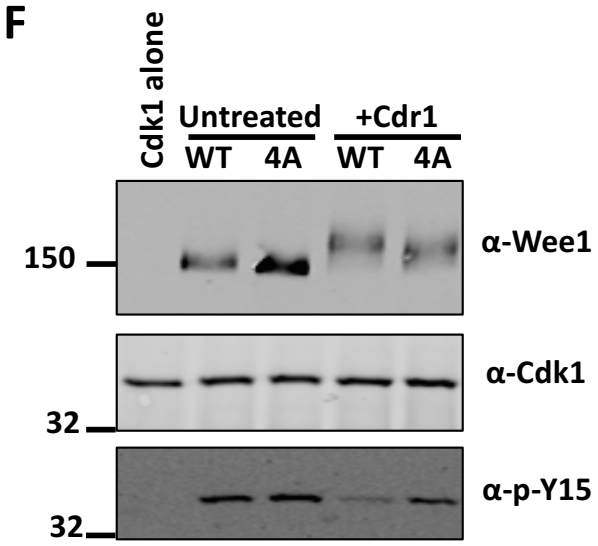


Figure 3

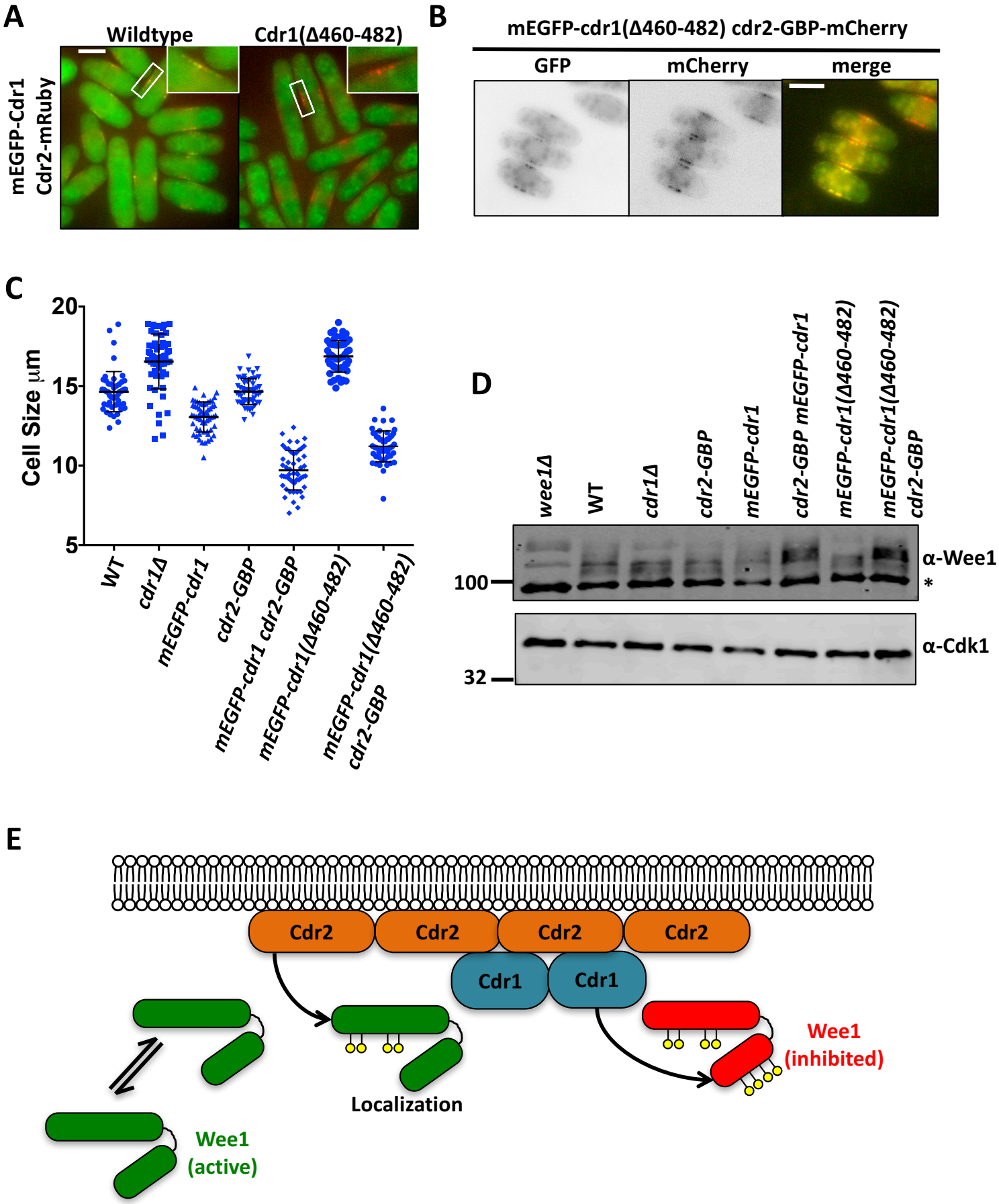

A
Wildtype
Cdr1(Δ460-482)
mEGFP-Cdr1
Cdr2-mRuby
B
mEGFP-cdr1(Δ460-482) cdr2-GBP-mCherry
GFP
mCherry
merge
C
Cell Size µm
20
15
10
5
WT
cdr1Δ
mEGFP-cdr1
cdr2-GBP
mEGFP-cdr1 cdr2-GBP
mEGFP-cdr1(Δ460-482)
mEGFP-cdr1(Δ460-482) cdr2-GBP
D
wee1Δ
WT
cdr1Δ
cdr2-GBP
mEGFP-cdr1
cdr2-GBP mEGFP-cdr1
mEGFP-cdr1(Δ460-482)
mEGFP-cdr1(Δ460-482) cdr2-GBP
100
32
α-Wee1
*
α-Cdk1
E
Cdr2
Cdr2
Cdr2
Cdr2
Cdr1
Cdr1
Localization
Wee1
(inhibited)
Wee1
(active)

Figure 4

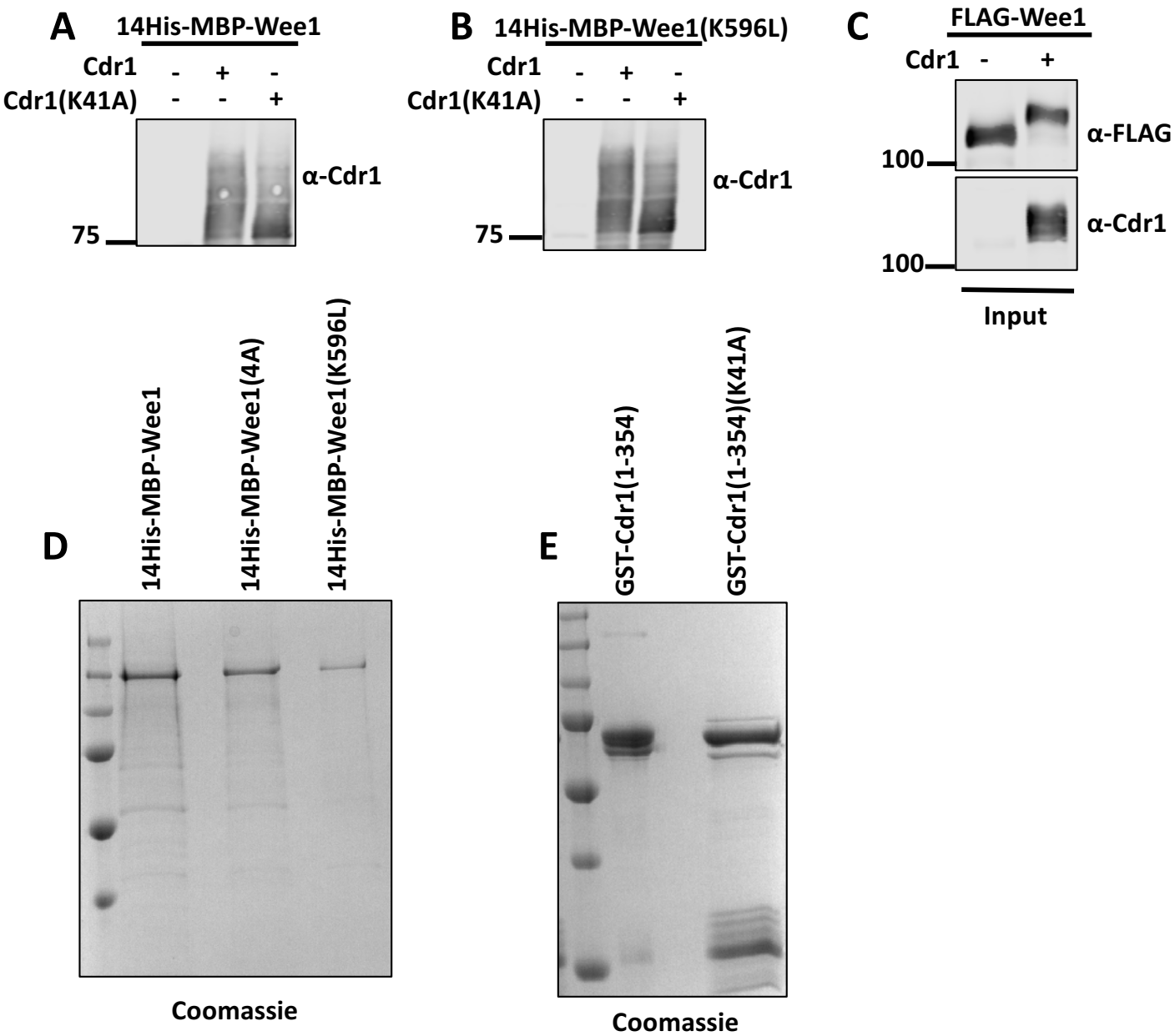

A
14His-MBP-Wee1
Cdr1 - + -
Cdr1(K41A) - - +
α-Cdr1
75
B
14His-MBP-Wee1(K596L)
Cdr1 - + -
Cdr1(K41A) - - +
α-Cdr1
75
C
FLAG-Wee1
Cdr1 - +
α-FLAG
100
α-Cdr1
100
Input
D
14His-MBP-Wee1
14His-MBP-Wee1(4A)
14His-MBP-Wee1(K596L)
Coomassie
E
GST-Cdr1(1-354)
GST-Cdr1(1-354)(K41A)
Coomassie

Figure S1

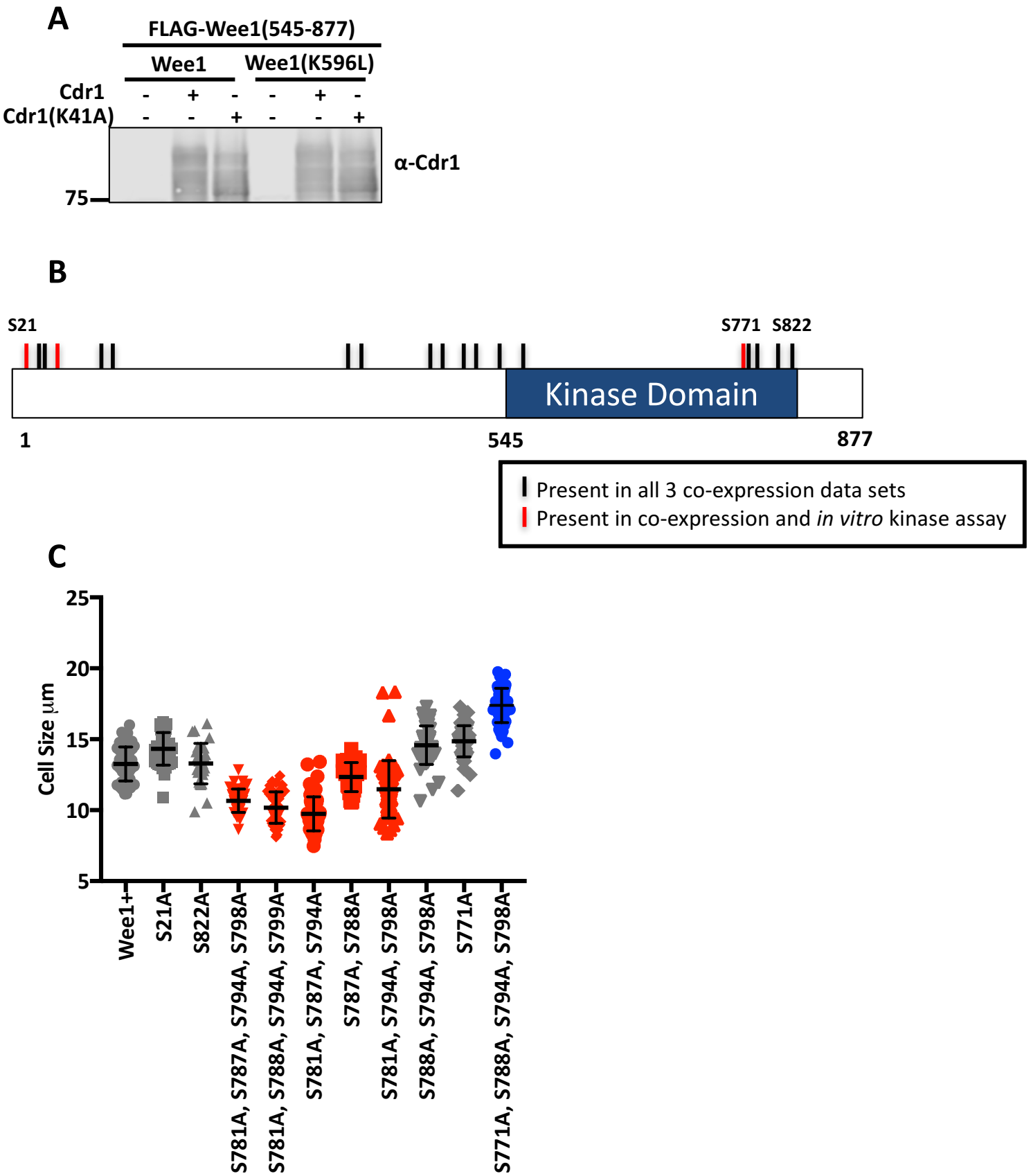

A
FLAG-Wee1(545-877)
Wee1
Wee1(K596L)
Cdr1
Cdr1(K41A)
α-Cdr1
75
B
S21
S771
S822
Kinase Domain
1
545
877
Present in all 3 co-expression data sets
Present in co-expression and in vitro kinase assay
C
Cell Size µm
25
20
15
10
5
Wee1+
S21A
S822A
S781A, S787A, S794A, S798A
S781A, S788A, S794A, S799A
S781A, S787A, S794A
S787A, S788A
S781A, S794A, S798A
S788A, S794A, S798A
S771A
S771A, S788A, S794A, S798A

Figure S2

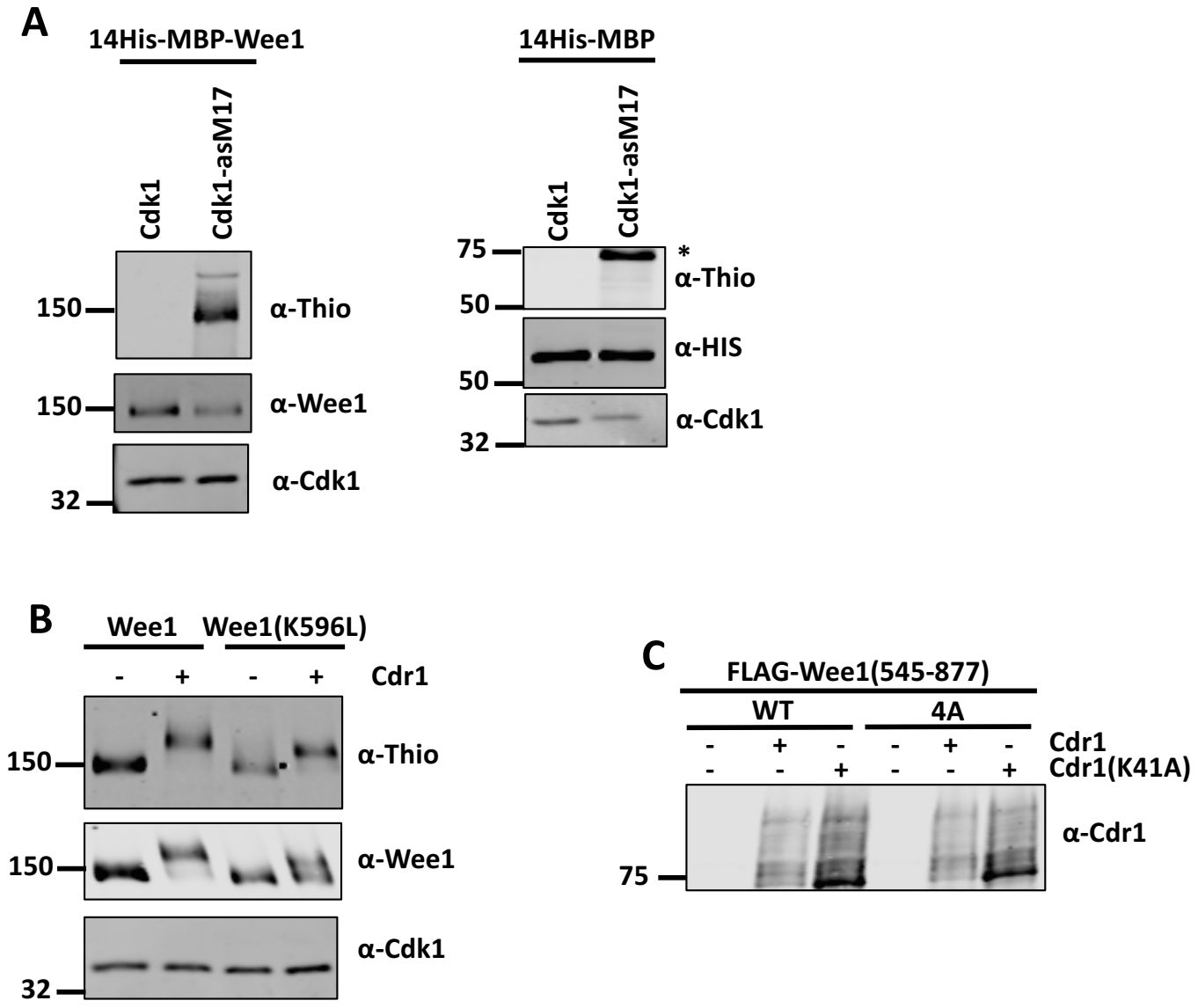

A
14His-MBP-Wee1
Cdk1
Cdk1-asM17
150
α-Thio
150
α-Wee1
32
α-Cdk1
14His-MBP
Cdk1
Cdk1-asM17
75
*
α-Thio
50
α-HIS
50
α-Cdk1
32
B
Wee1
Wee1(K596L)
- + - +
Cdr1
150
α-Thio
150
α-Wee1
α-Cdk1
32
C
FLAG-Wee1(545-877)
WT
4A
- + - - + -
Cdr1
- - + - - +
Cdr1(K41A)
α-Cdr1
75

Figure S3