Negative regulation of APC/C activation by MAPK-mediated attenuation of Cdc20^{Slp1} under stress

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One-sentence summary: Inhibitory effect on the activation of anaphase promoting complex/cyclosome (APC/C) by MAPK Pmk1

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Abstract

Mitotic anaphase onset is a key cellular process tightly regulated by multiple kinases. The involvement of mitogen-activated protein kinases (MAPKs) in this process has been established in *Xenopus* egg extracts. However, the detailed regulatory cascade remains elusive, and also it is unknown whether the MAPKs-dependent mitotic regulation is evolutionarily conserved in the single cell eukaryotic organism such as fission yeast (*Schizosaccharomyces pombe*). Here we show that two MAPKs in *S. pombe* indeed act in concert to restrain anaphase-promoting complex/cyclosome (APC/C) activity upon activation of the spindle assembly checkpoint (SAC). One MAPK, Pmk1, binds and phosphorylates $Slp1^{Cdc20}$, the co-activator of APC/C. Phosphorylation of $Slp1^{Cdc20}$ by Pmk1, but not by Cdk1, promotes its subsequent ubiquitylation and degradation. Intriguingly, Pmk1-mediated phosphorylation event is also required to sustain SAC under environmental stress. Thus, our study establishes a new underlying molecular mechanism of negative regulation of APC/C by MAPK upon stress stimuli, and provides an unappreciated framework for regulation of anaphase entry in eukaryotic cells.

INTRODUCTION

The evolutionarily conserved mitogen-activated protein kinase (MAPK) signaling pathways regulate multiple cellular functions in eukaryotic organisms in response to a wide variety of environmental cues (Plotnikov et al., 2011). However, different MAPK pathways have been evolved in an organism to integrate diverse signals and fulfill different regulations on various effectors (Cansado et al., 2021; Ronkina and Gaestel, 2022). This makes specifying the MAPKs and substrates involved in a specific physiological process rather complex and challenging.

In late mitosis, the timely polyubiquitylation and subsequent degradation of securin and cyclin B by anaphase-promoting complex/cyclosome (APC/C) play a critical role for anaphase onset and chromosome segregation (Peters, 2006; Sullivan and Morgan, 2007; Yamano, 2019). Given the essential role of APC/C in triggering chromosome segregation, it is not surprising that APC/C is a key molecular target of the spindle assembly checkpoint (SAC), which is an intricate surveillance mechanism that prolongs mitosis until all chromosomes achieve correct bipolar attachments to spindle microtubules (McAinsh and Kops, 2023; Murray, 2011; Musacchio, 2015). Previous studies have revealed that the active MAPK is required for spindle checkpoint activation and APC/C inhibition in *Xenopus* egg extracts or tadpole cells (Chen, 2004; Chung and Chen, 2003; Minshull et al., 1994; Takenaka et al., 1997; Zhao and Chen, 2006). These studies suggested that phosphorylation of several components of SAC, including Cdc20, Mps1 and Bub1, by MAPK serves as regulatory signals to block APC/C activation upon SAC activation (Chen, 2004; Chung and Chen, 2003; Zhao and Chen, 2006). However, all these studies used either the anti-MAPK antibodies or small molecule inhibitor UO126 to inhibit MAPK or MAPK-specific phosphatase MKP-1 to antagonize MAPK activity, these reagents could not distinguish the contributions of different MAPKs with high specificity. Thus, those seemingly defined mechanisms might suffer the ambiguity due to potential specificity issues.

The fission yeast *Schizosaccharomyces pombe* has three MAPK-signaling cascades: the pheromone signaling pathway (PSP), the stress-activated pathway (SAP) and the cell integrity pathway (CIP), with Spk1, Sty1 or Pmk1 as MAPK respectively (Figure 1A) (Cansado et al., 2021; Perez and Cansado, 2010). In *S. pombe*, whether MAPKs play key roles in regulation of the APC/C and spindle checkpoint remains largely unexplored. Intriguingly, all genes encoding the major components of three MAPK-signaling cascades in fission yeast are non-essential (Kim et al., 2010), this provides the possibility for investigations on their potential functions in a genetic background with complete deletion of relevant genes.

In this work, we set out to directly examine the requirement of the fission yeast MAPKs in mitotic APC/C activation and anaphase entry. Our results uncovered a previously unappreciated mechanism in which Pmk1, the MAPK of CIP, phosphorylates the APC/C activator Slp1 (the Cdc20 homologue in *S. pombe*) upon SAC activation. Phosphorylation of $Slp1^{Cdc20}$ results in its ubiquitylation and degradation and thus subsequently impedes APC/C activation and anaphase entry. This mechanism also operates in response to spindle defects under environmental stress. Therefore, our finding extends cell-cycle control stages linked to MAPKs from previously recognized G_2/M transition and cytokinesis to anaphase entry and mitotic exit in fission yeast. It will also be interesting to address whether this MAPK-mediated Cdc20 regulation exists as a general mechanism for balancing spindle checkpoint activity in higher eukaryotes.

RESULTS

SAP and CIP signaling components are required for spindle checkpoint activity

As the first step to examine the possible requirement of the fission yeast MAPKs in mitotic progression, we tested the sensitivity of deletion mutants of three fission yeast MAPKs to microtubule-destabilizing drug thiabendazole (TBZ), which has been routinely used as an indicator of defective kinetochore or spindle checkpoint (Akera et

al., 2015; Saitoh et al., 1997). We noticed that *pmk1*Δ and *sty1*Δ but not *spk1*Δ cells were extremely sensitive to TBZ, and constitutively activated CIP signaling by overexpressing Pek1^{DD} (Pek1-S234D;T238D) (Sugiura et al., 1999) or activated SAP signaling by overexpressing Wis1^{DD} (Wis1-S469D;T473D) (Shiozaki et al., 1998) also caused yeast cells to be sensitive to TBZ (Figure 1B; Supplementary Figure 1A). These observations indicated that Pmk1 and Sty1 pathways might be involved in mitosis-related processes.

To extend our analysis and precisely quantify whether the SAC is properly activated and maintained in MAPK mutants, we adopted one well-established assay using the cold-sensitive β-tubulin mutant *nda3-KM311*, which compromises the kinetochore-spindle microtubule attachment upon cold treatment (Hiraoka et al., 1984), to analyze the ability of cells to arrest in mitosis in the absence of spindle microtubules and enter anaphase when spindle microtubules are reassembled (Bai et al., 2022; May et al., 2017; Vanoosthuyse and Hardwick, 2009). In this assay, the SAC was first robustly activated by the *nda3-KM311* mutant at 18 °C and then inactivated simply by shift mitotically arrested cells back to permissive temperature (30 °C) (Figures 1C). Because Cdc13 (cyclin B in *S. pombe*) localizes to the spindle pole bodies (SPBs) in early mitosis and should be degraded by APC/C to promote metaphase-anaphase transition, accumulation of Cdc13-GFP on SPBs after cold treatment serves as the read-out of the SAC activation, and the disappearance rate of Cdc13-GFP spot under permissive temperature reflects the SAC inactivation efficacy (Figures 1C). It is noteworthy that the ATP analogue-sensitive mutant *sty1-T97A* (i.e. *sty1-as2*) (Zuin et al., 2010), instead of *sty1Δ* deletion mutant, was used in this assay. That was due to the G_2 delay that has been demonstrated in the *sty1Δ* deletion mutant (Shiozaki and Russell, 1995a), which could interfere mitotic timing for further analysis in this assay. We found that inactivation of CIP or SAP signaling by *pmk1*Δ or *sty1-T97A* compromised full SAC activation, as these two mutants had only less than 60% of cells with Cdc13-GFP on SPBs after 6 hours of cold treatment, whereas the percentage in wild-type and *spk1*Δ cells after the same treatment was roughly 90%

(Figure 1D; Supplementary Figure 1C). Consistently, the disappearance of Cdc13-GFP from SPBs in *pek1*^{DD} and *wis1*^{DD} mutant cells released from metaphase-arrest was severely delayed (Figure 1D; Supplementary Figure 1B), suggesting delayed SAC inactivation and anaphase onset after release from spindle checkpoint arrest in these mutants.

All above data suggested that MAPK pathways are evolutionarily conserved in the fission yeast to participate in spindle checkpoint activation and maintenance, of which the CIP and SAP signaling pathways, but not the Spk1 pathway, are actively involved.

CIP and SAP signaling attenuate protein levels of Slp1^{Cdc20} and facilitate association of MCC with APC/C respectively

The mitotic checkpoint complex (MCC, consisting of Mad3-Mad2-Slp1^{Cdc20} in *S. pombe*) is the most potent inhibitor of the APC/C prior to anaphase (Kapanidou et al., 2017; Primorac and Musacchio, 2013; Sudakin et al., 2001). Previous study has revealed that *Xenopus* MAPK phosphorylates Cdc20 and facilitates its binding by Mad2, BubR1 (Mad3 in *S. pombe*) and Bub3 to form a tight MCC to prevent Cdc20 from activating the APC/C (Chung and Chen, 2003). In fission yeast, the key SAC components Mad2 and Mad3 and one molecule of Slp1^{Cdc20} form MCC which binds to APC/C through another molecule of Slp1^{Cdc20} upon checkpoint arrest, and the recovery from mitotic arrest accompanies the loss of MCC-APC/C binding (May et al., 2017; Sczaniecka et al., 2008; Sewart and Hauf, 2017; Vanoosthuyse and Hardwick, 2009). Since we found that constitutive activation of CIP or SAP signaling in fission yeast by *pek1*^{DD} or *wis1*^{DD} mutations caused prolonged SAC activation, we were suspicious that these two MAPK pathways might also execute their functions cooperatively through a similar mechanism to that in *Xenopus*.

To test this possibility, we analyzed the MCC occupancy on APC/C by immunoprecipitations of the APC/C subunit Lid1 (Yoon et al., 2002) in *pek1*^{DD} and *wis1*^{DD} cells arrested by activated checkpoint. Compared to wild-type, more MCC

components (Mad2 plus Mad3 and Slp1^{Cdc20}) were co-immunoprecipitated in *wis1*^{DD} cells, while less of them were co-immunoprecipitated in *pek1*^{DD} cells (Figure 2A), suggesting that activation of the SAP but not the CIP pathway enhanced the association of MCC with APC/C.

The decrease of co-immunoprecipitated Mad2 and Mad3 in *pek1*^{DD} cells was largely out of expectation. Further analysis of the inputs of these immunoprecipitations showed that Slp1^{Cdc20} levels were significantly reduced in *pek1*^{DD} but not *wis1*^{DD} cells (Figure 2A). Our immunoblotting analyses using the total lysate of *pek1*^{DD} or *wis1*^{DD} cells arrested at metaphase also confirmed these results (Figure 2B). Consistently, the Slp1^{Cdc20} level was significantly increased in *pmk1Δ* and *pmk1Δ sty1-T79A* cells but not in *sty1-T79A* cells (Figure 2C). The elevated Slp1^{Cdc20} level in *pmk1Δ* cells is similar to that we observed in cells harboring two copies of *slp1*$^{+}$, which leads to artificially increased Slp1^{Cdc20} level and also causes a SAC strength defect (Bai et al., 2022). It is noteworthy that attenuated levels of Slp1^{Cdc20} in *pek1*^{DD} cells was not due to decreased transcription of *slp1*$^{+}$ gene (Figure 2D). These results collectively revealed a negative effect of CIP but not SAP signaling on Slp1^{Cdc20} levels, which potentially results in the weakened MCC-APC/C interaction detected in CIP mutants (Figure 2A).

Together, our above data suggested a new mechanism involves division of labor between the two fission yeast MAPK pathways CIP (Pek1-Pmk1) and SAP (Wis1-Sty1) upon SAC activation, which is required in concert to lower Slp1^{Cdc20} levels and enhance MCC affinity for APC/C to strongly inhibit APC/C activity (Figure 2E). In this study, we only focused on characterizing the role of the CIP/Pmk1 in regulating SAC inactivation and anaphase onset hereafter.

Pmk1 directly binds Slp1^{Cdc20} to attenuate its levels upon SAC activation

To gain further insight into how Slp1^{Cdc20} levels are modulated by Pmk1, we examined the potential interaction of Pmk1 with Slp1^{Cdc20}. Through incubating

MBP-tagged Slp1^{Cdc20} with metaphase-arrested yeast cell lysates, we found that Pmk1 expressed from yeast cells could be pull-downed by Slp1^{Cdc20} (Figure 3A).

In previous reports, MAPK-docking sites, which are important for interaction with MAPKs, have been identified at the N termini of different MAPKKs from yeast to humans with a notable feature of a cluster of at least two basic residues (mainly Lys/K and Arg/R) separated by a spacer of 2-6 residues from a hydrophobic-X-hydrophobic sequence (Bardwell et al., 2001; Bardwell and Thorner, 1996). Thus, we attempted to identify the potential key residues in Slp1^{Cdc20} that mediate the Pmk1-Slp1^{Cdc20} interaction. By visual scanning of Slp1^{Cdc20} sequence, we noticed five basic-residue patches which are also present in several fungal species and loosely resemble the consensus sequence of the known MAPK-docking sites, and four of them are within N-terminal portion of the protein (Figure 3B and Supplementary Figure 2). However, only mutating either of the two most N-terminal basic-residue patches (19-KKR-21 and 47-KR-48) to glutamates (Glu, E) or alanines (Ala, A) in Slp1^{Cdc20} resulted in decreased amount of Slp1^{Cdc20} pulled down by Pmk1-GST *in vitro* using the bacterially expressed recombinant proteins, and mutating both patches or mutating only two arginine residues (R21 and R48) in those patches was sufficient to completely disrupt their interaction (Figure 3C and Supplementary Figure 3). Importantly, mutations of these Pmk1-docking sequences in Slp1 (PDSS for short) to alanines mimicked *pmk1Δ* mutant in both elevated Slp1^{Cdc20} protein levels (Figure 3D) and defective activation and maintenance of the SAC upon *nda3*-mediated checkpoint arrest (Figure 3E and Supplementary Figure 4). Also, similar to removal of Pek1 downstream MAPK Pmk1, the lowered Slp1^{Cdc20} protein levels and delayed SAC inactivation in *pek1*^{DD} mutant could be relieved by *slp1(DS-5A)* mutant, which is deficient in Pmk1-docking (Figure 3D, 3E and Supplementary Figure 4).

Previously, one conserved non-canonical Pmk1-docking sequence which bears positively charged residues and IYT motif has been identified in MAPK phosphatase Pmp1 (Sacristan-Reviriego et al., 2014). Very strikingly, insertion of a 15 amino acid

sequence containing the Pmk1-docking sequence in Pmp1 (or PDSP for short) right in front of the first basic-residue patch in Slp1(DS-5A) mutant protein restored its interaction with Pmk1 (Figure 3B and 3C). And this artificially forced Pmk1-Slp1^{Cdc20} interaction via PDSP was able to lower Slp1^{Cdc20} protein levels and restore higher percentage of metaphase arrest upon SAC activation in *slp1(DS-5A)* mutant, which is Pmk1-docking-deficient, although the rescuing effect was less efficient than the presence of genuine PDSS (Figure 3D, 3E and Supplementary Figure 4). These data firmly supported the idea that Pmk1 binds Slp1^{Cdc20} to downregulate its levels upon SAC activation.

Collectively, these results demonstrated that Pmk1 directly binds to Slp1^{Cdc20} through two short N-terminal basic patches serving as the major docking sites for Pmk1, and the physical interaction of Pmk1-Slp1^{Cdc20} is involved in attenuation of Slp1^{Cdc20} abundance upon SAC activation (Figure 3F).

Pmk1 synergizes with Cdk1 to phosphorylate Slp1^{Cdc20}

The association of Pmk1 with Slp1^{Cdc20} and the negative effect of constitutively activated CIP signaling on SAC inactivation relies on Pmk1 kinase suggested that Slp1^{Cdc20} may be a direct substrate of Pmk1. To test this possibility, we performed *in vitro* phosphorylation reactions and found that bacterially expressed recombinant GST-Slp1^{Cdc20} could be efficiently phosphorylated in the presence of Pmk1-HA-His purified from yeast cells (Figure 4A).

To identify potential phosphorylation sites *in vivo*, we purified Apc15-GFP from wild type, *pmk1Δ* and *pek1^{DD}* cells arrested by activated checkpoint with intention that the Slp1^{Cdc20}-containing APC/C complexes could be captured (Supplementary Figure 5A and 5B). Purifications were performed in the presence of phosphatase inhibitors to enrich for phosphopeptides. Mass spectrometry analysis revealed that the phosphorylation of Thr480 residue at the most C-terminus of Slp1^{Cdc20} was dependent on the presence of Pmk1, and this modification was present in *pek1^{DD}* but not in wild

type cells (Figure 4B, Supplementary Figure 5C-E and Supplementary Figure 6), indicating that phosphorylation of Thr480 is very likely triggered by CIP signaling. We raised phospho-specific antibodies against pT480, it could recognize Slp1^{Cdc20} fragment phosphorylated by Pmk1 *in vitro* and mutation of Thr480 to alanine (T480A) abolished site-specific phosphorylation (Figure 4C). We also confirmed that endogenous Slp1^{Cdc20} was indeed phosphorylated at Thr480 in metaphase-arrested *mts3-1* cells, which was enhanced in *pek1*^{DD} mutant and diminished in *pmk1Δ* mutant (Figure 4D). Thus, our results established Thr480 as a major Pmk1 phosphorylation site *in vivo* upon SAC activation.

In addition to pT480, our mass spectrometry analysis also identified Ser28, Thr31 and Ser59 as Pmk1-independent and Ser76 as Pmk1-dependent phosphorylation sites (Figure 4B, Supplementary Figure 5C-E and Supplementary Figure 6). All these four residues are located within the N-terminal unstructured region, fit with the Cdk1 core motif (SP or TP), and more importantly, they are conserved not only in four *Schizosaccharomyces* species but also in several other fungal species (Figure 4B and Supplementary Figure 2B). Interestingly, they were also proposed as Cdk1 phosphorylation sites based on quantitative phosphoproteomic analysis in a recent study (Swaffer et al., 2018) (Figure 4B). By using the phospho-specific antibodies against both pS28 and pT31, we could detect the phosphorylation of Ser28 and Thr31 *in vitro* by Cdc13 (cyclin B)-containing Cdk1 complexes purified from metaphase-arrested yeast cells, which was diminished when the kinase activity of ATP analogue-sensitive Cdk1 [cdc2-as1(F84G)] was inhibited by 1-NM-PP1 or when Slp1$^{(S28A;T31A)}$ was added as the substrate in *in vitro* kinase reactions (Figure 4E). Next, we examined whether Ser28 and Thr31 were phosphorylated *in vivo* using immunoblotting with phospho-specific antibodies to directly detect Slp1^{Cdc20}, which was co-immunoprecipitated with Apc15 from *nda3*-arrested yeast cells. We observed phosphorylation of Ser28/Thr31 in wild type cells, but it disappeared following phosphatase treatment of wild type samples after immunoprecipitation, 1-NM-PP1 treatment of *cdc2-as1* cells before being collected for immunoprecipitation, or when

cells expressing Slp1$^{(S28A;T31A)}$ were used (Figure 4F). Thus, Slp1^{Cdc20} is also phosphorylated by Cdk1 at least at Ser28 and Thr31 in fission yeast mitotic cells.

We wondered whether Slp1^{Cdc20} phosphorylation by Pmk1 affects its stability and APC/C activation. Indeed, phospho-deficient Slp1^{T480A} mutant showed elevated Slp1^{Cdc20} levels in both metaphase-arrested and asynchronously grown cells (Figure 4G and Supplementary Figure 7) and compromised SAC activation compared to wild type cells, likely due to more efficient APC/C activation (Figure 4H and Supplementary Figure 8). Furthermore, Slp1^{T480A} mutant also reversed the inhibitory effect of *pek1*^{DD} on Slp1^{Cdc20} stability and APC/C activation after release from SAC-mediated arrest (Figure 4G and 4H).

Ser76 was identified as a Pmk1-dependent phosphorylation residue in our mass spectrometry analysis, but it was considered as a Cdk1 targeting site in an earlier study (Swaffer et al., 2018). It is fairly possible that phosphorylation of Ser76 is promoted by both Cdk1 and Pmk1, which is conceivable as CDKs and MAPKs recognize the same core motif (SP/TP) (Pinna and Ruzzene, 1996), and crosstalk between CDK and MAPK has recently been reported for pheromone signaling in budding yeast (Repetto et al., 2018). We tested how the double phosphorylation sites (S76A;T480A) mutant affects Slp1^{Cdc20} levels or APC/C activity, and observed that the protein levels of Slp1$^{S76A;T480A}$ were further elevated and SAC activation efficiency in this mutant was further lowered compared to Slp1^{T480A} mutant (Figure 4G and 4H). Of note, Slp1^{T480A} and Slp1$^{S76A;T480A}$ mutant proteins became easily detected in interphase cells (Supplementary Figure 7), this is very different from wild type Slp1 which normally accumulates exclusively in mitosis (Yamada et al., 2000).

We further tested whether replacement of other identified phospho-serine/threonine residues with alanines affects Slp1^{Cdc20} protein levels and APC/C activity. We constructed strains carrying combinations of mutations of S28A, T31A and S59A with S76A, and also with confirmed Pmk1 phosphorylation-deficient T480A, and found

that mutations of S28A, T31A and S59A did not affect Slp1^{Cdc20} protein levels, though they could also profoundly compromise SAC activation efficiency similarly to S76A;T480A mutant (Figure 4G, 4H and Supplementary Figure 7). We noticed that the presence of mutations of S28A, T31A and S59A also abolished the elevated Slp1^{Cdc20} protein levels conferred by S76A;T480A mutations (Figure 4G and Supplementary Figure 8). Thus, phosphorylation of S28, T31 and S59 targeted by Cdk1 most likely influences APC/C activation through distinct mechanism from Pmk1-mediated S76/T480 phosphorylation.

Taken together, our data suggested that activated Pmk1 phosphorylates Slp1^{Cdc20} *in vivo*, which leads to lowered Slp1^{Cdc20} levels, delayed SAC inactivation and APC/C activation as we observed in *pek1^{DD}* cells. Also, Pmk1 can join Cdk1 to enhance the multi-site phosphorylation of their shared substrate Slp1^{Cdc20} and collaboratively dampen APC/C activity.

Pmk1- but not Cdk1-mediated Slp1^{Cdc20} phosphorylation promotes its ubiquitylation

Our above data support the notion that the direct binding of Slp1^{Cdc20} by Pmk1 mediates its phosphorylation, which possibly results its subsequent rapid turnover. It has been shown in both human and fission yeast cells that Apc15, one of the conserved components of APC/C, promotes Cdc20/Slp1^{Cdc20} autoubiquitylation and its turnover by APC/C (Mansfeld et al., 2011; May et al., 2017; Sewart and Hauf, 2017; Uzunova et al., 2012). The increased Slp1^{Cdc20} levels in *pmk1Δ* cells (Figure 2C) is reminiscent of the previous observations in *apc15Δ* mutant, which also shows elevated levels of Slp1^{Cdc20} (May et al., 2017; Sewart and Hauf, 2017). We reasoned that the absence of Apc15 may reverse the negative effect of *pek1^{DD}* on Slp1^{Cdc20} levels and spindle checkpoint inactivation and APC/C activation. As expected, we indeed observed that deletion of *apc15* recovered Slp1^{Cdc20} levels in *pek1^{DD}* cells (Figure 5A), and also lowered the percentage of cells with Cdc13-GFP retained on SPBs in *nda3-KM311 pek1^{DD}* cells after cold treatment (Figure 5B and Supplementary

Figure 9A). These observations are consistent with previous reports showing the relative abundance between checkpoint proteins and the checkpoint target Slp1^{Cdc20} is an important determinant of checkpoint robustness (Heinrich et al., 2013) and *apc15Δ* mutant is spindle checkpoint-defective (May et al., 2017).

During the course of our mass spectrometry analysis on Slp1^{Cdc20} phosphorylation, we also detected Lys 479 (K479) as an ubiquitin-modifying site (Figure 4B and Supplementary Figure 5 and 6). We wondered whether K479 is involved in Slp1^{Cdc20} autoubiquitylation in fission yeast. In agreement with the mass spectrometry analysis, mutation of K479 to arginine indeed conferred stability of Slp1^{Cdc20} (Figure 5C), even in asynchronously growing cells (Supplementary Figure 10), which is reminiscent of the stabilized Slp1^{Cdc20} in *apc15Δ* mutant cells at interphase (Sewart and Hauf, 2017). These results suggested that K479 is likely Slp1^{Cdc20} ubiquitylation-relevant. Furthermore, *nda3-KM311 slp1^{K479R}* cells also demonstrated lowered metaphase arrest rate after cold treatment (Figure 5D and Supplementary Figure 9B), indicating defective SAC activation. Previous studies have identified two neighbouring lysine residues close to the carboxy terminus of human Cdc20, Lys485 and Lys490, as ubiquitylation sites in prometaphase cells, and mutating these two lysines to arginine completely prevented Cdc20 ubiquitylation (Danielsen et al., 2011; Mansfeld et al., 2011). Very interestingly, a second lysine residue, K472, is also present in fission yeast Slp1^{Cdc20} adjacent to K479 (Figure 4B and Supplementary Figure 11). Although K472 was not identified as an ubiquitin-modifying residue in our mass spectrometry analysis, replacement of K472 with arginine also efficiently stabilized Slp1^{Cdc20}, similar to K479R mutant (Figure 5C and Supplementary Figure 10). More strikingly, simultaneously mutating both K472 and K479 caused additive effects in elevating Slp1^{Cdc20} levels and lowering SAC activation rate in both wild type and *pek1^{DD}* strain backgrounds (Figure 5C, 5D and Supplementary Figure 10), suggesting that likely both these two lysine residues at the C-terminal tail of Slp1^{Cdc20} contribute to its turnover via ubiquitylation, which is very similar to the mechanism in human Cdc20. Consistently, using TUBEs (tandem ubiquitin-binding entities) (Hjerpe et al., 2009) to

enrich for ubiquitinated proteins in metaphase-arrested *mts3-1* cells expressing sfGFP-Slp1, we were able to demonstrate that mutating both K472 and K479 largely removed higher molecular weight bands detected by immunoblotting, which were corresponding to ubiquitylated species of Slp1^{Cdc20} (Figure 5E, F). We also noticed that Slp1$^{K472R;\ K479R}$ rendered more severely compromised viability than either single mutants (Supplementary Figure 12), indicating blocking Slp1^{Cdc20} ubiquitylation could bring about detrimental consequence to the cells.

Our observation that Slp1^{Cdc20} levels were lowered in *pek1^{DD}* mutant (Figure 2B) suggested that attenuation of Slp1^{Cdc20} levels upon constitutive activation of CIP signaling is very likely through facilitating its autoubiquitylation. To test this possibility, we examined and compared the ubiquitylation levels of sfGFP-Slp1 in wild type, *pmk1Δ* and *pek1^{DD}* cells by affinity pull-down assays using TUBEs (Figure 5E). Indeed, we observed a much weaker or stronger discrete laddering pattern of sfGFP-Slp1 in *pmk1Δ* and *pek1^{DD}* cells than that of wild type cells, indicating abated or enhanced ubiquitylation respectively (Figure 5F). Furthermore, mutations of T480A, S76A/T480A and Pmk1-docing site, but not S28A/T31A- or S28A/T31A/S59A-containing mutants, also reduced polyubiquitylation of Slp1^{Cdc20} (Figure 5F). All these results confirmed that Pmk1- but not Cdk1-mediated Slp1^{Cdc20} phosphorylation promotes its ubiquitylation.

Together, these findings lent further support to the idea that the CIP signaling pathway restrain APC/C activity through a cascade mechanism, in which Pmk1 directly binds and phosphorylates Slp1^{Cdc20} to facilitate its autoubiquitylation, and eventually lowers its levels (Figure 5G).

Osmotic stress and cell wall damage trigger rapid Slp1^{Cdc20} downregulation and mitotic exit delay

It has been well established that two MAPKs Sty1 and Pmk1 can be activated by environmental perturbations including osmotic stress, cell wall damage, thermal stress,

oxidative stress, and glucose limitation, among others (Cansado et al., 2021; Madrid et al., 2006; Shiozaki and Russell, 1995b). We wondered whether the phosphorylation of Slp1^{Cdc20} by Pmk1 could tune the strength of SAC activation and APC/C activity in response to extracellular stress. To investigate this possibility, we performed arrest-and-release experiments with *nda3-KM311* mutants and added 0.6 M KCl or 2 μg/mL of caspofungin 1 hr before release from metaphase arrest to elicit strong osmotic saline stress (KCl) or cell wall damage stress (caspofungin) response (Madrid et al., 2006; Madrid et al., 2016), respectively (Figure 6A). As previously reported, treatment with KCl or caspofungin resulted in full activation of both Pmk1 and Sty1 (for KCl treatment) or only Pmk1 (for caspofungin treatment) as determined using anti-phospho p42/44 and anti-phospho p38 antibodies, which serve as indicative of respective Pmk1 and Sty1 activation (Figure 6B). Intriguingly, treatment with KCl or caspofungin also correlated with a marked and rapid decrease in Slp1^{Cdc20} levels (Figure 6B).

Surprisingly, we did not see any alteration of APC/C-MCC interaction accompanying Pmk1 activation when cells were treated by caspofungin (Figure 6C), which was different from what we observed in P_{adh11}-*pek1*^{DD} cells (Figure 2A). Also, we failed to detect enhanced APC/C-MCC association like we observed in P_{adh11}-*wis1*^{DD} cells when cells were treated by KCl, where Sty1 was activated (Figure 6C). The latter observation was most likely due to the disruptive effect of high concentration of KCl on protein-protein interactions, because we observed similar effect when we immunoprecipitated APC/C in the presence of 0.6 M KCl in the lysis buffer (Figure 6C, right panel). With this data, we could not exclude the possibility that activated Sty1 transiently enhanced APC/C-MCC association in yeast cells, which was soon counteracted by the disruptive effect of saline. Nevertheless, the high SAC activation efficiency in mitotis-arrested cells exposed to osmotic stress (KCl) or cell wall damage stress (caspofungin) was largely lowered by *pmk1* deletion (Figure 6D), indicating that at least activated Pmk1 exerts regulation of the spindle checkpoint and APC/C activation under environmental stimuli. Further, Pmk1-docking site mutant

slp1(DS-5A) and Pmk1 phosphorylation-deficient mutants *slp1*^{T480A} and *slp1*$^{S76A;T480A}$ could partially suppress the sustained SAC activation responding to osmotic and cell wall damage stress (Figure 6E and Supplementary Figure 13).

DISCUSSION

Involvement of MAPKs in cell cycle regulation

So far, the only known cell-cycle control stages linked to MAPKs in fission yeast are at G_2/M transition and during cytokinesis (Gomez-Gil et al., 2020; Petersen and Hagan, 2005; Petersen and Nurse, 2007). The p38 MAPK family member Sty1 either arrests or promotes mitotic commitment in unperturbed, stressed or resumed cell cycles after stress depending on the downstream kinases it associates with. Sty1 can phosphorylate kinase Srk1 at threonine 463 (T463), which further phosphorylates and inhibits Cdc25 phosphatase to negatively regulate the onset of mitosis (Lopez-Aviles et al., 2005; Lopez-Aviles et al., 2008; Smith et al., 2002). Sty1 also mediates phosphorylation of Polo kinase Plo1 at serine 402 (S402), which then promotes its recruitment to the spindle pole bodies (SPBs), where it positively modulates Cdc25 control of Cdc2 (fission yeast Cdk1) activity for mitotic entry (Petersen and Hagan, 2005; Petersen and Nurse, 2007). During cytokinesis, two fission yeast formins For3 and Cdc12 cooperate to nucleate actin filaments for assembly of contractile actomyosin ring (CAR) (Coffman et al., 2013). It has been shown that Sty1 activity negatively regulates protein levels of For3 in response to actin cytoskeleton damage to downregulate CAR assembly (Gomez-Gil et al., 2020). Very recently, Pmk1 has also been proposed to act as a negative regulator of cytokinesis, which delays CAR constriction upon cell wall stress (e.g. treatment with blankophor, caspofungin and caffeine) through a novel checkpoint mechanism (Edreira et al., 2020). However, the detailed mechanisms how Sty1 and Pmk1 pose negative effect on cytokinesis have not been elucidated.

In the current study, we have expanded MAPK signaling-controlled cell-cycle stage to

metaphase-anaphase transition in fission yeast. In response to unattached or tensionless kinetochores, the SAC generates the MCC, which inhibits the APC/C and block anaphase onset by suppressing APC/C-catalyzed ubiquitylation and subsequent degradation of securin and cyclin B. As a critical APC/C inhibitor, MCC fulfills its function through the physical binding of BubR1/Mad3 and Mad2 to two molecules of Cdc20/Slp1^{Cdc20} (*i.e.* Cdc20^{MCC} and Cdc20$^{APC/C}$), and this mechanism has been confirmed at least in both humans and fission yeast (Alfieri et al., 2016; Chao et al., 2012; Izawa and Pines, 2015; Sewart and Hauf, 2017; Yamaguchi et al., 2016). In this study, we have uncovered a dual mechanism, in which two fission yeast MAPKs Pmk1 and Sty1 restrain APC/C activity through distinct manners, either by phosphorylating Slp1^{Cdc20} to lower its levels or by phosphorylating unknown substrate(s) to promote MCC-APC/C association and thus SAC signaling strength, respectively (Figure 7). Therefore, our study has established another critical APC/C-inhibitory mechanism in the spindle checkpoint, though it will be important for future studies to identify the phosphorylation target(s) posed by SAP signaling pathway. Nevertheless, our current study together with earlier report (Edreira et al., 2020) have implicated fission yeast Pmk1 in both the mitotic and cytokinesis surveillance mechanisms in response to unfavorable environmental stimuli.

The regulation of mitotic entry by MAPK in fission yeast is triggered by many environmental insults, including high osmolarity, oxidative stress, heat shock, centrifugation, nutrient starvation and rapamycin treatment (Hartmuth and Petersen, 2009; Petersen and Hagan, 2005; Petersen and Nurse, 2007; Shiozaki and Russell, 1995a; Shiozaki et al., 1998). In this study, we found that treatment of fission yeast cells with KCl or caspofungin, which provokes a strong osmotic stress or cell wall damage response and quick Pmk1 activation, correlated with an attenuated Slp1^{Cdc20} protein levels and delayed APC/C activation. Deletion of *pmk1*$^{+}$ largely overrides the negative effects of KCl or caspofungin treatment on APC/C activation (Figure 6D), confirming that activated Pmk1 is responsible for prolonged metaphase arrest under environmental stress. Thus, our finding implicates MAPK Pmk1 as an important

regulator for anaphase entry in response to adverse extracellular conditions. Intriguingly, one previous study showed that treatment of human cells with the carcinogen cadmium activates MAPK p38 (fission yeast Sty1 homologue) and triggers accelerated ubiquitination and proteolysis of Cdc20, which is essential for prometaphase arrest under SAC (Yen and Yang, 2010). Therefore, although different MAPKs might be employed to fulfil the task in yeast and higher eukaryotes, it is still possible that phosphorylation and regulation of Cdc20 levels by MAPKs is an evolutionarily conserved mechanism to adjust mitotic exit to extracellular or environmental stimuli.

Negative phospho-regulation of Cdc20 by MAPK as a spindle assembly checkpoint-dependent APC/C-inhibitory mechanism

APC/C activity can be regulated at multiple levels, Cdk1-dependent APC/C phosphorylation is one of the major prerequisites for APC/C activation and anaphase onset, in which Apc3 and Apc1 are key subunits for positive phospho-regulation (Fujimitsu et al., 2016; Kraft et al., 2003; Qiao et al., 2016; Steen et al., 2008; Zhang et al., 2016). However, it has been shown that Cdk1-dependent phosphorylation of APC/C co-activator Cdc20 lessens its interaction with APC/C, thus serves as a negative phospho-regulation (Labit et al., 2012). In addition to its phosphorylation by Cdk1, phosphorylation of Cdc20 by multiple other kinases, such as Bub1, PKA, MAPK and Plk1, has also been demonstrated or suggested in *Xenopus*, humans or budding yeast to be inhibitory to APC/C activation and improper anaphase onset upon SAC activation ((Jia et al., 2016) and reviewed in (Yu, 2007)). Therefore, Cdc20 generally serves as an integrator of multiple intracellular signaling cascades that regulate progression through mitosis. In this work, we demonstrated that phosphorylation of $Slp1^{Cdc20}$ by Pmk1 but not Cdk1 promotes its autoubiquitylation, this mechanism is distinct from that reported in *Xenopus* egg extracts and tadpole cells, in which phosphorylation of Cdc20 by MAPK is instead responsible for facilitated Cdc20 binding by MCC to prevent it from activating the APC/C (Chung and Chen, 2003).

Although multiple kinases have been proposed to be directly involved in phosphorylation of Cdc20 proteins (Yu, 2007), precisely identifying the *in vivo* phosphorylation sites in Cdc20 has turned out to be challenging, most likely due to its protein scarcity and instability. Based on known Cdk1 core consensus (SP/TP), 9 residues in human Cdc20 were originally suggested as putative Cdk1 sites, but among them only Ser41 has been proven to be phosphorylated *in vivo* by mass spectrometry analysis (Kramer et al., 2000; Yudkovsky et al., 2000). However, subsequent mass spectrometry analyses identified Ser41 as one of the Bub1 phosphorylation sites both *in vivo* and *in vitro* and another putative Cdk1 site Thr106 as one of the Plk1 sites (Jia et al., 2016; Tang et al., 2004). It is very likely that there are not as many genuine Cdk1 sites in Cdc20 as originally assumed. One supporting evidence is that mutation of only one (Thr32) of the three putative Cdk sites to alanine in N terminus of *C. elegans* CDC-20 is sufficient to phenocopy 3A mutant for accelerated APC/C activation (Kim et al., 2017). Interestingly, Thr32 of *C. elegans* CDC-20 is conserved as Thr 59 and Thr68 in human and *Xenopus* Cdc20, respectively (see Supplementary Figure 14). Based on two-dimensional tryptic phosphopeptide analysis, Thr68 in *Xenopus* Cdc20 has also been regarded as a MAPK target site (Chung and Chen, 2003). These studies in diverse organisms raised an issue that whether multiple kinases share the same phosphorylation sites in Cdc20? It would not be so surprising if it turns out to be the case eventually, at least for Cdk1 and MAPKs, because CDKs and MAPKs recognize the same core motif (SP/TP) (Pinna and Ruzzene, 1996).

By purifying Apc15-aasociated fission yeast $Slp1^{Cdc20}$ proteins from checkpoint-arrested $pek1^{DD}$ and *pmk1*Δ cells, we were able to identify Thr480 and Ser76 as *in vivo* Pmk1-relevant phosphorylation sites and 3 Pmk1-independent phosphorylation sites (S28, T31, S59) based on mass spectrometry analyses. All our identified sites except Thr480 are located in the N terminal disordered portion of $Slp1^{Cdc20}$ sequence, this is similar to the phosphorylation residues identified by biochemical and functional analyses in other organisms, in which the major

phosphorylation sites all fall within the N-terminus of Cdc20 homologues. We further provided evidence that S28/T31 are phosphorylated by Cdk1 both *in vitro* and *in vivo*, and T480 phosphorylated by Pmk1 both *in vitro* and *in vivo*. We noticed that there is a tendency for reported phosphorylation residues to be concentrated in the N terminus of Cdc20 proteins (Jia et al., 2016; Tang et al., 2004; Yu, 2007). However, due to the lack of clear sequence homology in their N-terminal and C-terminal tails between $Slp1^{Cdc20}$ and its Cdc20 homologues in higher eukaryotes (see Supplementary Figure 14), it is impossible to certainly assign any identified sites from different organisms as homologous sites. But it is possible that multiple kinases modify shared sites in Cdc20, this collaborative effect may be especially helpful under adverse environmental stress. Nevertheless, all reported Cdc20 phosphorylation events constitute an APC/C-inhibitory mechanism, no matter what kinases are responsible for the modifications (Chung and Chen, 2003; D'Angiolella et al., 2003; Jia et al., 2016; Kim et al., 2017; Kramer et al., 2000; Labit et al., 2012; Tang et al., 2004; Yu, 2007; Yudkovsky et al., 2000).

The current model of inhibitory signaling from Cdc20 posits that its phosphorylation either promotes the formation of MCC or inhibits APC/C^{Cdc20} catalytically, both are required for proper spindle checkpoint signaling (Jia et al., 2016; Labit et al., 2012; Yamano, 2019). Interestingly, our current study suggests $Slp1^{Cdc20}$ phosphorylation by fission yeast MAPK Pmk1 may represent another critical APC/C-inhibitory mechanism in the spindle checkpoint through reducing the protein levels of $Slp1^{Cdc20}$, which is achieved via autoubiquitylation-mediated degradation (Figure 7). Actually, previous studies in fission and budding yeast have underlined the importance of accurate relative abundance between checkpoint proteins and $Cdc20/Slp1^{Cdc20}$, which sets an important determinant of checkpoint robustness (Heinrich et al., 2013; Pan and Chen, 2004). A benefit of allowing the association of MCC-bound Cdc20 with APC/C is to provide an opportunity for Cdc20 autoubiquitylation by APC/C, a process aided by APC/C subunit Apc15 (Mansfeld et al., 2011; May et al., 2017; Sewart and Hauf, 2017; Uzunova et al., 2012). Notably, the depletion of $apc15^{+}$ restores $Slp1^{Cdc20}$ levels

in *pek1*^{DD} cells (Figure 5A), consistent with the idea that Pmk1-mediated Slp1^{Cdc20} phosphorylation very likely facilitates its autoubiquitylation. Indeed, our biochemical and functional analyses further establish K479 and K472 in Slp1^{Cdc20} as two key residues directly involved in its ubiquitylation, which likely follows its phosphorylation, particularly at adjacent Thr480. Although K485 and K490 are similarly located at C terminus of human Cdc20 and can be ubiquitylated and responsible for Cdc20 degradation (Danielsen et al., 2011; Mansfeld et al., 2011), there has been no reports on phosphorylation-modified residues in this region. However, although the sequence homology within N- and C-terminal regions between Slp1^{Cdc20} and Cdc20 homologues in higher eukaryotes is extremely low, we do notice that there are several lysine-flanking serine or threonine residues within Cdc20 C-terminal tails of higher eukaryotes (see Supplementary Figure 11), this raises a possibility that this region may be involved in phosphorylation-coupled ubiquitylation. Currently, we do not know how T480 phosphorylation accelerate ubiquitylation of K479 and K472 in Slp1^{Cdc20}. It is possible that phosphorylation of Slp1^{Cdc20} might alter the mode of Cdc20 binding to APC/C which becomes conducive to catalyze Slp1^{Cdc20} ubiquitylation.

In summary, our studies revealed a distinct dual mechanism of MAPK-dependent anaphase onset delay imposed directly on APC/C co-activator Slp1^{Cdc20} and an unidentified substrate, which may emerge as an important regulatory layer to fine tune APC/C and MCC activity upon SAC activation. Importantly, although this mechanism of APC/C regulation by two MAPKs has not been examined in other organisms, it is very likely widespread across eukaryotes including humans, given the involvement of MAPKs in general response to wide range of adverse environmental situations and similar regulations of APC/C and SAC in eukaryotes characterized so far. It is also plausible to assume that the MAPK-mediated APC/C inhibition pathway may also contribute to the pathogenesis of multiple cancers. Indeed, Cdc20 upregulation has been demonstrated in several types of cancers, including breast and colon cancer, and it contributes to aggressive tumor progression and poor prognosis in gastric cancer

and primary non-small cell lung cancer (Chi et al., 2019). It would be interesting to address whether the altered levels of Cdc20 in these cancers are MAPK relevant. Nevertheless, inhibition of APC/C^{Cdc20} activity or induction of Cdc20 degradation have already been listed as alternative therapeutic strategies to control cancer (Greil et al., 2022; Jeong et al., 2022). Thus, although the detailed molecular mechanisms may vary, further studies on MAPK-mediated APC/C^{Cdc20} inhibition mechanism in human would lead to better understanding of its oncogenic role in tumor progression.

Materials and Methods

Fission yeast strains, media and genetic methods

Fission yeast cells were grown in either YE (yeast extract) rich medium or EMM (Edinburgh minimal medium) containing the necessary supplements. G418 disulfate (Sigma-Aldrich; A1720), hygromycin B (Sangon Biotech; A600230) or nourseothiricin (clonNAT; Werner BioAgents; CAS#96736-11-7) was used at a final concentration of 100 μg/ml and thiabendazole (TBZ) (Sigma-Aldrich; T8904) at 5-15 μg/ml in YE media where appropriate. For serial dilution spot assays, 10-fold dilutions of a mid-log-phase culture were plated on the indicated media and grown for 3 to 5 days at indicated temperatures.

To create strain with an ectopic copy of *slp1*$^+$ at *lys1*$^+$ locus on chromosome 1, the open reading frame of *slp1*$^+$ (1467 bp) and its upstream 1504 bp sequence was first cloned into the vector pUC119-P_{adh21}-MCS-*hphMX6-lys1** with *adh21* promoter (P_{adh21}) removed using the 'T-type' enzyme-free cloning method (Chen et al., 2017). The sequence of *hphMX6* in above plasmid was replaced by fragment corresponding to nourseothiricin resistance gene sequence by *Bgl*II-*EcoR*I cut and re-ligation, generating pUC119-P_{slp1}-*slp1*$^+$-T_{adh1}*::natMX6-lys1**. The resultant plasmids were linearized by *Apa*I and integrated into the *lys1*$^+$ locus, generating the strains *lys1Δ::*P_{slp1}-*slp1*$^+$-T_{adh1}*::hphMX6* and *lys1Δ::*P_{slp1}-*slp1*$^+$-T_{adh1}*::nat*^{R}.

To generate the mutant strains harboring lysine/arginine (K/R) or serine/threonine to glutamic acid (E) or alanine (A) mutations, or lysine (K) to arginine (R) mutations within Slp1, the desired mutations were introduced into pUC119-P_{slp1}-*slp1*$^{+}$-T_{adh1}*::hphMX6* using standard methods of site-directed mutagenesis.

To generate the strains carrying superfolder GFP (sfGFP)-tagged wild type or mutant Slp1, a fragment corresponding to the sequence of *sfGFP* was inserted in front of *slp1* in pUC119-P_{slp1}-*slp1*-T_{adh1}*::hphMX6-lys1** using the 'T-type' enzyme-free cloning method. The resulting plasmids were linearized and integrated into the *lys1*$^{+}$ locus of chromosome 1 using the *hyg*^{r} marker. Then endogenous *slp1*$^{+}$ was deleted and replaced with *ura4*$^{+}$.

To generate the strains expressing *ade6::*P_{adh1}*-GST-slp1(456-488aa)::natR*, C-terminal sequence of *slp1*$^{+}$ corresponding to 456-488aa was first cloned into the vector pUC119-P_{adh1}-MCS-*natMX6-lys1** and the sequence of *lys1** was replaced with *ade6*$^{+}$ from pAde6-NotI-hphMX (a kind gift of Li-lin Du), and then the sequence of GST from pGEX-4T-1 was inserted in front of *slp1*. The resulting plasmid pUC119-P_{adh1}*-GST-slp1(456-488aa)-natMX6-ade6* was linearized and integrated into the *ade6*$^{+}$ locus of chromosome III using the *nat*^{r} marker.

To generate the constitutively active MAPKK strains *pek1*^{DD} or *wis1*^{DD}, the Ser234 and Thr238 of *pek1*$^{+}$ in pUC119-P_{adh21}*-6HA-pek1*$^{+}$-T_{adh1}*-hphMX6-lys1**, pUC119-P_{adh11}*-6HA-pek1*$^{+}$-T_{adh1}*-hphMX6-lys1** and pUC119-P_{adh11}*-6HA-pek1*$^{+}$-T_{adh1}*-kanMX6-ura4*$^{+}$ and the Ser469 and Thr473 of *wis1*$^{+}$ in pUC119-P_{adh21}*-6HA-wis1*$^{+}$-T_{adh1}*-hphMX6-lys1**, pUC119-P_{adh11}*-6HA-wis1*$^{+}$-T_{adh1}*-hphMX6-lys1** and pUC119-P_{adh11}*-6HA-wis1*$^{+}$-T_{adh1}*-kanMX6-ura4*$^{+}$ were changed to aspartic acids (D) by standard methods of site-directed mutagenesis. The resulting plasmids were linearized and integrated into the *lys1*$^{+}$ locus of chromosome I or the *ura4*$^{+}$ locus of

chromosome III using the *kan*^{r} marker. A list of the yeast strains used is in Supplementary Table S1.

Fission yeast cell synchronization methods

For *nda3-KM311* strains, cells were grown at the permissive temperature for *nda3-KM311* (30 °C) to mid-log phase, synchronized at S phase by adding HU (Sangon Biotech; A600528) to a final concentration of 12 mM for 2 hours followed by a second dose of HU (6 mM final concentration) for 3.5 hours. HU was then washed out and cells were released at specific temperatures as required by subsequent experiments.

Spindle checkpoint activation and silencing assays in fission yeast

For checkpoint silencing assay in the absence of microtubules, mid-log *cdc13-GFP nda3-KM311* cells were first synchronized with HU at 30 °C and then arrested in early mitosis by shifting to 18 °C for 6 hours, followed by incubation at 30 °C to allow spindle reformation and therefore spindle checkpoint inactivation. For osmotic stress or cell wall damage stress experiments, 0.6 M KCl or 2 μg/mL caspofungin was added into cultures during 18 °C incubation 1 hr before being shifted to 30 °C. Cells were withdrawn at certain time intervals and fixed with cold methanol and stained with DAPI. 200-300 cells were analyzed for each time point. Each experiment was repeated at least three times.

Immunoblotting and immunoprecipitation

For routine Western blot and immunoprecipitation experiments, yeast cells were collected from *nda3-KM311* arrested and released cultures, followed by lysing with glass bead disruption using Bioprep-24 homogenizer (ALLSHENG Instruments, Hangzhou, China) in NP40 lysis buffer (6 mM Na_2HPO_4, 4 mM NaH_2PO4, 1% NP-40, 150 mM NaCl, 2 mM EDTA, 50 mM NaF, 0.1 mM Na_3VO_4) plus protease inhibitors as previously described (Wang et al., 2012). Proteins were immunoprecipitated by IgG Sepharose beads (GE Healthcare; 17-0969-01) (for Lid1-TAP) or anti-GFP

nanobody agarose beads (AlpalifeBio, Shenzhen, China; KTSM1301) (for Mad3-GFP). Immunoblot analysis of cell lysates and immunoprecipitates was performed using appropriate antibodies at 1:500 - 1:5000 dilutions and was read out using chemiluminescence.

Detection of *in vivo* phosphorylation of Slp1^{Cdc20} at Thr480 and Ser28/Thr31

For detection of phosphorylation of Slp1^{Cdc20} at Thr480 *in vivo*, *mts3-1* mutant strains expressing *ade6::P$_{adh1}$-GST-slp1(456-488aa)::natR* in wild type, *pmk1*Δ and *lys1*Δ*::P$_{adh11}$-6xHA-pek1^{DD}* background were arrested at 36℃ for 3.5 hours, followed by pull-down of GST-Slp1(456-488aa) by glutathione Sepharose 4B (GE Healthcare). Purified GST-Slp1(456-488aa) was detected with either goat anti-GST HRP-conjugated antibodies (GE Healthcare) or rabbit polyclonal anti-pThr480 antibodies.

For detection of phosphorylation of Slp1^{Cdc20} at Ser28/Thr31 *in vivo*, *nda3-KM311 apc15-13myc::kanR* strains expressing *slp1^{+}* or *slp1(S28A;T31A)* in wild type or *cdc2-asM17* background were first synchronized with HU at 30 °C and then arrested in early mitosis by shifting to 18 °C for 6 hours. For *cdc2-asM17* strains, 1 μM 1-NM-PP1 (Toronto Research Chemicals; A603003) was added into one of the cultures to inactivate Cdc2 (Cdk1) 20 min before being collected. Apc15-13myc was immunoprecipitated by anti-c-Myc magnetic beads (MedChemExpress (MCE); HY-K0206). To remove Slp1^{Cdc20} phosphorylation, one of the immunoprecipitated samples was treated with 400 units of Lambda protein phosphatase (New England Biolabs, P0753S) at 30 °C for 30 min. Co-immunoprecipitated Slp1^{Cdc20} was detected with rabbit polyclonal anti-Slp1 antibodies, and the phosphorylated form of Slp1^{Cdc20} was detected with rabbit polyclonal anti-pSer28/pThr31 antibodies respectively.

Purification of APC/C and analyses by mass spectrometry

To prepare APC/C for mass spectrometry analyses, Apc15-GFP was purified from 6-liter cultures of *nda3-KM311* arrested cells with desired genetic background. Cells

were disrupted and cell lysates were prepared as described above for routine immunoprecipitation experiments.

For mass spectrometry analyses, purified samples were first run on PAGE gels, after staining of gels with Coomassie blue, excised gel segments were subjected to in-gel trypsin (Promega, V5111) digestion and dried. Samples were then analyzed on a nanoElute (plug-in V1.1.0.27; Bruker, Bremen, Germany) coupled to a timsTOF Pro (Bruker, Bremen, Germany) equipped with a CaptiveSpray source. Peptides were separated on a 15cm X 75µm analytical column, 1.6 µm C18 beads with a packed emitter tip (IonOpticks, Australia). The column temperature was maintained at 55 °C using an integrated column oven (Sonation GmbH, Germany). The column was equilibrated using 4 column volumes before loading sample in 100% buffer A (99.9% MilliQ water, 0.1% FA) (Both steps performed at 980 bar). Samples were separated at 400 nL/min using a linear gradient from 2% to 25% buffer B (99.9% ACN, 0.1% FA) over 90 min before ramping to 37% buffer B (10min), ramp to 80% buffer B (10min) and sustained for 10 min (total separation method time 120 min). The timsTOF Pro (Bruker, Bremen, Germany) was operated in PASEF mode using Compass Hystar 6.0. Mass Range 100 to 1700m/z, 1/K0 Start 0.6 V·s/cm^2 End 1.6 V·s/cm^2, Ramp time 110.1ms, Lock Duty Cycle to 100%, Capillary Voltage 1600V, Dry Gas 3 L/min, Dry Temp 180°C, PASEF settings: 10 MS/MS scans (total cycle time 1.27sec), charge range 0-5, active exclusion for 0.4 min, Scheduling Target intensity 10000, Intensity threshold 2500, CID collision energy 42eV. All raw files were analyzed by PEAKS Studio Xpro software (Bioinformatics Solutions Inc., Waterloo, ON, Canada). Data was searched against the *S. pombe* proteome sequence database (Uniprot database with 5117 entries of protein sequences at https://www.uniprot.org/proteomes/UP000002485). *De novo* sequencing of peptides, database search and characterizing specific PTMs were used to analyze the raw data; false discovery rate (FDR) was set to ≤1%, and [-10*log(p)] was calculated accordingly where p is the probability that an observed match is a random event. The PEAKS used the following parameters: (i) precursor ion mass tolerance, 20 ppm; (ii)

fragment ion mass tolerance, 0.05 Da (the error tolerance); (iii) tryptic enzyme specificity with two missed cleavages allowed; (iv) monoisotopic precursor mass and fragment ion mass; (v) a fixed modification of cysteine carbamidomethylation; and (vi) variable modifications including N-acetylation of proteins and oxidation of Met.

Ubiquitin affinity pull-down assays

For detection of *in vivo* ubiquitylation of $Slp1^{Cdc20}$, strains all carried *mts3-1* mutation were used. sfGFP-tagged wild type Slp1 or Slp1 mutants were expressed in the genomically integrated form of P_{slp1}*-sfGFP-slp1(WT or mutants)::hyg*^{R} in wild type, *pmk1*Δ or P_{adh11}*-6xHA-pek1*^{DD} background. Cultures were first grown at 25 °C to mid-log phase and then shifted to 36°C for 3.5 hours to block cells in mitosis prior to harvesting and cell lysis. Ubiquitinated proteins were pulled down from yeast lysates using Tandem Ubiquitin Binding Entities (TUBEs) (LifeSensors, UM402) according to the manufacturer's instructions. The bound proteins were eluted from washed Agarose-TUBE2 beads and analyzed by western blotting using a mouse monoclonal anti-GFP antibody (Beijing Ray Antibody Biotech; RM1008). Cell extracts were also immunoblotted for GFP and Cdc2 as loading controls (inputs).

Expression and purification of recombinant proteins

GST or MBP fusion constructs of fission yeast full-length or truncated $Slp1^{Cdc20}$, Pmk1, Pek1 and Atf1 were generated by PCR of the corresponding gene fragments from yeast genomic DNA or cDNA and cloned in frame into expression vectors pGEX-4T-1 (GE Healthcare) or pMAL-c2x (New England Biolabs), respectively. Site-directed mutagenesis was done by standard methods to generate vectors carrying Pek1-DD(S234D, T238D) or $Slp1^{Cdc20}$ harboring K(Lys)/R(Arg) to E(Glu) or A(Ala) mutations at individual or combined sites including K19, K20, R21, K47, R48, K140, K141, K161, R162, R163, K472, R473. Integrity of cloned DNA was verified by sequencing analysis. All recombinant GST- or MBP-fusion proteins were expressed in *Escherichia coli* BL21 (DE3) cells. The recombinant proteins were purified by glutathione Sepharose 4B (GE Healthcare) or Amylose Resin High Flow (New

England Biolabs) respectively according to the manufacturers' instructions.

In vitro pulldown assay

The recombinant MBP-Slp1^{Cdc20} and MBP, or GST-Pmk1 and GST proteins were produced in bacteria and purified as described above. To detect the interaction between bacterially expressed Slp1^{Cdc20} and Pmk1-HA expressed in yeast, about 1 μg of MBP-Slp1^{Cdc20} or MBP immobilized on amylose resin was incubated with cleared cell lysates prepared from yeast strains *nda3-KM311 pmk1-6His-HA*, which were HU synchronized and arrested at prometaphase by being grown at 18 °C for 6 hours. To detect the direct interaction between bacterially expressed Slp1 and Pmk1, about 1 μg of GST-Pmk1 or GST immobilized on glutathione Sepharose 4B resin was incubated with cleared bacterial lysates expressing MBP-Slp1^{Cdc20}. Samples were incubated for 1-3 hours at 4 °C. Resins were washed 3 times with lysis buffer (6 mM Na_2HPO_4, 4 mM NaH_2PO_4, 1% NP-40, 150 mM NaCl, 2 mM EDTA, 50 mM NaF, 0.1 mM Na_3VO_4, plus protease inhibitors), suspended in SDS sample buffer, and then subject to SDS-PAGE electrophoresis and Coomassie brilliant blue (CBB) staining.

Non-radioactive *in vitro* kinase assay

GST-Slp1^{Cdc20}, GST-Atf1, GST-Pmk1, and GST-Pek1^{DD} fusions were expressed and purified from *Escherichia coli* with glutathione Sepharose 4B as described above. Pmk1-HA-His and Cdc13-GFP were purified from yeast lysates by immuprecipitation with anti-HA or anti-GFP antibodies respectively.

For detection of phosphorylation of Slp1^{Cdc20} and Atf1 by yeast derived Pmk1, glutathione Sepharose 4B beads bound with GST-Slp1^{Cdc20} or GST-Atf1 and agarose beads bound with Pmk1-HA-His were mixed and washed 3 times with kinase buffer (50 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 1 mM EGTA), then the reactions were incubated in kinase buffer with 20 μM ATPγS (Sigma-Aldrich; A1388) at 30°C for 45 min. The kinase reactions were stopped by adding 20 mM EDTA, and the reaction mixture was alkylated after incubation at room temperature with 2.5 mM

p-nitrobenzyl mesylate (PNBM) (Abcam; ab138910) for 1 hr. The alkylation reaction was stopped by boiling in SDS-PAGE loading buffer. Phosphorylated proteins were detected with rabbit monoclonal anti-Thiophosphate ester antibody (Abcam, ab239919).

For detection of phosphorylation of $Slp1^{Cdc20}$ at Thr480 by bacterially expressed Pmk1, beads bound with GST-Pmk1, GST-Pek1(DD) and fused substrates $GST\text{-}Slp1^{(456\text{-}488aa)}$ or $GST\text{-}Slp1^{(456\text{-}488aa,\ T480A)}$ were washed and incubated in kinase buffer (50 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 1 mM EGTA) with 10 mM ATP (Sangon Biotech; A600020-0005) at 30°C for 45 min. The reaction was stopped by boiling in SDS-PAGE loading buffer. Phosphorylated proteins were detected with rabbit polyclonal anti-pThr480 antibodies.

For detection of phosphorylation of $Slp1^{Cdc20}$ at Ser28/Thr31 by Cdk1, beads bound with bacterially expressed $MBP\text{-}Slp1^{(1\text{-}190aa)}$ or $MBP\text{-}Slp1^{(1\text{-}190aa,S28A;T31A)}$ and agarose beads bound with Cdc13-GFP/Cdc2 from yeast lysate were mixed and washed 3 times with kinase buffer (10 mM Tris-HCl, pH 7.4, 10 mM $MgCl_2$, 1 mM DTT), then the reactions were incubated in kinase buffer with 10 mM ATP (Sangon Biotech; A600020-0005) at 30°C for 45 min. To inactivate Cdc2-as1, 5 μM 1-NM-PP1 (Toronto Research Chemicals; A603003) was added into the reaction. The reaction was stopped by boiling in SDS-PAGE loading buffer. Phosphorylated proteins were detected with rabbit polyclonal anti-pSer28/pThr31 antibodies.

Antibodies for immunoblotting

The phospho-site specific antibodies were made in an in-house facility (for anti-Slp1-pS28/pT31) or by ABclonal Technology Co., Ltd. (Wuhan, China) (for anti-Slp1-pT480) by immunizing rabbits with pS28/pT31- or pT480-containing peptides (LVFPN(S-p)PI(T-p)PLHQQ or PITK(T-p)PSSS, respectively) coupled to haemocyanin (Sigma). The purified antibodies were concentrated to 0.2 mg/mL and used at 1:100 dilution. The following antibodies used for immunoblot analyses were

purchased from the indicated commercial sources and were used at the indicated dilution: peroxidase-anti-peroxidase (PAP) soluble complex (Sigma-Aldrich; P1291; 1:10,000); rabbit polyclonal anti-Myc (GeneScript; A00172-40; 1:2,000); mouse monoclonal anti-GFP (Beijing Ray Antibody Biotech; RM1008; 1:2,000); rat monoclonal anti-HA (Roche, Cat. No. 11 867 423 001; 1:2,000); rabbit polyclonal anti-Slp1 (Kim et al., 1998) (1:500); rabbit polyclonal anti-phospho-p44/42 (detecting activated Pmk1) (Cell Signaling Technology; #9101; 1:1,000); rabbit monoclonal anti-phospho-p38 (Cell Signaling Technology; #4511; 1:1,000); rabbit polyclonal anti-PSTAIRE (detecting Cdc2) (Santa Cruz Biotechnology; sc-53; 1:1,000); rabbit monoclonal anti-Thiophosphate ester antibody (Abcam; ab239919; 1:5,000); goat anti-GST HRP-conjugated antibody (RRID:AB_771429; GE Healthcare; 1:10,000). Secondary antibodies used were goat anti-mouse or goat anti-rabbit polyclonal IgG (H+L) HRP conjugates (Thermo Fisher Scientific; #31430 or #32460; 1:10,000).

RT-qPCR

For RT-qPCR of *slp1*$^{+}$ mRNA, *nda3-KM311* strains were grown, synchronized by HU and arrested at metaphase with SAC being activated as described above. Cells were collected and total cellular RNA was isolated by using TRIZOL method. cDNA was prepared using oligo(dT) and PrimeScript reverse transcriptase (Takara, D2680A). Real-time PCR was performed in the presence of SYBR Green using an Applied Biosystems 7900HT light cycler. Relative RNA levels were calculated using the *ΔCT* method and normalized to *act1*$^{+}$ levels.

Fluorescence microscopy

Cdc13-GFP proteins were observed in cells after fixation with cold methanol. For DAPI staining of nuclei, cells were fixed with cold methanol, washed in PBS and resuspended in PBS plus 1 μg/ml DAPI. Photomicrographs of cells were obtained using a Nikon 80i fluorescence microscope coupled to a cooled CCD camera (Hamamatsu, ORCA-ER) or a Perkin Elmer spinning-disk confocal microscope (UltraVIEW® VoX) with a 100x NA 1.49 TIRF oil immersion objective (Nikon)

coupled to a cooled CCD camera (9100-50 EMCCD; Hamamatsu Photonics) and spinning disk head (CSU-X1, Yokogawa). Image processing and analysis were carried out using Element software (Nikon), ImageJ software (National Institutes of Health) and Adobe Photoshop.

Statistical analyses and reproducibility

No statistical methods were used to predetermine sample size. Sample size was as large as practicable for all experiments. The experiments were randomized and the investigators were blinded to group allocation during experiments but not blinded to outcome assessment and data analysis.

All experiments were independently repeated two to more than three times with similar results obtained. For quantitative analyses of each time course experiment, 300 cells were counted for each time point or sample. The same sample was not measured or counted repeatedly. No data were excluded from our studies. Data collection and statistical analyses were performed using Microsoft Office Excel or GraphPad Prism 6 softwares. Data are expressed as mean values with error bars (± standard deviation/s.d.) and were compared using two-tailed Student's *t*-tests unless indicated otherwise.

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Author Contributions Statement:

W.S., J.C., and C.J. performed all microscopy experiments with the help from Z.L.. L.S., X.C., W.S., C.S., L.L., S.B. and S.W. performed all immunoblotting, co-immunoprecipitation experiments, *in vitro* binding assays and *in vitro* kinase aassays. S.W. and X.W. performed large-scale protein purifications. X.C. and S.W. carried out genetic analyses. Q.J. and Y.W. conceived the study. Q.J. acquired funding. Y.W. and Q.J. designed the experiments and supervised the research. Y.W. and Q.J. wrote the paper with the inputs from Z.L..

Competing Interests Statement:

The authors declare no competing or financial interests.

Data availability statement

The authors confirm that all data supporting the findings of this study are available within the manuscript main figures, supplementary figures, supplementary tables and source data files. The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository with the dataset identifier PXD048800.

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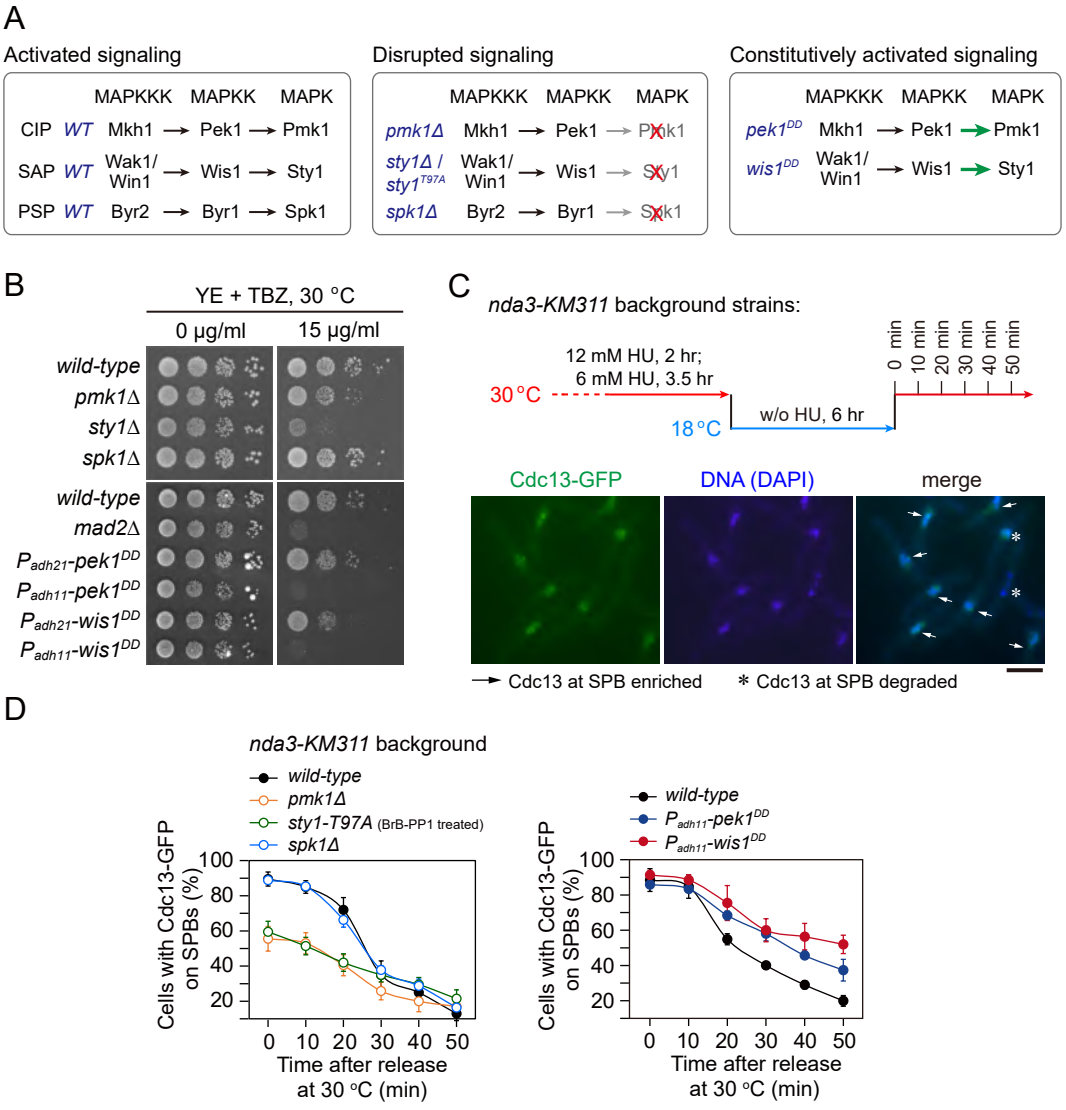
A
Activated signaling
MAPKKK MAPKK MAPK
CIP WT Mkh1 → Pek1 → Pmk1
SAP WT Wak1/ Win1 → Wis1 → Sty1
PSP WT Byr2 → Byr1 → Spk1
Disrupted signaling
MAPKKK MAPKK MAPK
pmk1Δ Mkh1 → Pek1 → Pmk1
sty1Δ / sty1T97A Wak1/ Win1 → Wis1 → Sty1
spk1Δ Byr2 → Byr1 → Spk1
Constitutively activated signaling
MAPKKK MAPKK MAPK
pek1DD Mkh1 → Pek1 → Pmk1
wis1DD Wak1/ Win1 → Wis1 → Sty1
B
YE + TBZ, 30 °C
0 μg/ml
15 μg/ml
wild-type
pmk1Δ
sty1Δ
spk1Δ
wild-type
mad2Δ
Padh21-pek1DD
Padh11-pek1DD
Padh21-wis1DD
Padh11-wis1DD
C
nda3-KM311 background strains:
12 mM HU, 2 hr;
6 mM HU, 3.5 hr
30 °C
18 °C
w/o HU, 6 hr
0 min
10 min
20 min
30 min
40 min
50 min
Cdc13-GFP
DNA (DAPI)
merge
Cdc13 at SPB enriched
* Cdc13 at SPB degraded
D
nda3-KM311 background
wild-type
pmk1Δ
sty1-T97A (BrB-PP1 treated)
spk1Δ
Cells with Cdc13-GFP on SPBs (%)
Time after release at 30 °C (min)
wild-type
Padh11-pek1DD
Padh11-wis1DD
Cells with Cdc13-GFP on SPBs (%)
Time after release at 30 °C (min)

Figure 1. *pmk1*Δ and *sty1-T97A* mutants display spindle checkpoint activation defects, while *pek1*^{DD} and *wis1*^{DD} mutants are defective in checkpoint silencing.

(**A**) Schematic of core modules of three *Schizosaccharomyces pombe* mitogen-activated protein kinase (MAPK) signaling pathways. Each cascade consists of three core kinases (MAP kinase kinase kinase (MAPKKK), MAP kinase kinase (MAPKK), and MAPK). CIP, the cell integrity pathway; SAP, the stress-activated pathway; PSP, pheromone signaling pathway. These pathways can be disrupted by *pmk1Δ*, *sty1Δ* or ATP analogue-sensitive mutation *sty1-T97A* or *spk1Δ*, and constitutively activated by mutations in MAPKKs Pek1 (*pek1*^{DD}, *pek1-S234D;T238D*) or Wis1 (*wis1*^{DD}, *wis1-S469D;T473D*) respectively.

(**B**) Serial dilution assay on TBZ sensitivity of all MAPK deletion mutants and *pek1*^{DD}- or *wis1*^{DD}-overexpressing mutants. *mad2*Δ is a positive control. Note P_{adh11} is a stronger version of P_{adh21} promoter.

(**C**) Schematic depiction of the experiment design for time-course analyses on SAC or APC/C activation. *nda3-KM311* cells carrying Cdc13-GFP were grown, synchronized with HU and treated at 18 ℃ for 6 hours to activate SAC, and finally shifted back to the permissive temperature 30 ℃. Samples were collected at 10 min intervals and subjected to microscopy analyses. Example pictures of cells with Cdc13-GFP signals enriched or disappeared at spindle pole bodies (SPBs) are shown. Scale bar, 5 μm.

(**D**) Time-course analyses of SAC activation and inactivation in *nda3-KM311 cdc13-GFP* strains with indicated genotypes. *sty1-T97A* was inactivated by 5μM 3-BrB-PP1.

Sun *et al*., Figure 2.

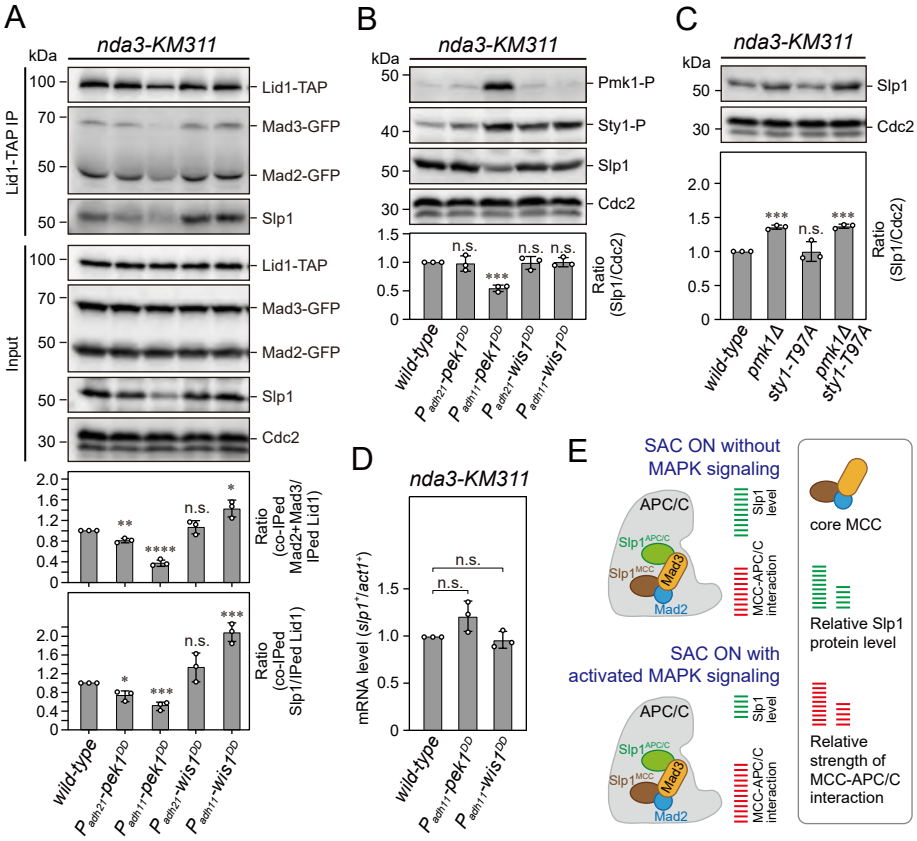

Figure 2. Upon spindle checkpoint activation, Slp1^{Cdc20} levels are reduced in *pek1^{DD}* and the MCC-APC/C association is enhanced in *wis1^{DD}* cells compared to wild-type cells.

(**A**) Co-immunoprecipitation analysis on MCC-APC/C association. Cells with indicated genotypes were grown at 30 ℃ to mid-log phase and arrested at 18 ℃ for 6 hours. Lid1-TAP was immunoprecipitated and associated Mad2, Mad3 and Slp1^{Cdc20} were detected by immunoblotting. The amount of co-immunoprecipitated Mad2, Mad3 and Slp1^{Cdc20} was quantified by being normalized to those of total immunoprecipitated Lid1 in each sample, with the relative ratio between Mad2-GFP plus Mad3-GFP or Slp1^{Cdc20} and Lid1-TAP in wild-type sample set as 1.0. Blots are representative of three independent experiments. *p* values were calculated against wild-type cells. *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$; ****, $p<0.0001$; n.s., not significant.

(**B, C**) Immunoblot analysis of Slp1^{Cdc20} abundance in *nda3-KM311* cells treated at 18 ℃ for 6 hr. Slp1^{Cdc20} levels were quantified with the relative ratio between Slp1^{Cdc20} and Cdc2 in wild-type strain set as 1.0. Phosphorylated Pmk1 (Pmk1-P) or phosphorylated Sty1 (Sty1-P) in (A) were detected using anti-phospho p42/44 and anti-phospho p38 antibodies and represents activated CIP or SAP signaling, respectively. *sty1-T97A* was inactivated by 5μM 3-BrB-PP1. Blots shown are the representative of three independent experiments. *p* values were calculated against wild-type cells. ***, $p<0.001$; n.s., not significant.

(**D**) RT-qPCR analysis of mRNA levels of *slp1*$^{+}$. Cells with indicated genotypes were grown and treated as in (A-C) before RNA extraction. The relative fold-change (*slp1*$^{+}$/*act1*$^{+}$) in mRNA expression was calculated with that in wild-type cells being normalized to 1.0. Note mRNA level of *slp1*$^{+}$ in *pek1^{DD}* mutant is not decreased. Error bars indicate mean ± standard deviation of three independent experiments. Two-tailed unpaired *t*-test was used to derive *p* values. n.s, not significant.

(**E**) Schematic summary of the negative effect of activated CIP and SAP signaling on APC/C activation based on primary phenotype characterization of *pmk1Δ*, *sty1-T97A*, *pek1^{DD}* and *wis1^{DD}* mutants.

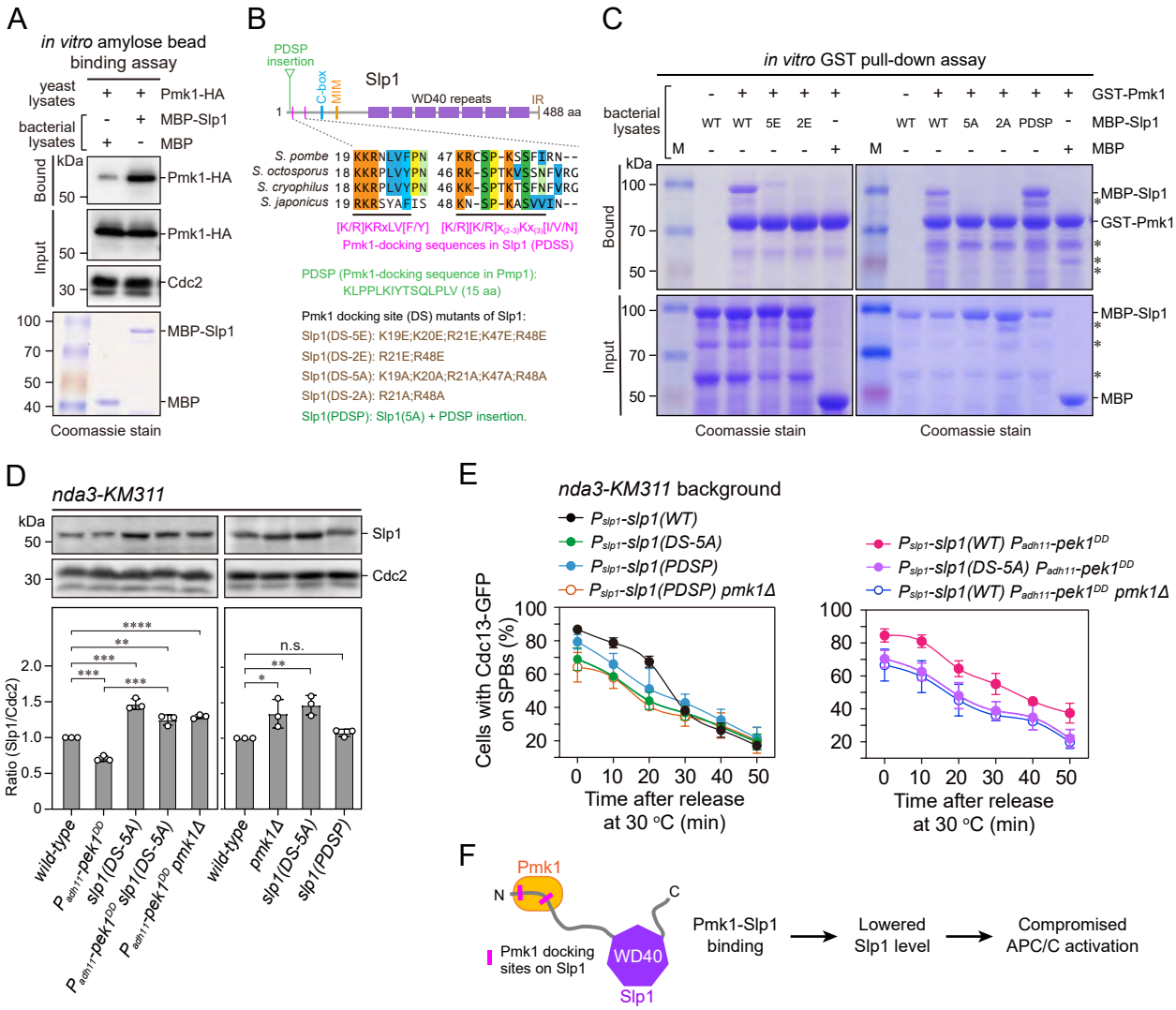
A
in vitro amylose bead binding assay
yeast lysates
Pmk1-HA
bacterial lysates
MBP-Slp1
MBP
Bound
Input
kDa
Pmk1-HA
Cdc2
MBP-Slp1
MBP
Coomassie stain
B
PDSP insertion
C-box
MIM
Slp1
WD40 repeats
IR
488 aa
S. pombe
S. octosporus
S. cryophilus
S. japonicus
[K/R]KRxLV[F/Y]
[K/R][K/R]x(2-3)Kx(3)[I/V/N]
Pmk1-docking sequences in Slp1 (PDSS)
PDSP (Pmk1-docking sequence in Pmp1):
KLPPLKIYTSQLPLV (15 aa)
Pmk1 docking site (DS) mutants of Slp1:
Slp1(DS-5E): K19E;K20E;R21E;K47E;R48E
Slp1(DS-2E): R21E;R48E
Slp1(DS-5A): K19A;K20A;R21A;K47A;R48A
Slp1(DS-2A): R21A;R48A
Slp1(PDSP): Slp1(5A) + PDSP insertion.
C
in vitro GST pull-down assay
bacterial lysates
GST-Pmk1
MBP-Slp1
MBP
WT
5E
2E
5A
2A
PDSP
Bound
Input
MBP-Slp1
GST-Pmk1
MBP
Coomassie stain
D
nda3-KM311
Slp1
Cdc2
Ratio (Slp1/Cdc2)
n.s.
wild-type
pmk1Δ
slp1(DS-5A)
slp1(PDSP)
E
nda3-KM311 background
Pslp1-slp1(WT)
Pslp1-slp1(DS-5A)
Pslp1-slp1(PDSP)
Pslp1-slp1(PDSP) pmk1Δ
Pslp1-slp1(WT) Padh11-pek1DD
Pslp1-slp1(DS-5A) Padh11-pek1DD
Pslp1-slp1(WT) Padh11-pek1DD pmk1Δ
Cells with Cdc13-GFP on SPBs (%)
Time after release at 30 °C (min)
F
Pmk1
N
C
Pmk1 docking sites on Slp1
WD40
Slp1
Pmk1-Slp1 binding
Lowered Slp1 level
Compromised APC/C activation

Figure 3. Pmk1 directly binds Slp1^{Cdc20} to attenuate its levels upon spindle checkpoint activation.

(**A**) *In vitro* binding assay using bacterially expressed MBP-Slp1^{Cdc20} and yeast lysates prepared from *nda3-KM311 pmk1-HA-6His* cells arrested at 18 ℃ for 6 hr. Note that weak band detected in MBP sample was due to unspecific background binding to amylose beads. Coomassie blue staining shows inputs for MBP and MBP-Slp1^{Cdc20}.

(**B**) Schematic depiction of the *S. pombe* Slp1^{Cdc20} protein structure with the positions of two confirmed basic-residue patches mediating Slp1^{Cdc20}-Pmk1 association indicated by pink bars. Alignment highlights the conservation of basic-residue patches within 4 *Schizosaccharomyces* species. The deduced Pmk1-docking motifs and different versions of motif mutations are shown. MIM, Mad2-interaction motif; IR, isoleucine-arginine tail.

(**C**) *In vitro* GST pull-down assays with bacterially expressed recombinant GST-Pmk1 and MBP-fusions of wild-type Slp1^{Cdc20} or Slp1^{Cdc20} mutants harboring Pmk1-docking motif mutations. An aliquot of the same amount of MBP-Slp1^{Cdc20} as that added in each GST pull-down reactions was immobilized by amylose resin as the input control. Asterisks indicate unspecific or degraded protein bands.

(**D**) Immunoblot analysis of Slp1^{Cdc20} abundance in *nda3-KM311* cells with indicated genotypes treated at 18 ℃ for 6 hr. Slp1^{Cdc20} levels were quantified as in Figure 2B and 2C. The experiment was repeated 3 times and the mean value for each sample was calculated. *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$; ****, $p<0.0001$; n.s., not significant.

(**E**) Time-course analyses of SAC activation and inactivation in *nda3-KM311 cdc13-GFP* strains with indicated genotypes. The experiment was repeated 3 times and the mean value for each sample was calculated as in Figure 1D.

(**F**) Schematic summarizing the negative effect of Pmk1-Slp1^{Cdc20} association on Slp1^{Cdc20} abundance and APC/C activation.

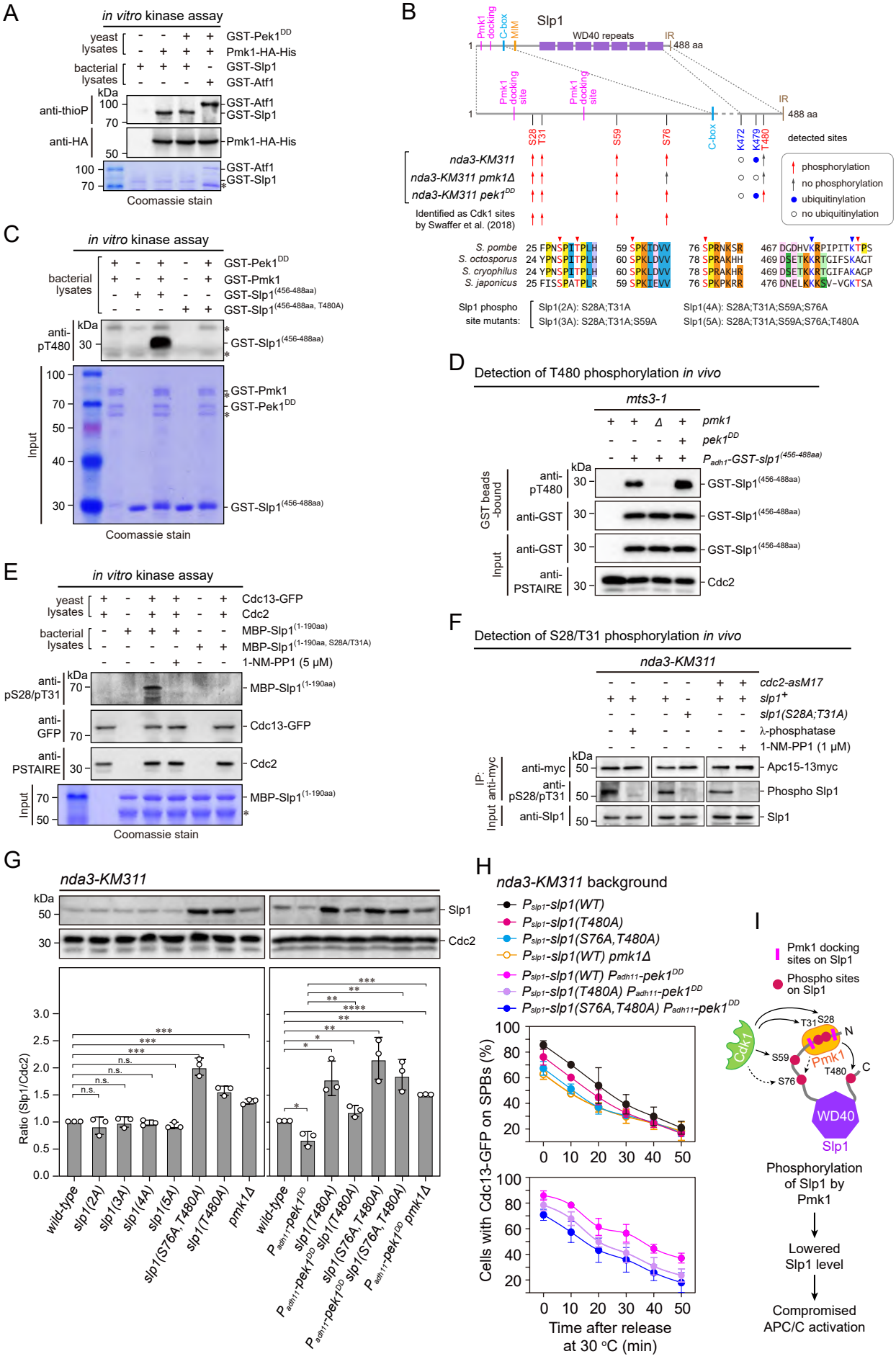
A
in vitro kinase assay
yeast lysates
GST-Pek1DD
Pmk1-HA-His
bacterial lysates
GST-Slp1
GST-Atf1
kDa
anti-thioP
anti-HA
GST-Atf1
GST-Slp1
Pmk1-HA-His
Coomassie stain
B
Slp1
Pmk1 docking
C-box
MIM
WD40 repeats
IR
488 aa
Pmk1 docking site
S28
T31
S59
S76
K472
K479
T480
detected sites
nda3-KM311
nda3-KM311 pmk1Δ
nda3-KM311 pek1DD
Identified as Cdk1 sites by Swaffer et al. (2018)
phosphorylation
no phosphorylation
ubiquitinylation
no ubiquitinylation
S. pombe
S. octosporus
S. cryophilus
S. japonicus
Slp1 phospho site mutants:
Slp1(2A): S28A;T31A
Slp1(3A): S28A;T31A;S59A
Slp1(4A): S28A;T31A;S59A;S76A
Slp1(5A): S28A;T31A;S59A;S76A;T480A
C
in vitro kinase assay
bacterial lysates
GST-Pek1DD
GST-Pmk1
GST-Slp1(456-488aa)
GST-Slp1(456-488aa, T480A)
anti-pT480
Input
GST-Pmk1
GST-Pek1DD
GST-Slp1(456-488aa)
Coomassie stain
D
Detection of T480 phosphorylation in vivo
mts3-1
pmk1
pek1DD
Padh1-GST-slp1(456-488aa)
GST beads -bound
anti-pT480
anti-GST
Input
anti-PSTAIRE
GST-Slp1(456-488aa)
Cdc2
E
in vitro kinase assay
yeast lysates
Cdc13-GFP
Cdc2
bacterial lysates
MBP-Slp1(1-190aa)
MBP-Slp1(1-190aa, S28A/T31A)
1-NM-PP1 (5 μM)
anti-pS28/pT31
anti-GFP
anti-PSTAIRE
Input
Coomassie stain
F
Detection of S28/T31 phosphorylation in vivo
nda3-KM311
cdc2-asM17
slp1+
slp1(S28A;T31A)
λ-phosphatase
1-NM-PP1 (1 μM)
IP: anti-myc
Input
anti-myc
anti-pS28/pT31
anti-Slp1
Apc15-13myc
Phospho Slp1
Slp1
G
nda3-KM311
Slp1
Cdc2
Ratio (Slp1/Cdc2)
wild-type
slp1(2A)
slp1(3A)
slp1(4A)
slp1(5A)
slp1(S76A,T480A)
slp1(T480A)
pmk1Δ
Padh11-pek1DD
slp1(T480A) slp1(T480A)
slp1(S76A,T480A) slp1(S76A,T480A)
Padh11-pek1DD pmk1Δ
H
nda3-KM311 background
Pslp1-slp1(WT)
Pslp1-slp1(T480A)
Pslp1-slp1(S76A,T480A)
Pslp1-slp1(WT) pmk1Δ
Pslp1-slp1(WT) Padh11-pek1DD
Pslp1-slp1(T480A) Padh11-pek1DD
Pslp1-slp1(S76A,T480A) Padh11-pek1DD
Cells with Cdc13-GFP on SPBs (%)
Time after release at 30 °C (min)
I
Pmk1 docking sites on Slp1
Phospho sites on Slp1
Cdk1
Pmk1
WD40
Slp1
Phosphorylation of Slp1 by Pmk1
Lowered Slp1 level
Compromised APC/C activation

Figure 4. Pmk1 synergizes with Cdk1 to phosphorylate and reduce Slp1^{Cdc20} abundance.

(**A**) Non-radioactive *in vitro* phosphorylation assays with bacterially expressed recombinant GST-Slp1^{Cdc20} and Pmk1-HA-His purified from yeast cells. The incorporation of the thiophosphate group was determined using anti-thiophosphate ester antibodies (anti-thioP) as indicative of phosphorylation. Note that the presence or absence of GST-Pek1^{DD} does not affect Slp1^{Cdc20} phosphorylation efficiency. A known Pmk1 substrate Atf1 was used as a positive control (Atf1-P). Asterisks indicate bands corresponding to unspecific or likely degraded proteins.

(**B**) Summary of mass spectrometry data on Slp1^{Cdc20} phosphorylation and ubiquitylation *in vivo* in *nda3-KM311*-arrested cells. Red arrows and filled blue circles denote all detected phosphorylated or ubiquitylated sites respectively, while gray arrows and unfilled circles indicate the absence of phosphorylation or ubiquitylation in some of these sites respectively. Alignment highlights the conservation of most of the detected phosphorylation and ubiquitylation sites within 4 *Schizosaccharomyces* species.

(**C**) *In vitro* phosphorylation assays with bacterially expressed recombinant GST-fusion of Slp1^{Cdc20} fragment (456-488aa), GST-Pmk1 and GST-Pek1^{DD}. The reactions were blotted with pT480 antibodies. Asterisks indicate bands corresponding to unspecific or likely degraded proteins.

(**D**) Immunoblot detection of Slp1^{Cdc20} phosphorylation at T480 *in vivo*. GST-slp1(456-488aa) was purified from *mts3-1* cells with indicated genotypes arrested at 36℃ for 3.5 hours, and detected with anti-GST and anti-pThr480 antibodies. Note that pThr480 is absent in *pmk1*Δ cells, and enhanced in *pek1^{DD}* cells relative to that in wild type cells.

(**E**) *In vitro* phosphorylation assays with bacterially expressed recombinant MBP-fusion of Slp1^{Cdc20} fragment (1-190aa) and Cdc13 (cyclin B)-containing Cdk1 complexes purified from metaphase-arrested *nda3-KM311* yeast cells. 1-NM-PP1 was added as inhibitor for analogue-sensitive Cdc2-as. The reactions were blotted with pS28/pT31 antibodies. Asterisks indicate bands corresponding to unspecific or likely degraded proteins.

(**F**) Immunoblot detection of Slp1^{Cdc20} phosphorylation at S28/T31 *in vivo*. Apc15-13myc was immunoprecipitated from *nda3-KM311* cells treated at 18 ℃ for 6 hr and the samples were blotted with pS28/pT31 antibodies. One IP sample from wild

type background was treated with λ-phosphatase. For *cdc2-asM17* cells, 1-NM-PP1 was added to inactivate Cdc2 during culturing.

(**G**) Immunoblot analysis of $Slp1^{Cdc20}$ abundance in *nda3-KM311* cells treated at 18 ℃ for 6 hr. $Slp1^{Cdc20}$ levels were quantified as in Figure 2B and 2C. The experiment was repeated 3 times and the mean value for each sample was calculated. *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$; ****, $p<0.0001$; n.s., not significant.

(**H**) Time-course analyses of SAC activation and inactivation in *nda3-KM311 cdc13-GFP* strains with indicated genotypes. The experiment was repeated 3 times and the mean value for each sample was calculated as in Figure 1D.

(I) Schematic summarizing the negative effect of $Slp1^{Cdc20}$ phosphorylation by Pmk1 on its abundance and APC/C activation.

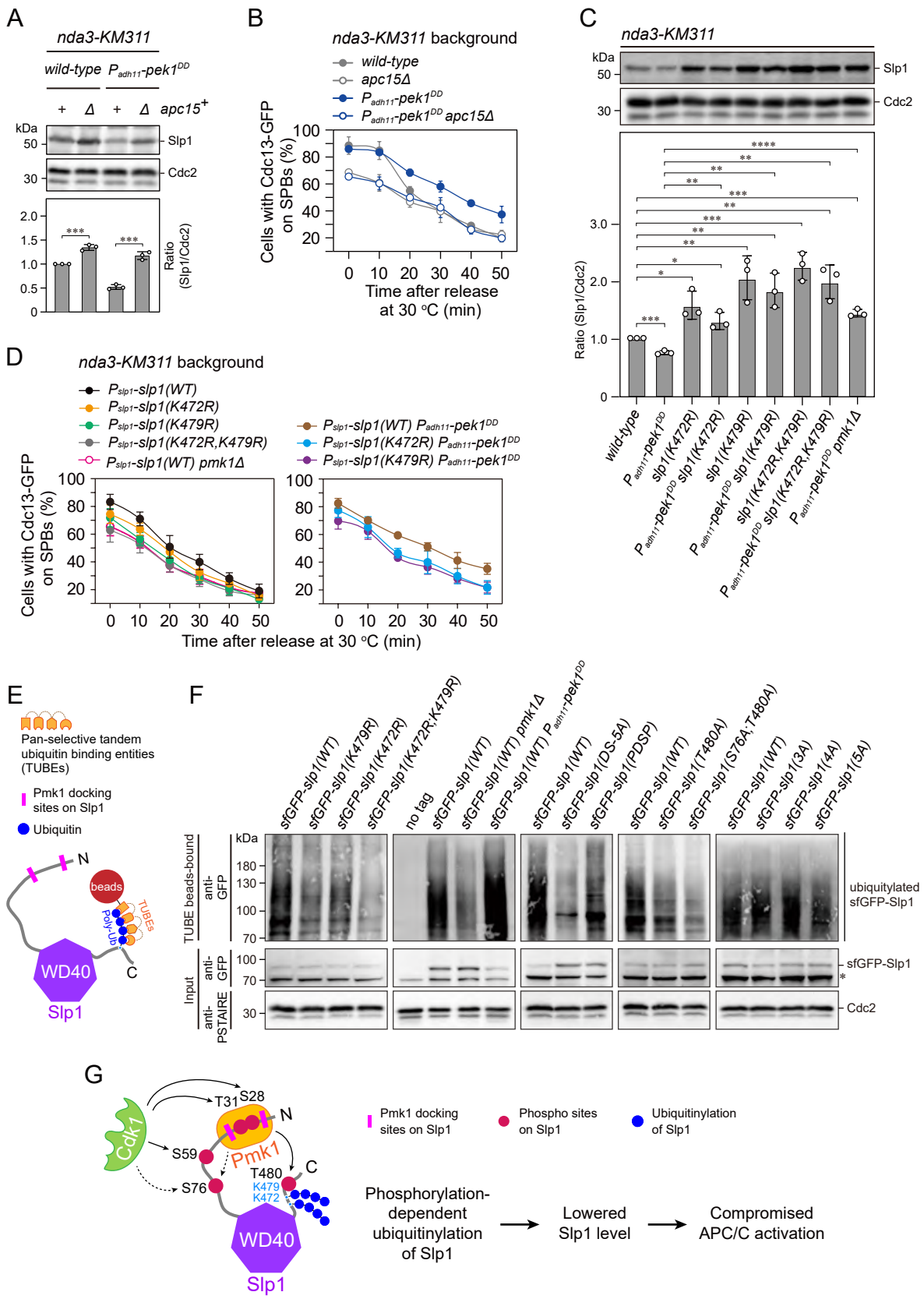
A
nda3-KM311
wild-type
Padh11-pek1DD
+ Δ + Δ apc15+
kDa
50
30
Slp1
Cdc2
2.0
1.5
1.0
0.5
0
***
***
Ratio (Slp1/Cdc2)
B
nda3-KM311 background
wild-type
apc15Δ
Padh11-pek1DD
Padh11-pek1DD apc15Δ
Cells with Cdc13-GFP on SPBs (%)
100
80
60
40
20
0 10 20 30 40 50
Time after release at 30 °C (min)
C
nda3-KM311
kDa
50
30
Slp1
Cdc2
3.0
2.0
1.0
0
Ratio (Slp1/Cdc2)
wild-type
Padh11-pek1DD
slp1(K472R)
Padh11-pek1DD slp1(K472R)
slp1(K479R)
Padh11-pek1DD slp1(K479R)
slp1(K472R,K479R)
Padh11-pek1DD slp1(K472R,K479R)
Padh11-pek1DD pmk1Δ
D
nda3-KM311 background
Pslp1-slp1(WT)
Pslp1-slp1(K472R)
Pslp1-slp1(K479R)
Pslp1-slp1(K472R,K479R)
Pslp1-slp1(WT) pmk1Δ
Pslp1-slp1(WT) Padh11-pek1DD
Pslp1-slp1(K472R) Padh11-pek1DD
Pslp1-slp1(K479R) Padh11-pek1DD
Cells with Cdc13-GFP on SPBs (%)
Time after release at 30 °C (min)
E
Pan-selective tandem ubiquitin binding entities (TUBEs)
Pmk1 docking sites on Slp1
Ubiquitin
N
beads
TUBEs
Poly-Ub
WD40
C
Slp1
F
sfGFP-slp1(WT)
sfGFP-slp1(K479R)
sfGFP-slp1(K472R)
sfGFP-slp1(K472R,K479R)
no tag
sfGFP-slp1(WT)
sfGFP-slp1(WT) pmk1Δ
sfGFP-slp1(WT) Padh11-pek1DD
sfGFP-slp1(WT)
sfGFP-slp1(DS-5A)
sfGFP-slp1(PDSP)
sfGFP-slp1(WT)
sfGFP-slp1(T480A)
sfGFP-slp1(S76A,T480A)
sfGFP-slp1(WT)
sfGFP-slp1(3A)
sfGFP-slp1(4A)
sfGFP-slp1(5A)
TUBE beads-bound
anti-GFP
kDa
180
130
100
70
ubiquitylated sfGFP-Slp1
Input
anti-GFP
100
70
sfGFP-Slp1
*
anti-PSTAIRE
30
Cdc2
G
Cdk1
T31
S28
N
S59
Pmk1
T480
C
S76
K479
K472
WD40
Slp1
Pmk1 docking sites on Slp1
Phospho sites on Slp1
Ubiquitinylation of Slp1
Phosphorylation-dependent ubiquitinylation of Slp1
Lowered Slp1 level
Compromised APC/C activation

Figure 5. Pmk1- but not Cdk1-mediated Slp1^{Cdc20} phosphorylation promotes its ubiquitylation.

(**A**) Immunoblot analysis of Slp1^{Cdc20} abundance in *apc15*$^{+}$ or *apc15*Δ background cells with indicated genotypes after being treated at 18 ℃ for 6 hr. Slp1^{Cdc20} levels were quantified as in Figure 2B. The experiment was repeated 3 times and the mean value for each sample was calculated. ***, $p<0.001$.

(**B**) Time-course analyses of SAC activation and inactivation in *nda3-KM311 cdc13-GFP* strains with indicated genotypes. The experiments were performed as in Figure 1D.

(**C**) Immunoblot analysis of Slp1^{Cdc20} abundance in K472R, K479R or K472R/K479R mutants. The experiment was repeated 3 times and the mean value for each sample was calculated. *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$; ****, $p<0.0001$.

(**D**) Time-course analyses of SAC activation and inactivation in *nda3-KM311 cdc13-GFP* strains with K472R, K479R or K472R/K479R mutations. The experiments were performed as in (B).

(E) Schematic depiction of affinity pull-down assays using TUBE (Tandem Ubiquitin Binding Entity) agarose beads to detect Slp1^{Cdc20} ubiquitylation.

(F) TUBE pull-down assays in *mts3-1* strains carrying sfGFP-tagged wild type or mutants of Slp1^{Cdc20} or *pmk1Δ* or *pek1*^{DD} mutations. The TUBE beads-bound samples were blotted with anti-GFP antibodies. Asterisk indicate unspecific bands recognized by anti-GFP antibodies.

(G) Schematic summarizing the negative effect of the coupling of Pmk1 phosphorylation and K472/K479-mediated ubiquitylation on Slp1^{Cdc20} abundance and APC/C activation.

Sun *et al*., Figure 6.

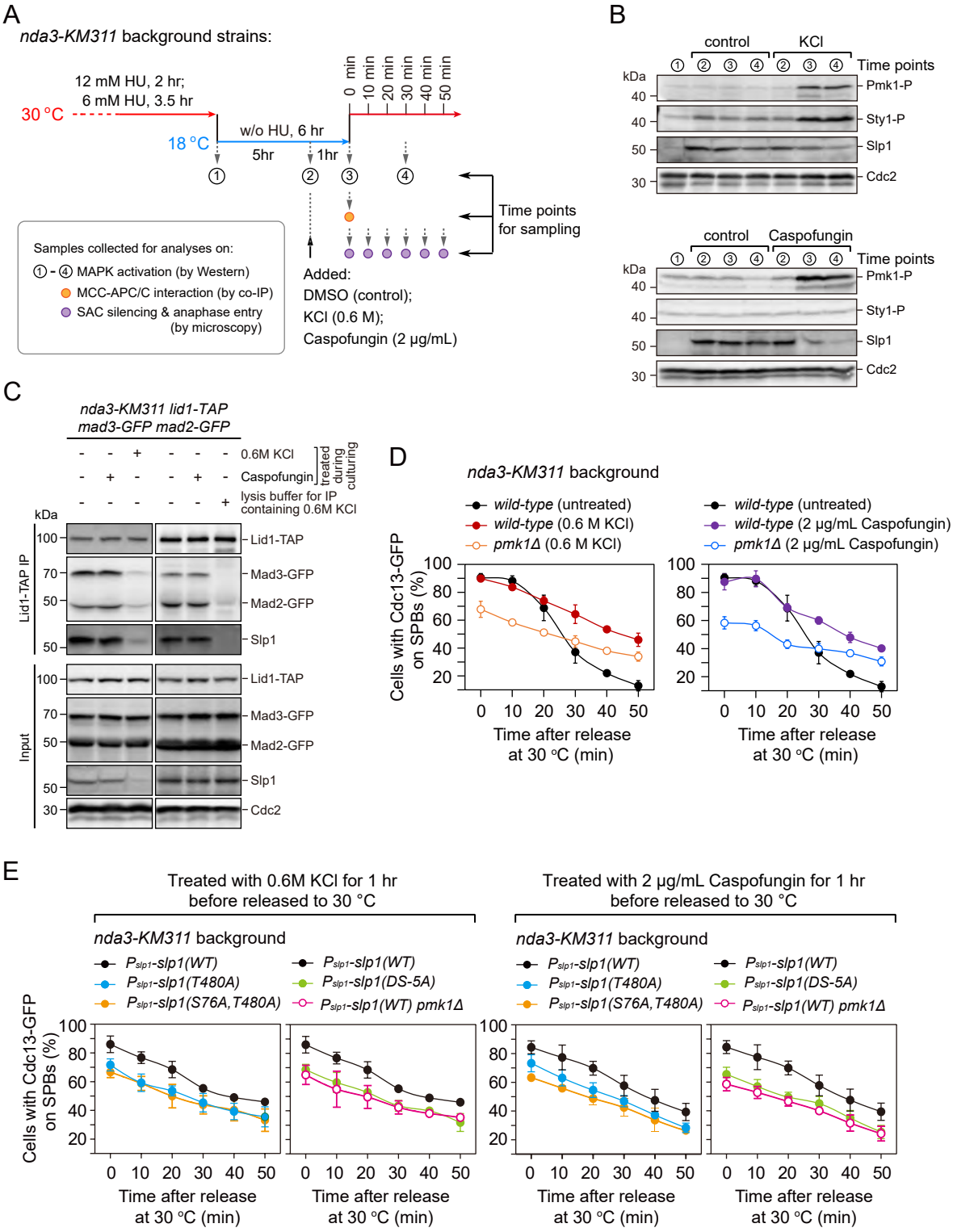

Figure 6. Osmotic stress and cell wall damage trigger rapid Pmk1 phosphorylation-dependent Slp1^{Cdc20} downregulation and mitotic exit delay.

(**A**) Schematic depiction of the experimental design for treatment with KCl or caspofungin during *nda3*-mediated spindle checkpoint activation to activate MAPKs. Samples were collected at indicated time points for subsequent analyses including immunoblotting, co-IP and time-course analysis on SAC or APC/C activation.

(**B**) Immunoblot analysis of activation of MAPKs and Slp1^{Cdc20} protein levels. Samples with or without indicated treatments were blotted with anti-phospho p42/44 and anti-phospho p38 antibodies as indicative of phosphorylated Pmk1 (Pmk1-P) or phosphorylated Sty1 (Sty1-P), respectively. Slp1^{Cdc20} levels were detected with anti-Slp1 antibodies and anti-Cdc2 was used as loading control.

(**C**) Co-immunoprecipitation analysis of APC/C-MCC association upon environmental stress. Lid1-TAP was immunoprecipitated from *nda3-KM311*-arrested cells and associated Mad2-GFP, Mad3-GFP and Slp1^{Cdc20} were detected as in Figure 2A. Note that APC/C-MCC association was disrupted when 0.6 M KCl was present during cell culturing or during immunoprecipitation procedures.

(**D**) Time-course analyses of SAC activation and inactivation in *nda3-KM311 cdc13-GFP* strains with indicated genotypes after arrest at 18 ℃ and KCl or caspofungin treatments.

(**E, F**) Immunoblot analyses of Slp1^{Cdc20} abundance and time-course analyses of SAC activation and inactivation efficiency in phosphorylation- and ubiquitylation-deficient mutants upon SAC activation and environmental stress.

Sun *et al*., Figure 7.

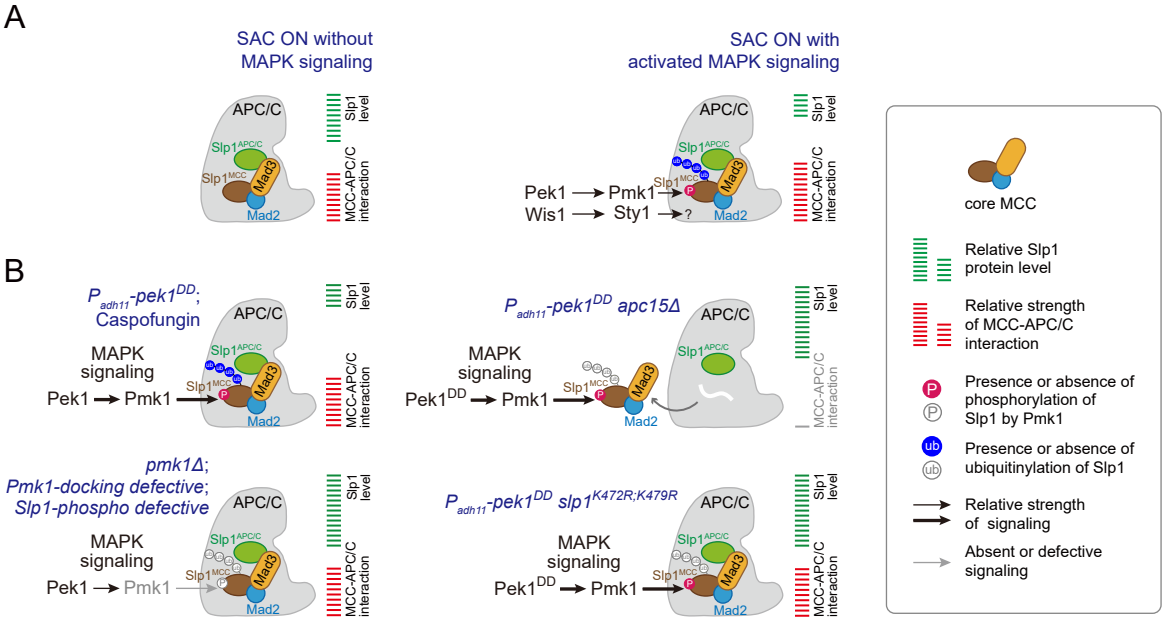

Figure 7. Summary of the mechanisms that how MAPKs negatively regulate APC/C activity in fission yeast.

(**A**) Schematic depiction of a possible dual mechanism that how activated MAPK signaling pathways are involved in delaying APC/C activation and SAC inactivation. Upon SAC and MAPK signaling activation, division of labor between the CIP and the SAP pathways enables the phosphorylation of $Slp1^{Cdc20}$ and unidentified substrate(s) by Pmk1 and Sty1 respectively, which leads to lowered $Slp1^{Cdc20}$ level and enhanced MCC affinity for APC/C.

(**B**) Summary of the alteration of $Slp1^{Cdc20}$ protein levels and MCC-APC/C association strength under various conditions examined in this study. White wavy line indicates the absence of Apc15.