Published in final edited form as:
Curr Biol. 2013 October 7; 23(19): 1920–1926. doi:10.1016/j.cub.2013.07.077.

CK1 is required for a mitotic checkpoint that delays cytokinesis

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Summary

Failure to accurately partition genetic material during cell division causes aneuploidy and drives tumorigenesis [1]. Cell cycle checkpoints safeguard cells from such catastrophes by impeding cell cycle progression when mistakes arise. FHA-RING E3 ligases, including human RNF8 [2] and CHFR [3] and fission yeast Dma1 [4], relay checkpoint signals by binding phosphorylated proteins via their FHA domains and promoting ubiquitination of downstream targets [5]. Upon mitotic checkpoint activation, *S. pombe* Dma1 concentrates at spindle pole bodies (SPBs) in an FHA-dependent manner and ubiquitinates Sid4, a scaffold of Polo kinase, to suspend cytokinesis [6]. However, the kinase(s) that phospho-prime Sid4 for Dma1-mediated ubiquitination are unknown. Here, we report that the highly conserved protein kinase CK1 transmits the signal necessary to stall cytokinesis by phospho-priming Sid4 for Dma1-mediated ubiquitination. Like Dma1, CK1 accumulates at SPBs during a mitotic arrest and associates stably with SPB components, including Sid4. Our results establish CK1 as an integral component of a mitotic, ubiquitin-mediated checkpoint pathway.

Results and Discussion

Sid4 phosphorylation on T275 recruits Dma1 via its FHA domain

Dma1 executes its checkpoint function by inhibiting the septation initiation network (SIN) [7], a *hippo* related signaling pathway that triggers cytokinesis after mitosis [8]. The SIN pathway resides at SPBs and is activated by the Polo kinase Plo1 [9, 10], which is recruited to the SIN via interaction with Sid4 [11]. We showed previously that Dma1-mediated ubiquitination of Sid4 obstructs Plo1's SPB localization, in a degradation independent mechanism, and thereby prevents Plo1 activation of the SIN [6, 7]. However, the upstream signals that control Dma1 function at SPBs are undefined.

Many checkpoint proteins harbor a phospho-dependent protein-protein interaction domain coupled to a catalytic domain, affording them the ability to modify substrates in a stimulus-dependent manner [12]. Because *S. pombe* Dma1 requires its phospho-threonine binding

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FHA domain to localize to SPBs and the cell division site [7], we surmised that Dma1-Sid4 interaction depends on the phospho-status of Sid4. Thus, we examined the SDS-PAGE mobility of Sid4 in checkpoint-activated cells using the cold-sensitive β-tubulin mutant *nda3*-KM311 [13]. In *dma1*$^{+}$ cells, many slower migrating Sid4 isoforms were detected, which collapsed into a discrete ladder upon phosphatase treatment (Figure 1A, lanes 1 and 2). These bands are ubiquitinated isoforms because they collapse into a single band in the absence of *dma1*$^{+}$ (Figure 1A, lane 4) and Dma1 is required for Sid4 ubiquitination [6]. In *dma1*Δ cells, a single slower migrating form of Sid4 was detected, which was collapsed by phosphatase treatment, indicating that Sid4 is phosphorylated in vivo (Figure 1A, lanes 3 and 4). In vivo radiolabeling experiments validated Sid4 as a phospho-protein and revealed that Sid4 is phosphorylated on serines and threonines (Figure S1A–C). The constitutive presence of an unmodified Sid4 isoform indicates that only a subpopulation of Sid4 is modified (Figure 1A). Collectively, these data indicate that Sid4 is ubiquitinated and phosphorylated in vivo.

Sid4 was hyper-phosphorylated in cells arrested in mitosis with an active spindle checkpoint (*nda3-KM311*) compared to all other cell cycle arrests (Figure 1B). Notably, Sid4 was not hyper-phosphorylated to the same extent in *mts3-1* mutants [14], which arrest in metaphase do to a proteasome defect (Figure 1B), suggesting that some Sid4 phosphorylation is specific to spindle checkpoint activity. To identify Sid4 phospho-site(s) required for Dma1 interaction, we employed a targeted mutagenesis approach. Because Dma1 interacts with the N-terminus of Sid4 (aa1-300) [7] and Dma1's FHA domain is predicted to bind phosphorylated threonines, we performed alanine scanning of the 15 threonines in Sid4's N-terminus, substituting *sid4* mutant alleles for the endogenous gene at its native locus (Figure S1D). T275, a site that is conserved in other *Schizosaccharomyces* species (Figure 1C), was the only threonine required for Sid4 ubiquitination (Figure 1D and S1E).

FHA domain binding studies indicate that residues in the pT+3 position contribute to FHA binding specificity [15]. Although mutating S278 to alanine abolished Sid4 ubiquitination (Figure 1D), mutating S278 to a glutamate did not affect Sid4 ubiquitination (Figure 1D). This indicates that a negative charge in the pT+3 position is required to stabilize Dma1 interaction with T275-phosphorylated Sid4 and thus S278 must also be phosphorylated. Mutating residues immediately adjacent to T275 and S278 did not affect Sid4 ubiquitination, implying that T275 and S278 are the only residues critical for this event (Figure 1E). Although Dma1-GFP still localized to SPBs in *sid4(T275A)* mutant cells (Figure S1F), combining *sid4(T275A)* with a mutation in the second SIN scaffold, *cdc11-123*, eliminated Dma1-GFP SPB localization, though each individual mutant did not affect its localization (Figure S1F–G). These data indicate that Dma1 has at least two binding partners at SPBs and that mutation of T275 on Sid4 specifically abrogates Dma1-Sid4 interaction.

Because T275 and S278 are necessary for Sid4 ubiquitination, we examined whether phosphorylation of these two sites was sufficient to foster binding to Dma1's FHA domain. Sid4 phospho-peptides spanning the putative Dma1-binding region (aa271-282) were incubated with a recombinant Dma1 fragment containing the FHA domain (aa 1-143; His-FHA) and tested for their ability to interact. While the unphosphorylated peptide, or peptides phosphorylated on either T275 or S278 alone, did not support His-FHA binding, a peptide

with both T275 and S278 phosphorylated (pT275, pS278) bound the His-FHA fragment (Figure 1F). This interaction requires a functional FHA domain because a mutation in the FHA domain (R64A) predicted to disrupt interaction with the phosphorylated target site abolished the association (Figure 1F). A phospho-mimetic peptide (T275E, S278E) did not bind the FHA domain, indicating that negatively charged amino acids do not effectively mimic phosphorylation in this context (Figure S1H). This is consistent with our finding that Sid4(T275E) mutants are not ubiquitinated in vivo (Figure S1E). Thus, phosphorylation on both T275 and S278 is necessary and sufficient to support binding of the Dma1 FHA domain to Sid4 and Sid4 ubiquitination.

Sid4 phosphorylation on T275 and S278 is required for the Dma1-dependent checkpoint

Given that phosphorylation of T275 and S278 recruits Dma1 to Sid4 for subsequent Sid4 ubiquitination, we examined whether phosphorylation of these sites was stimulated in response to spindle checkpoint activation. To detect phosphorylation of T275 and S278 in vivo, we generated a phospho-specific antibody to these two phospho-sites (pT275, S278) (Figure 2A). Indeed, phosphorylation of these sites was detected at increased levels in checkpoint-stimulated cells (*nda3-KM311*) compared to cells growing asynchronously or cells arrested in mitosis in the absence of a spindle checkpoint (*mts3-1*) (Figure 2B). Thus, Sid4 phosphorylation on T275 and S278 is stimulated in response to a mitotic checkpoint.

To examine whether the checkpoint was negatively affected in *sid4(T275A)* mutants as we would predict, *nda3-KM311* cells were synchronized in S-phase with hydroxyurea treatment, released to 19°C to activate the mitotic checkpoint and monitored for their ability to maintain the arrest. *nda3-KM311* cells held a checkpoint arrest for 6-7 hrs, whereas *nda3-KM311 sid4(T275A)* cells bypassed the arrest after 5 hrs (Figure 2C). This is comparable to *nda3-KM311 dma1*Δ cells, which also evaded the arrest after 5 hrs. Importantly, *nda3-KM311 sid4(T275A) dma1*Δ cells did not exhibit additive defects, indicating that both mutations function in the same pathway (Figure 2C). These data indicate that mutating T275 eliminates Dma1-dependent checkpoint signaling.

In corroboration of these findings, *sid4(T275A)* mutants were refractory to *dma1* over-expression lethality (Figure 2D). Furthermore, *sid4(T275A)* mutants were synthetically sick with mutation of another SIN inhibitor *cdc16-116* and suppressed the temperature-sensitive lethality of positive SIN regulator mutants *spg1-106, sid2-250, cdc11-136, plo1-1* and *plo1-24c* (Figure S2). These genetic data imply that eliminating T275 phosphorylation produces a hypermorphic *sid4* allele due to loss of cytokinesis inhibition by Dma1-mediated ubiquitination.

Dma1 functions upstream of the SIN inhibitor Cdc16, whose *S. cerevisiae* homolog Bub2 functions in a mitotic checkpoint independently of the kinetochore-based proteins: Mad1-3, Bub1 and Bub3 [16–19]. Over-expression of the kinetochore-based SAC activator, *mph1*, does not drive Dma1 to SPBs (data not shown), implying that Dma1 similarly functions in a mitotic checkpoint pathway independent of Mad1-3, Bub1 and Bub3. To test this, we compared the checkpoint defects of *sid4(T275A)* and *dma1*Δ mutants to *mad2*Δ mutants. *mad2*Δ cells bypassed the checkpoint arrest with similar kinetics as both *dma1*Δ and *sid4(T275A)* and a double *dma1*Δ *mad2*Δ mutant displayed an additive checkpoint defect

phenotype (Figure 2C). Thus, similar to *S. cerevisiae*, a mitotic checkpoint exists in *S. pombe*, which is dependent on *dma1*, but independent of Mad1-3, Bub1 and Bub3.

CK1 is required for Sid4 ubiquitination and associates with the SIN pathway during a mitotic checkpoint

To identify the protein kinase(s) directing Dma1-Sid4 interaction, we screened a comprehensive non-essential protein kinase deletion collection [20] for loss of Sid4 ubiquitination and found that deleting any single kinase did not abolish Sid4 ubiquitination (Figure S3A). Similarly, we screened all available essential temperature-sensitive or analog-sensitive kinase mutants and did not identify any that eliminated Sid4 ubiquitination (Figure S3B). Finally, we screened several multi-kinase deletions based on sequence homology within their kinase domains [20], as these should have similar phosphorylation consensus sites (Figure S3C). The results of this screen indicated that only *S. pombe* CK1 homologs, *hhp1* and *hhp2*, are required for Sid4 ubiquitination (Figure 3A).

CK1 is a conserved kinase important for many cellular processes including cell proliferation and chromosome segregation [21]. Human CK1δ/ε, which are most related to Hhp1/2, localize to centrosomes and inhibition of these two isoforms results in aberrant mitoses [22]. In cells growing asynchronously, both Hhp1-GFP and Hhp2-GFP localized to the nucleus, SPBs, and the cell division site, although Hhp2-GFP was more prominent at the division site compared to Hhp1-GFP (Figure 3B). Hhp1-GFP localization varied in different cell cycle arrests (Figure S3D), implying that Hhp1-GFP localization is dynamic. When the checkpoint was activated, Hhp1-GFP and Hhp2-GFP localized predominantly to SPBs (Figure 3C), a pattern that mirrors Dma1 localization [7].

Consistent with the localization analyses, Hhp1-HA_3-TAP and Hhp2-HA_3-TAP co-purified several SPB proteins from checkpoint-activated cells, including many SIN proteins (Figure 3D). When purified from *nda3-KM311 hhp1*Δ cells, Hhp2-HA_3-TAP co-purified more SPB proteins compared to *nda3-KM311 hhp1*$^+$ cells (Figure 3D), suggesting that Hhp1 is the dominant kinase at SPBs, but Hhp2 may compensate if Hhp1 is absent. Hhp1-GFP localization at SPBs is Sid4 independent (Figure S3E), but requires Ppc89, a protein that tethers Sid4 at SPBs (Figure S3F). Interestingly, human CK1δ/ε is tethered to centrosomes by the scaffold protein CG-NAP [23], which forms a complex with another centrosomal scaffold protein Kendrin [24], a putative Sid4 homolog. Thus, CK1's centrosomal tethering mechanism may be conserved.

Since CK1 localizes in the nucleus and SPBs, it is in a prime position to transmit signals from the nucleus to SPBs. Accordingly, Hhp1-GFP became less pronounced at SPBs and re-accumulated in the nucleus as *nda3-KM311* cells released from a checkpoint block (Figure 3E). This prompted us to examine whether Hhp1's strong mitotic SPB localization depended on spindle checkpoint activation. Indeed, compared to mitotic cells with no checkpoint activation, Hhp1-GFP intensity at SPBs was significantly higher when a mitotic checkpoint was activated (Figure 3F–G). These data indicate that Hhp1/2 display dynamic localization patterns and accumulate at SPBs in response to spindle checkpoint activation.

CK1 phospho-primes Sid4 for Dma1-mediated ubiquitination

The canonical CK1 consensus sequence is p(S/T)X_{1-2}(S/T) and a negatively charged amino acid can sometimes substitute for the N-terminal phospho-amino acid [21]. However, CK1 does not always require N-terminally phosphorylated or acidic amino acids [25–27]. Because CK1 is required for Sid4 phosphorylation on T275 and S278 in vivo (Figure 4A), we examined whether CK1 directly phosphorylates these sites in vitro. Full length Sid4-myc_6 was produced through an in vitro transcription/translation system and phosphorylated by recombinant CK1δ, which we found to have the same specificity toward Sid4 as Hhp1/2 (data not shown). CK1 phosphorylation of Sid4-myc_6 was detected with the phospho-T275, S278 antibody, indicating that CK1δ directly phosphorylates one or both sites (Figure 4B). As expected, Sid4(T275A, S278A)-myc_6 incubated with CK1δ was not detected by the phospho-T275, S278 antibody (Figure 4B). Sid4(T275A)-myc_6 and Sid4(S278A)-myc_6 single mutants incubated with CK1δ were detected by the phospho-antibody, albeit to a lesser extent, indicating that CK1 phosphorylates both sites in vitro (Figure 4B).

Surprisingly, we found that whereas CK1 phosphorylated Sid4 on T275 and S278 in the context of the full-length protein, it did not phosphorylate these sites when the C-terminus was removed (Figure S4A–C). Accordingly, a Sid4N-Ppc89C fusion that lacks Sid4's endogenous C-terminus and was not ubiquitinated in vivo [6] is also not phosphorylated on T275 or S278 (Figure S4D). Thus, the Sid4 C-terminus is required for N-terminal CK1-mediated phosphorylation. Although atypical, a tertiary structural requirement for CK1-mediated phosphorylation was previously described [25]. Specifically, CK1ε phosphorylates full-length SV40 large T-antigen on two sites in the N-terminus of T-antigen, but does not phosphorylate a C-terminally truncated mutant even though it retains these sites [26, 27]. Thus, similar to CK1ε phosphorylation of T-antigen, CK1 recognizes its target sites on Sid4 through an unconventional mechanism requiring non-linear elements of the protein's structure.

Because CK1 phosphorylates Sid4 on T275 and S278 directly, we next examined whether CK1 was required for the Dma1-dependent checkpoint. Because *hhp1-as hhp2* mutants exhibit a significant delay in G2 due to unrelated cell cycle defects, we were precluded from examining their mitotic checkpoint competency directly. However, *hhp1-as hhp2* cells were refractory to *dma1* over-expression (Figure 4C), suggesting that CK1 is required for Dma1-dependent signaling. Furthermore, Dma1-GFP localization at SPBs in checkpoint-activated cells is dependent on *hhp1/2*(Figure 4D). Collectively, these data establish CK1 as the major proximal upstream signaling component that recruits Dma1 to Sid4 during a mitotic checkpoint.

Conclusions

We have discovered a novel function of the highly conserved protein kinase CK1 in the Dma1 signaling pathway that delays cytokinesis when cells encounter mitotic stress. Our data support a model (Figure 4E) wherein *S. pombe* CK1-mediated phosphorylation of Sid4 generates a binding motif (pTXXpS) that recruits Dma1 via its phospho-threonine binding FHA domain. Subsequently, Dma1 ubiquitinates Sid4 to antagonize Plo1 recruitment and consequently prevents Plo1 from activating the SIN and cytokinesis [6, 7].

In the DNA damage response pathway, ATM and ATR are the major sensors for DNA damage and phosphorylation of their targets recruit downstream checkpoint proteins and repair factors that amplify the checkpoint response [28]. Similarly, several protein kinases including Mps1, Bub1 and Aurora B have been implicated in sensing microtubule-kinetochore attachments and recruiting spindle checkpoint proteins to kinetochores to inhibit the anaphase promoting complex/cyclosome (APC/C) [29]. Our identification of CK1 as an upstream activator of the Dma1-dependent checkpoint pathway is the first insight into a mitotic molecular sensor for the FHA-RING E3 ligase family. It was recently discovered that CK1δ phospho-primes a viral E3 ligase, which binds and hijacks RNF8 away from its cellular target MDC1 [30]. Taken together, these studies might represent a paradigm for CK1 phosphoregulation of FHA-RING E3 ligase targeting.

We have also established CK1 and Dma1 as components of a mitotic checkpoint that operates in parallel to Mad1-3, Bub1 and Bub3. In contrast to these kinetochore-based SAC components that monitor successful MT-chromosome attachments, it has been proposed that the Bub2/Cdc16-dependent checkpoint, which is based at SPBs, monitors chromosome segregation by sensing MT-SPB tension or kinetochore-SPB interactions [16–19]. CK1 is ideally situated in the nucleus and at SPBs to detect MT-SPB attachments/tension and transmit signals to SPBs, where Dma1 executes its checkpoint function. Because human CK1δ/ε also localizes in the nucleus and at centrosomes [31], our identification of CK1 as a prospective sensor of the Bub2/Cdc16-dependent checkpoint pathway could help to reveal the mechanical and/or biochemical signals that trigger a centrosome-based SAC pathway in multiple organisms.

Experimental procedures

Yeast methods

Yeast strains (Table S2) were grown in yeast extract media supplemented with appropriate amino acids [25]. For over-expression studies, cells harboring the pREP41 plasmid were first grown in minimal media containing thiamine and then in minimal media lacking thiamine to induce expression.

S. pombe protein methods

Immunoprecipitation experiments were performed as previously described [7], except that cells were lysed using a Fastprep© cell homogenizer (MP biomedicals). The Sid4 anti-serum was raised against recombinant GST-Sid4(1-300) (Cocalico). GFP antibody was purchased from Roche and fluorescently labeled streptavidin and all secondary antibodies were from Licor. Lambda phosphatase (New England Biolabs) treatment of immunoprecipitated proteins was performed in 25 mM HEPES-NaOH pH 7.4, 150 mM NaCl, and 1 mM $MnCl_2$ for 45 minutes at 30°C.

In vitro binding assay

5 μm of synthetic biotinylated phospho-peptides (Genscript) were conjugated to streptavidin beads. Recombinant His-FHA was produced in bacteria and purified on Ni^{2+}-charged His-bind resin (Novagen) using the manufacturer's recommendation. Proteins were eluted and

0.5 μg was used in each binding reaction. Binding was performed in column buffer (20mM Tris, 200mM NaCl, 1mM EDTA, 1mM DTT, 0.1% NP-40, pH 7.4) for 1hr at 4°C, washed 3× with column buffer and bound proteins were detected on an immunoblot.

In vitro kinase assays

Sid4-myc$_6$ (pCS2FA2R-Sid4-myc$_6$) was produced using a TNT® quick coupled in vitro transcription/translation reticulocyte system (Promega) and immunoprecipitated with a myc antibody (9E10). Immunoprecipitated proteins were left on beads and washed in CK1 reaction buffer (50 mM Tris pH 7.5, 10 mM $MgCl_2$, and 5 mM DTT). Washed beads were incubated with 10uM ATP and recombinant CK1δ (New England Biolabs) in CK1 kinase buffer (50 mM Tris pH 7.5, 10 mM $MgCl_2$, and 5 mM DTT) at 30°C for 30 min. Peptides were separated on a 4–12% polyacrylamide gel and visualized by immunoblot.

Microscopy methods

Live cell imaging was conducted as previously described [7] using a spinning disk confocal microscope (Ultraview LCI; PerkinElmer). Fluorescence quantiation was performed using ImageJ software available at http://rsbweb.nih.gov/ij/as previously discussed [7]. For DAPI and methyl blue staining, cells were fixed in 70% ethanol before staining and imaged on a personal DeltaVision System equipped with an Olympus IX71 microscope using a 100x NA 1.40 UPlansApo oil immersion objective.

Checkpoint assay

Cells were synchronized in S-phase using hydroxyurea (Sigma) at a final concentration of 12 mM for 3–3.5 hrs at 32°C. After the arrest, cells were filtered and immediately incubated at 19°C to activated the spindle checkpoint. Septation indices were measured periodically for 9 hrs by methyl blue staining, which stains the septa.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We are grateful to Dan McCollum and the members of the Gould Lab, especially Adam Bohnert and Janel McLean, for many helpful discussions and critically reading the manuscript. We also thank Mohan Balasubramanian for generously providing many of the kinase deletion strains used in this study. AEJ was supported by the Cellular, Biochemical and Molecular Sciences training program, NIH T32 GM08554. This work was supported in part by the Howard Hughes Medical Institute.

References

1. Kops GJPL, Weaver BAA, Cleveland DW. On the road to cancer: aneuploidy and the mitotic checkpoint. Nature reviews Cancer. 2005; 5:773–85.
2. Tuttle RL, Bothos J, Summers MK, Luca FC, Halazonetis TD. Defective in mitotic arrest 1/ring finger 8 is a checkpoint protein that antagonizes the human mitotic exit network. Molecular cancer research: MCR. 2007; 5:1304–11. [PubMed: 18171988]
3. Scolnick DM, Halazonetis TD. Chfr defines a mitotic stress checkpoint that delays entry into metaphase. Nature. 2000; 406:430–5. [PubMed: 10935642]

4. Murone M, Simanis V. The fission yeast dma1 gene is a component of the spindle assembly checkpoint, required to prevent septum formation and premature exit from mitosis if spindle function is compromised. The EMBO journal. 1996; 15:6605–16. [PubMed: 8978687]
5. Brooks L, Heimsath EG, Loring GL, Brenner C. FHA-RING ubiquitin ligases in cell division cycle control. Cellular and molecular life sciences: CMLS. 2008; 65:3458–66. [PubMed: 18597043]
6. Johnson AE, Gould KL. Dma1 ubiquitinates the SIN scaffold, Sid4, to impede the mitotic localization of Plo1 kinase. The EMBO journal. 2011; 30:341–54. [PubMed: 21131906]
7. Guertin DA, Venkatram S, Gould KL, McCollum D. Dma1 prevents mitotic exit and cytokinesis by inhibiting the septation initiation network (SIN). Developmental cell. 2002; 3:779–90. [PubMed: 12479804]
8. Johnson AE, McCollum D, Gould KL. Polar opposites: Fine-tuning cytokinesis through SIN asymmetry. Cytoskeleton (Hoboken, NJ). 2012; 69:686–99.
9. Tanaka K, Petersen J, MacIver F, Mulvihill DP, Glover DM, Hagan IM. The role of Plo1 kinase in mitotic commitment and septation in Schizosaccharomyces pombe. The EMBO journal. 2001; 20:1259–70. [PubMed: 11250892]
10. Mulvihill DP, Hyams JS. Cytokinetic actomyosin ring formation and septation in fission yeast are dependent on the full recruitment of the polo-like kinase Plo1 to the spindle pole body and a functional spindle assembly checkpoint. Journal of cell science. 2002; 115:3575–86. [PubMed: 12186944]
11. Morrell JL, Tomlin GC, Rajagopalan S, Venkatram S, Feoktistova AS, Tasto JJ, Mehta S, Jennings JL, Link A, Balasubramanian MK, et al. Sid4p-Cdc11p assembles the septation initiation network and its regulators at the S. pombe SPB. Current biology: CB. 2004; 14:579–84. [PubMed: 15062098]
12. Mohammad DH, Yaffe MB. 14-3-3 proteins, FHA domains and BRCT domains in the DNA damage response. DNA repair. 2009; 8:1009–17. [PubMed: 19481982]
13. Toda T, Umesono K, Hirata A, Yanagida M. Cold-sensitive nuclear division arrest mutants of the fission yeast Schizosaccharomyces pombe. Journal of molecular biology. 1983; 168:251– 70. [PubMed: 6887244]
14. Seeger M, Gordon C, Ferrell K, Dubiel W. Characteristics of 26 S proteases from fission yeast mutants, which arrest in mitosis. Journal of molecular biology. 1996; 263:423–31. [PubMed: 8918598]
15. Durocher D, Taylor IA, Sarbassova D, Haire LF, Westcott SL, Jackson SP, Smerdon SJ, Yaffe MB. The molecular basis of FHA domain:phosphopeptide binding specificity and implications for phospho-dependent signaling mechanisms. Molecular cell. 2000; 6:1169–82. [PubMed: 11106755]
16. Fraschini R, Formenti E, Lucchini G, Piatti S. Budding yeast Bub2 is localized at spindle pole bodies and activates the mitotic checkpoint via a different pathway from Mad2. The Journal of cell biology. 1999; 145:979–91. [PubMed: 10352016]
17. Li R. Bifurcation of the mitotic checkpoint pathway in budding yeast. Proceedings of the National Academy of Sciences of the United States of America. 1999; 96:4989–94. [PubMed: 10220406]
18. Alexandru G, Zachariae W, Schleiffer A, Nasmyth K. Sister chromatid separation and chromosome re-duplication are regulated by different mechanisms in response to spindle damage. The EMBO journal. 1999; 18:2707–21. [PubMed: 10329618]
19. Gardner RD, Burke DJ. The spindle checkpoint: two transitions, two pathways. Trends in cell biology. 2000; 10:154–8. [PubMed: 10740270]
20. Bimbó A, Jia Y, Poh SL, Karuturi RKM, Den Elzen N, Peng X, Zheng L, O'Connell M, Liu ET, Balasubramanian MK, et al. Systematic deletion analysis of fission yeast protein kinases. Eukaryotic cell. 2005; 4:799–813. [PubMed: 15821139]
21. Knippschild U, Gocht A, Wolff S, Huber N, Löhler J, Stöter M. The casein kinase 1 family: participation in multiple cellular processes in eukaryotes. Cellular signalling. 2005; 17:675–89. [PubMed: 15722192]
22. Behrend L, Milne DM, Stöter M, Deppert W, Campbell LE, Meek DW, Knippschild U. IC261, a specific inhibitor of the protein kinases casein kinase 1-delta and -epsilon, triggers the mitotic checkpoint and induces p53-dependent postmitotic effects. Oncogene. 2000; 19:5303–13. [PubMed: 11103931]

23. Sillibourne JE, Milne DM, Takahashi M, Ono Y, Meek DW. Centrosomal anchoring of the protein kinase CK1delta mediated by attachment to the large, coiled-coil scaffolding protein CG-NAP/AKAP450. Journal of molecular biology. 2002; 322:785–97. [PubMed: 12270714]
24. Takahashi M, Yamagiwa A, Nishimura T, Mukai H, Ono Y. Centrosomal proteins CG-NAP and kendrin provide microtubule nucleation sites by anchoring gamma-tubulin ring complex. Molecular biology of the cell. 2002; 13:3235–45. [PubMed: 12221128]
25. Swiatek W, Kang H, Garcia BA, Shabanowitz J, Coombs GS, Hunt DF, Virshup DM. Negative regulation of LRP6 function by casein kinase I epsilon phosphorylation. The Journal of biological chemistry. 2006; 281:12233–41. [PubMed: 16513652]
26. Cegielska A, Moarefi I, Fanning E, Virshup DM. T-antigen kinase inhibits simian virus 40 DNA replication by phosphorylation of intact T antigen on serines 120 and 123. Journal of virology. 1994; 68:269–75. [PubMed: 8254738]
27. Cegielska A, Gietzen KF, Rivers A, Virshup DM. Autoinhibition of casein kinase I epsilon (CKI epsilon) is relieved by protein phosphatases and limited proteolysis. The Journal of biological chemistry. 1998; 273:1357–64. [PubMed: 9430669]
28. Ciccia A, Elledge SJ. The DNA damage response: making it safe to play with knives. Molecular cell. 2010; 40:179–204. [PubMed: 20965415]
29. Musacchio A, Salmon ED. The spindle-assembly checkpoint in space and time. Nature reviews Molecular cell biology. 2007; 8:379–93.
30. Chaurushiya MS, Lilley CE, Aslanian A, Meisenhelder J, Scott DC, Landry S, Ticau S, Boutell C, Yates JR, Schulman BA, et al. Viral E3 ubiquitin ligase-mediated degradation of a cellular E3: viral mimicry of a cellular phosphorylation mark targets the RNF8 FHA domain. Molecular cell. 2012; 46:79–90. [PubMed: 22405594]
31. Milne DM, Looby P, Meek DW. Catalytic activity of protein kinase CK1 delta (casein kinase 1delta) is essential for its normal subcellular localization. Experimental cell research. 2001; 263:43–54. [PubMed: 11161704]

Highlights

- Dma1's FHA domain binds to a pTXXpS phospho-motif on Sid4
- CK1 is required for Dma1-dependent Sid4 ubiquitination
- CK1 phosphorylation of Sid4 is required for the Dma1-dependent mitotic checkpoint
- CK1 concentrates at SPBs and phospho-primes Sid4 for Dma1-mediated ubiquitination

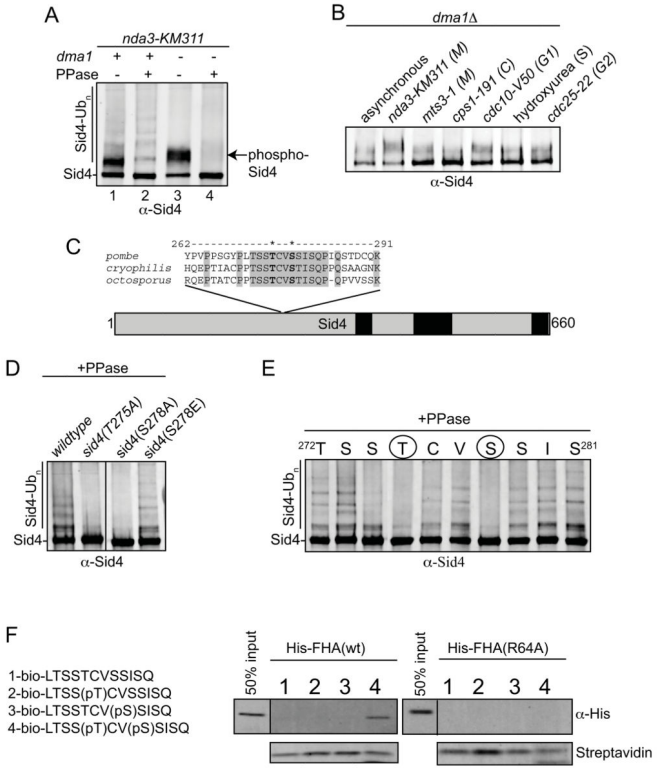

Figure 1. Sid4 phosphorylation on T275 and S278 recruit Dma1 via its FHA domain

A. Sid4 was immunoprecipitated in the presence and absence of *dma1*$^{+}$ and lambda phosphatase treatment and detected by immunoblot. B. Sid4 was immunoprecipitated from the indicated strains and detected by immunoblot. C. Schematic diagram of Sid4 with the relative position of the Dma1 binding site. ClustalW alignment of the Dma1 binding sequence from *S. pombe*, *S. cryophilis* and *S. octosporus*. Black regions represent the predicted coil-coil regions. D. Sid4 from the indicated strains was immunoprecipitated from denatured cell lysates, treated with phosphatase and visualized by immunoblotting. E. In vivo ubiquitination status of Sid4 mutants. Circled amino acids indicate T275 and S278 residues that are required for Sid4 ubiquitination. F. Synthetic peptides conjugated to streptavidin beads were incubated with recombinant His-FHA or His-FHA(R64A) proteins and bound proteins were detected by immunoblotting. See also Figure S1.

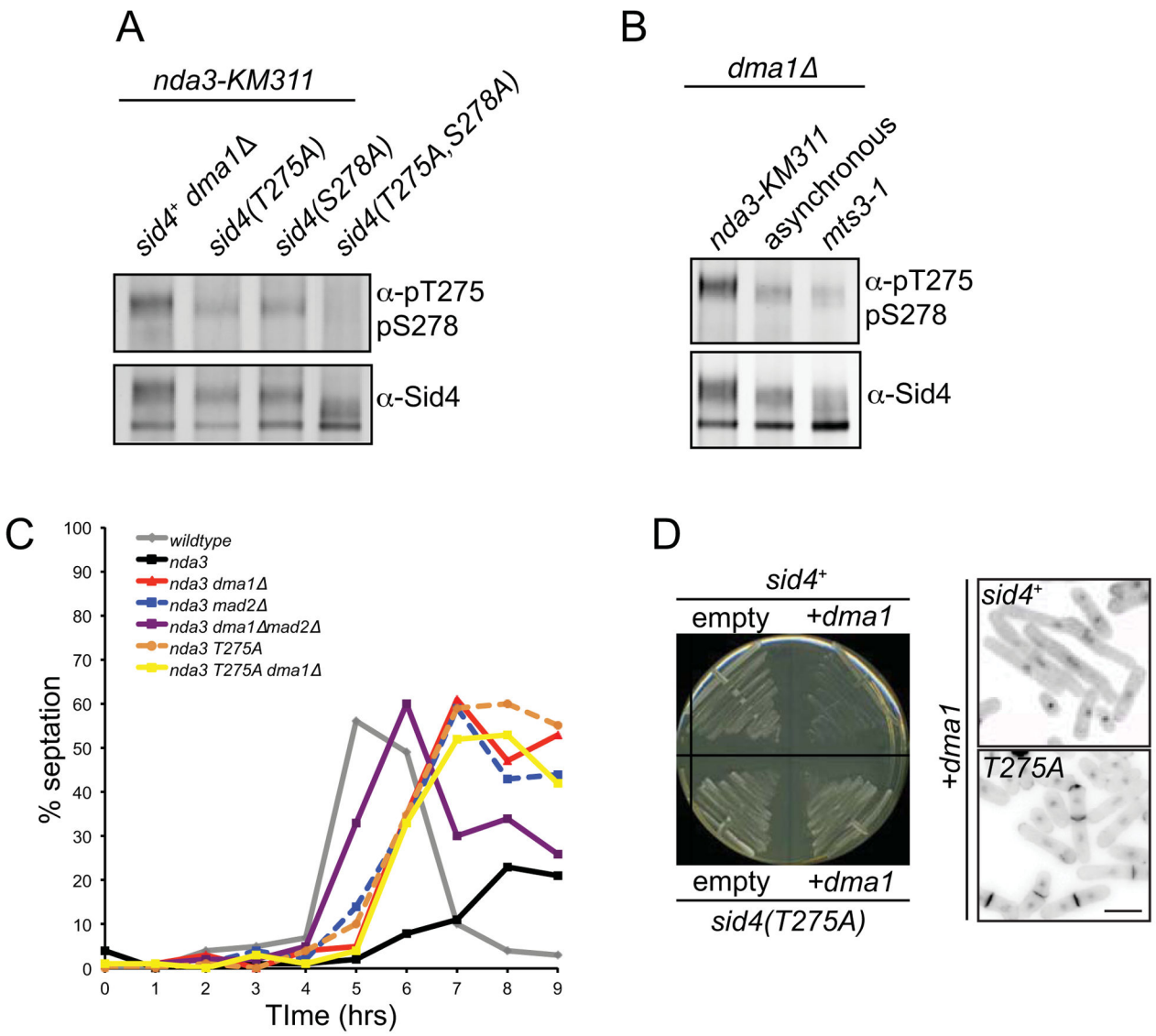

Figure 2. Sid4 phosphorylation on T275 is required for the Dma1-dependent checkpoint

A–B. Sid4 was immunoprecipitated under denatured conditions with a Sid4 antibody and detected via immunoblot using a phospho-T275, S278 or Sid4 antibody. C. Spindle checkpoint assay. For each strain indicated, cells were synchronized in S-phase with hydroxyurea, shifted to 19°C to activate the spindle checkpoint and septation indices were measured periodically for 9 hrs. D. Over-expression of *dma1* in *sid4*$^{+}$ or *sid4(T275A)* mutant cells. Growth of the transformants was observed on agar plates (left) after de-repression of the *nmt41* promoter and their phenotypes were analyzed by DAPI (DNA) and methyl blue (septa) staining (right). Inverted grayscale images are shown, scale bar, 5 μm. See also Figure S2.

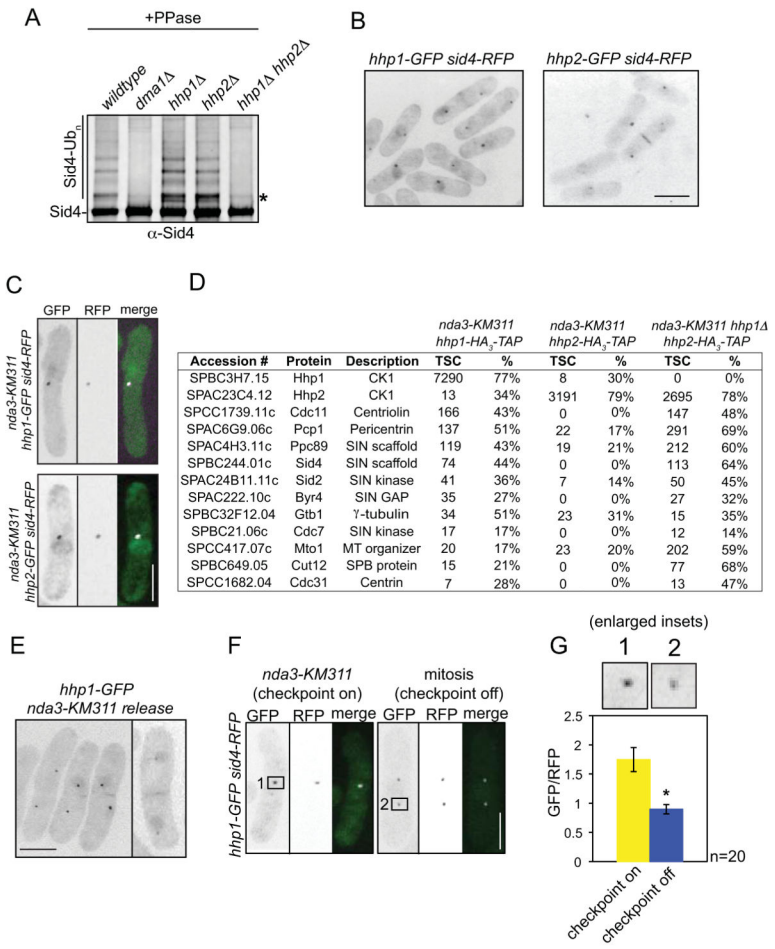

Figure 3. CK1 is required for Sid4 ubiquitination and associates with the SIN pathway during a mitotic checkpoint

A. Sid4 was immunoprecipitated from the indicated strains, treated with phosphatase and visualized by immunoblotting. B. Hhp1-GFP and Hhp2-GFP localization in cells growing asynchronously. Inverted grayscale images are shown, scale bar 5 μm. C. Hhp1-GFP co-imaged with Sid4-RFP in checkpoint-stimulated cells. Inverted grayscale images are shown for GFP and RFP, scale bar, 5 μm. D. Hhp1-HA_3-TAP or Hhp2-HA_3-TAP was purified from checkpoint-activated cells and interacting proteins were identified by 2D-LC/MS. TSC=total spectral counts, %=percent sequence coverage. For a complete list of co-purifying proteins, see Table S1. E. Hhp1-GFP imaged in cells released from a pro-metaphase arrest. Inverted grayscale images are shown, scale bar, 5 μm. F. Hhp1-GFP imaged in checkpoint-activated and asynchronously growing mitotic cells. Inverted grayscale images are shown for GFP and RFP, scale bar, 5 μm. G. Quantitation of Hhp1-GFP at SPBs in the strains used in 3F. Values are represented as GFP/RFP ratios. n=20 cells, *p<0.001 compared to “checkpoint off” cells. See also Figure S3.

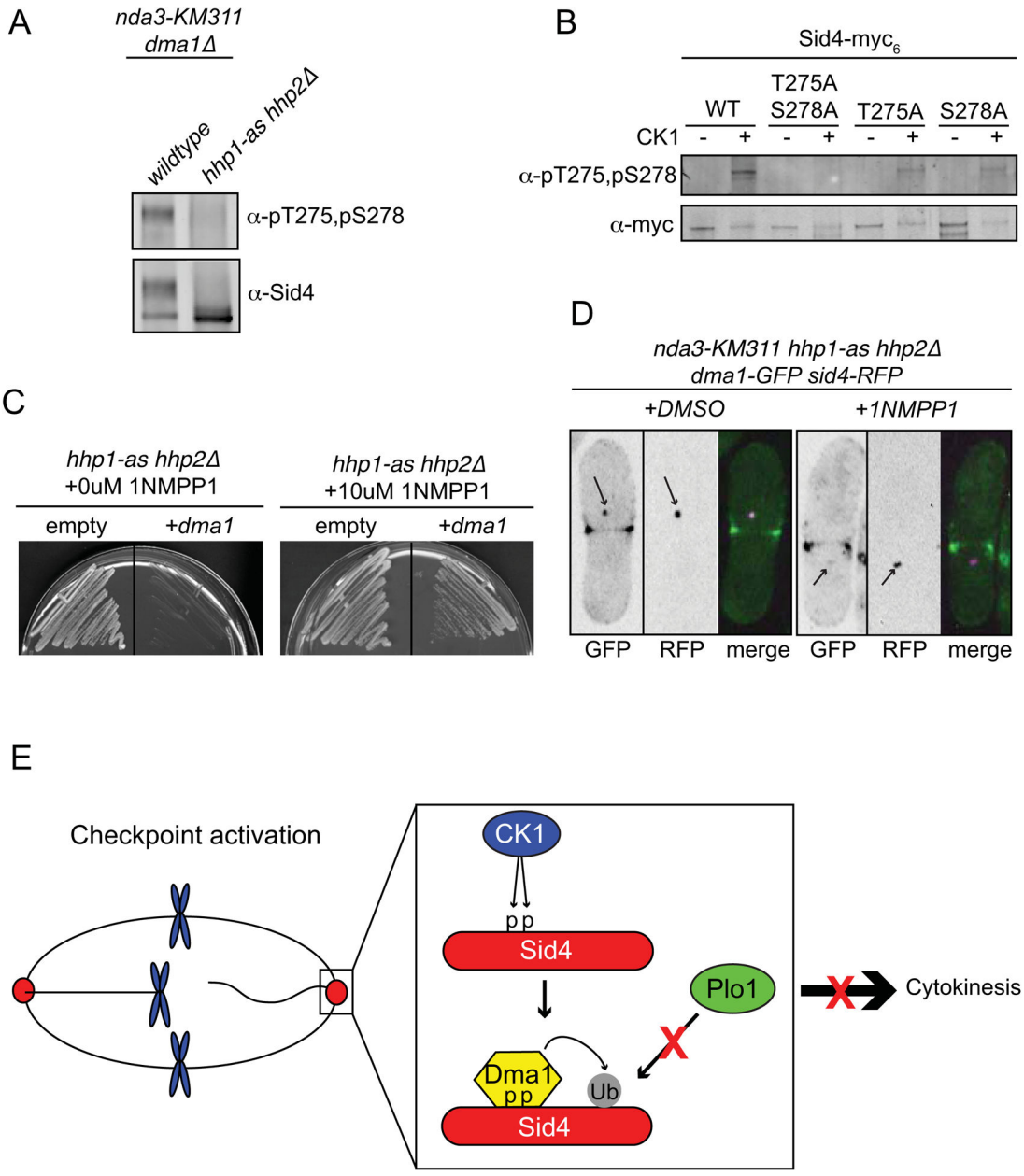

Figure 4. CK1 phospho-primes Sid4 for Dma1-mediated ubiquitination

A. Sid4 was immunoprecipitated under denatured conditions with a Sid4 antibody and detected via immunoblot using a phospho-T275, S278 or Sid4 antibody. B. Sid4-myc$_6$ proteins were produced via a coupled in vitro transcription/translation reaction, immunoprecipitated with a myc antibody and phosphorylated with CK1. Phosphorylated proteins were detected by immunoblot analyses using myc or phospho-T275, S278 antibodies. C. Over-expression of *dma1* in *hhp1-as hhp2*Δ cells. Growth of transformants was observed on agar plates in the presence or absence of the ATP analog 1NMPP1. D. Dma1-GFP co-imaged with Sid4-RFP in *hhp1-as hhp2*Δ cells in the presence of DMSO or the ATP analog 1NMPP1. E. Model for CK1 activation of the Dma1-dependent checkpoint pathway. Upon

checkpoint activation, CK1 concentrates at SPBs and phosphorylates the SIN scaffold Sid4 on T275 and S278. CK1 mediated phosphorylation of Sid4 recruits Dma1 via its FHA domain, which subsequently ubiquitinates Sid4. Sid4 ubiquitination impedes Plo1 localization to SPBs, restricting Plo1's ability to promote cytokinesis. See also Figure S4.