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Report

Spatiotemporal Regulation of Ipl1/Aurora Activity by Direct Cdk1 Phosphorylation

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Summary

Oscillating cyclin-dependent kinase 1 (Cdk1) activity is the major regulator of cell-cycle progression [1], whereas the Aurora B kinase, as part of the chromosome passenger complex (CPC), controls critical aspects of mitosis such as chromosome condensation and biorientation on the spindle [2]. How these kinases mechanistically coordinate their important functions is only partially understood. Here, using budding yeast, we identify a regulatory mechanism by which the Cdk1 kinase Cdc28 directly controls the Aurora kinase Ipl1. We show that Cdk1 phosphorylates Ipl1 on two serine residues in the N-terminal domain, thereby suppressing its association with the microtubule plus-end tracking protein Bim1 until the onset of anaphase. Failure to phosphorylate Ipl1 leads to its premature targeting to the metaphase spindle and results in constitutive Bim1 phosphorylation, which is normally restricted to anaphase. Cells expressing an Ipl1-Sli15 complex that cannot be phosphorylated by Cdk1 display a severe growth defect. Our work shows that Ipl1/Aurora is not only the catalytic subunit of the CPC but also an important regulatory target that allows Cdk1 to coordinate chromosome biorientation with spindle morphogenesis.

Results and Discussion

Aurora B/Ipl1 is part of a conserved chromosome passenger complex (CPC: Ipl1/Aurora, Sli15/INCENP [inner centromere protein], Bir1/Survivin, Nbl1/Borealin) that displays a dynamic subcellular localization over the cell cycle. In metaphase, Aurora B/CPC associates with centromeres and regulates kinetochore-microtubule (MT) attachments in the process of chromosome biorientation [3–6]. During anaphase, however, Aurora B delocalizes to the spindle midzone where it is involved in timely spindle disassembly and cytokinesis [7–10]. The highly conserved process of CPC translocation is critical to sustain stable kinetochore-MT attachments and silence the mitotic checkpoint in anaphase [11, 12]. One mechanism thought to control the relocalization of Aurora/Ipl1 is cyclin-dependent kinase 1 (Cdk1) phosphorylation of the regulatory subunit Sli15/INCENP. At the metaphase-anaphase transition, Cdk1 activity decreases, and the release of the counteracting Cdc14 phosphatase from the nucleolus allows dephosphorylation of Sli15 and its binding to the anaphase spindle [13]. Aurora kinase itself may also prevent premature CPC metaphase spindle association by phosphorylating additional sites on Sli15 [14]. Several lines of evidence point out that the control of Sli15 MT-binding activity by Cdk1 and Ipl1 phosphorylation is not sufficient to explain the mechanism of CPC translocation. First, mutations in Sli15 that completely prevent Cdk1 or Ipl1 phosphorylation have little effect on cell viability, suggesting that important control mechanisms are still active in these mutants [13, 14]. In addition, it is an open question how association of the CPC with a specific subset of MTs within the spindle can be achieved. We have recently shown that the Ipl1 kinase associates with the MT plus-end-binding protein Bim1 [9], the yeast end-binding 1 (EB1) homolog [15–18]. Here we investigate the function and regulation of this association.

The Aurora Kinase Ipl1 Is a Novel Yeast +TIP

To elucidate the Ipl1-Bim1-binding mechanism, we narrowed down the Ipl1 domain responsible for the association by testing several truncation mutants in a GST (glutathione S-transferase)-Bim1-binding assay (see Figure S1A available online). Our analysis revealed that the binding interface is localized within the unstructured N-terminal part (aa 1–87) preceding the kinase domain, and we identified two potential EB1-targeting motifs (Ser-X-Ile-Pro) [19, 20] in this region (residues 46–49 and 72–75) (Figure 1A). Because some EB1-binding proteins employ tandem SxIP motifs [21], we next tested the functionality of these motifs. Elimination of either putative EB1 motif resulted in a 2-fold reduction of Ipl1 binding to Bim1. Association of Ipl1 and Bim1, however, was completely abolished upon mutating both motifs simultaneously, indicating that they are indeed functional EB1-binding sites (Figures S1A and S1B). We also confirmed that in the context of the Ipl1-Sli15 complex, the SxIP motifs in the Ipl1 N terminus are critical for binding to Bim1 (Figure S1C).

Cdk1 Phosphorylates Ipl1 and Inhibits Its Binding to Bim1

To understand whether the interaction between Ipl1 and Bim1 is subject to mitotic regulation, we purified the kinase complex from cells arrested in metaphase. Tandem affinity purification (TAP)-tag purification of the Sli15 subunit allowed efficient isolation of the entire CPC (Figure 1B). Inspection of detected phosphorylation sites (Table S1) on Ipl1 confirmed activating T loop autophosphorylation at position T260 and three additional sites located within the N terminus [22]. One of them, Ser-76, was previously described as a Cdk1 site [23], whereas Ser-50 also displays a full consensus motif for Cdk1. In vitro kinase assays with Cdk1/Cdc28 and recombinant Ipl1/Aurora confirmed that Cdk1 phosphorylates Ipl1 directly (Figure 1C), and this phosphorylation was reduced upon mutating Ser-50 and -76 to alanine (Figure S2B). Notably, these two sites on Ipl1 mapped immediately adjacent to the identified SxIP motifs mediating the interaction with Bim1, suggesting that the physical interaction between these two conserved proteins might be regulated by Cdk1 phosphorylation. In support of this, binding assays with GST-Bim1 and Ipl1 fused to enhanced green fluorescent protein (EGFP) phosphorylated by Cdc28/Clb2 in vitro showed a significant reduction in kinase affinity for Bim1 (Figure 1D).

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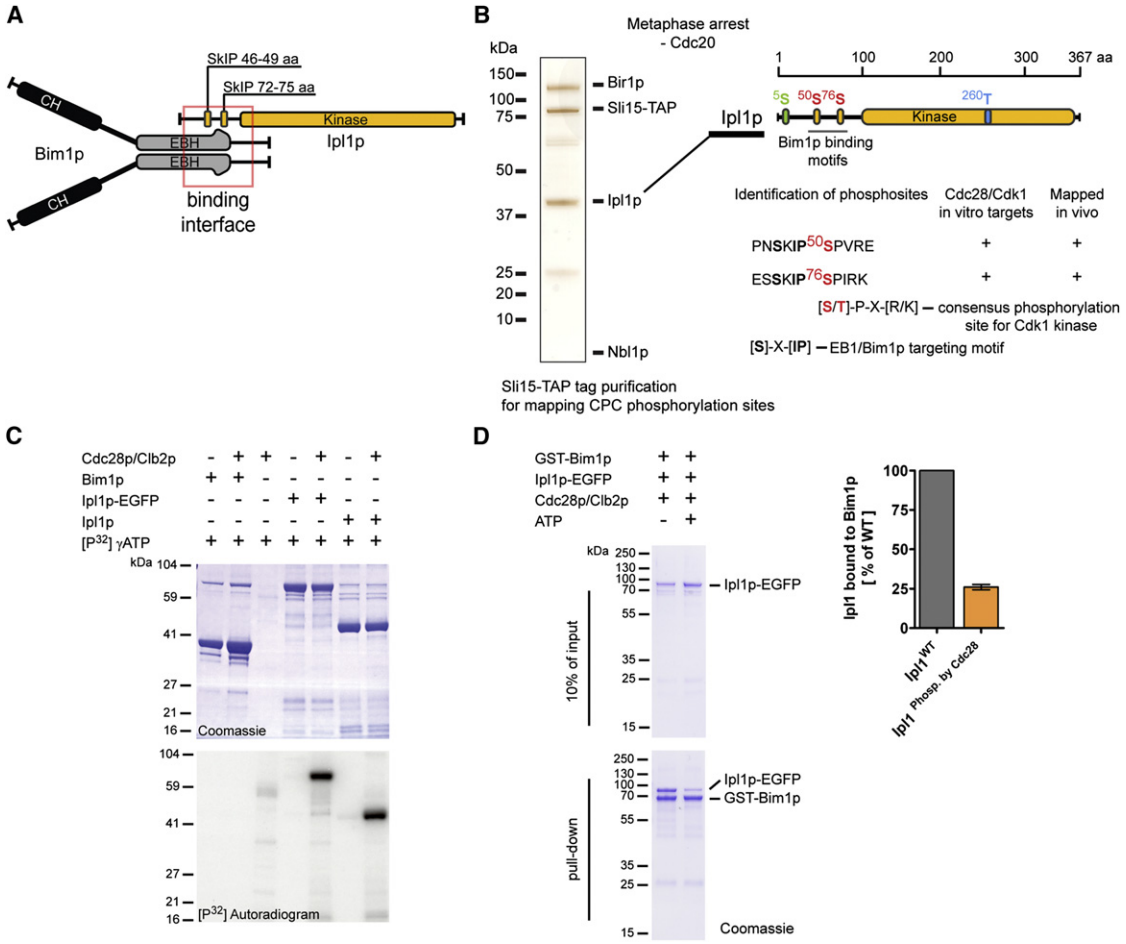

Figure 1. Ipl1 Is a Target for Cdk1-Dependent Phosphorylation

(A) Model representation depicting the Ipl1-Bim1-binding interface.

(B) Sli15-Stag-TEV-ZZ tandem-affinity purification from yeast cells arrested at metaphase and subsequent mass spectrometry. Schematic representation of the mapped phosphorylation sites on the Ipl1 kinase and depiction of two Cdk1 sites at the N terminus of Ipl1 adjacent to the detected EB1-binding motifs.

(C) Cdk1/Cdc28 phosphorylates Ipl1 in vitro. Under conditions of undetectable Ipl1 autophosphorylation, recombinant Ipl1 is efficiently phosphorylated by Cdk1. The specificity of the reaction was confirmed by using the Bim1 as a negative control.

(D) Ipl1 kinase phosphorylated by Cdk1/Cdc28 displays reduced binding to GST-Bim1 in a pull-down assay. Cdk1/Cdc28 phosphorylation of Ipl1 affects its binding to Bim1. Quantification of Ipl1 binding from Coomassie-stained gels in three independent pull-downs. Error bars represent SEM.

See also Figure S1.

Tandem SxIP Motifs Mediate Docking of One Ipl1 Molecule to a Bim1 Homodimer

To provide a quantitative analysis of Bim1-Ipl1 complex formation, we performed isothermal titration calorimetry (ITC) analysis with the Bim1 dimerization domain (197–283 aa) and the 2× SxIP-containing Ipl1 N terminus (1–87 aa) (Figure 2A). These fragments interacted with an apparent dissociation constant (K_D) of 0.12 μM (Figure 2B), which indicates an unusually tight EB1-cargo interaction [19]. We found that one molecule of Ipl1 formed a complex with one Bim1 homodimer. This suggests that the two SxIP motifs from a single Ipl1 molecule may together occupy both hydrophobic-binding pockets on Bim1, which could explain the observed high-affinity interaction. Mutation of the first SxIP motif increased the K_D to 2.34 μM, whereas elimination of the second one even more strongly decreased its affinity for Bim1 (Figure 2B). As shown before, elimination of both motifs abrogated binding completely.

To quantify the effects of Cdk1 phosphorylation, we mutated Ser-50 and Ser-76 on Ipl1 to two alanines (phospho null) or aspartic acids (phospho-mimicking). ITC measurements indicated a strong decrease in Bim1-binding affinity of the Ipl1-2D mutant (Figure 2C). To test whether Cdk1 phosphorylation mutants are also affected in Ipl1-Bim1 complex formation in vivo, we used whole-cell extracts from yeast cells with mutated *ipl1* alleles at the endogenous *IPL1* locus and performed pull-down experiments with GST-Bim1. Compared to wild-type (WT) *Ipl1* and the *2A* mutant, *2D* and *2× SxNN* variants displayed reduced binding to Bim1, similar to what we observed with recombinant proteins (Figure S2C).

Dephosphorylation of Ipl1 at the Onset of Anaphase Triggers Its Binding to Bim1

Analysis of the Ipl1 migration pattern on Phos-Tag gels [24] in samples collected every 15 min after a G1 release showed a phosphorylation shift that paralleled the expression of Securin/Pds1 (Figure 2D). Whole-cell extracts were prepared from the same time points and subjected to pull-down experiments with GST-Bim1. Most interesting, maximum Ipl1 cell-cycle phosphorylation (S phase to metaphase transition)

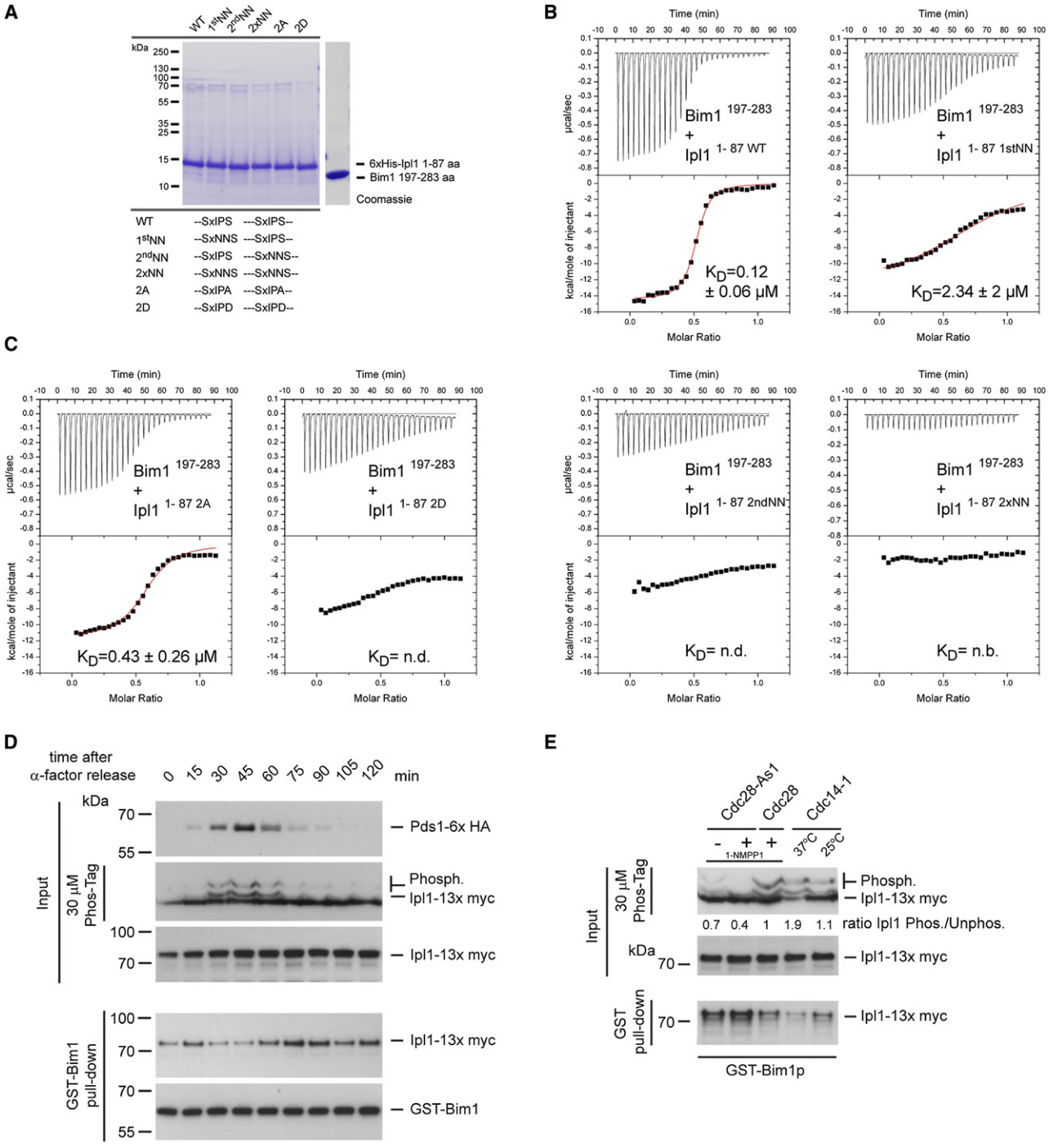

Figure 2. Phosphorylation of Two SxIP Motifs Regulates Docking of One Ipl1 Molecule to a Bim1 Homodimer

(A) Coomassie-stained gel of the Ipl1 variants mutated in SxIP motifs and the cargo-binding domain of Bim1 purified from *E. coli*.

(B) ITC analysis of the Ipl1-Bim1 interaction. A total of 15 μM of Bim1 (residues 197–283) was titrated with 0.15 mM Ipl1 variants defective in single or both SxIP motifs. K_D was estimated from nonlinear curve fitting to the binding isotherms. n.b., not binding; n.d., not determined.

(C) Binding affinity measurements between Bim1 197–283 aa and Ipl1 N-terminal part containing two serine residues mimicking Cdk1 phosphorylation using ITC.

(D) Interaction analysis between Ipl1 and Bim1 in a time course α factor arrest/release experiment. Western blot analysis of Ipl1 bound to GST-Bim1 displayed reduced association of the kinase in a time window of maximal Ipl1 phosphorylation, detectable before degradation of the yeast Securin Pds1. SDS-PAGE was performed in the presence of 30 μM Mn^{2+} Phos-Tag.

(E) Cdc14-dependent dephosphorylation of Ipl1 regulates its affinity for Bim1. Analysis of Ipl1 phosphorylation after inhibition of Cdk1 activity with the *cdc28-as1* allele or after inactivation of the phosphatase Cdc14. The ratio between phosphorylated and unphosphorylated Ipl1 was quantified using the band intensities on the Phos-Tag gel. Yeast extracts from cells compromised in Cdk1 kinase or Cdc14 phosphatase were used in GST-Bim1-binding assays. Bound Ipl1-13xmyc was analyzed with western blot after SDS-PAGE performed in the presence of 30 μM Mn^{2+} Phos-Tag.

See also Figure S2.

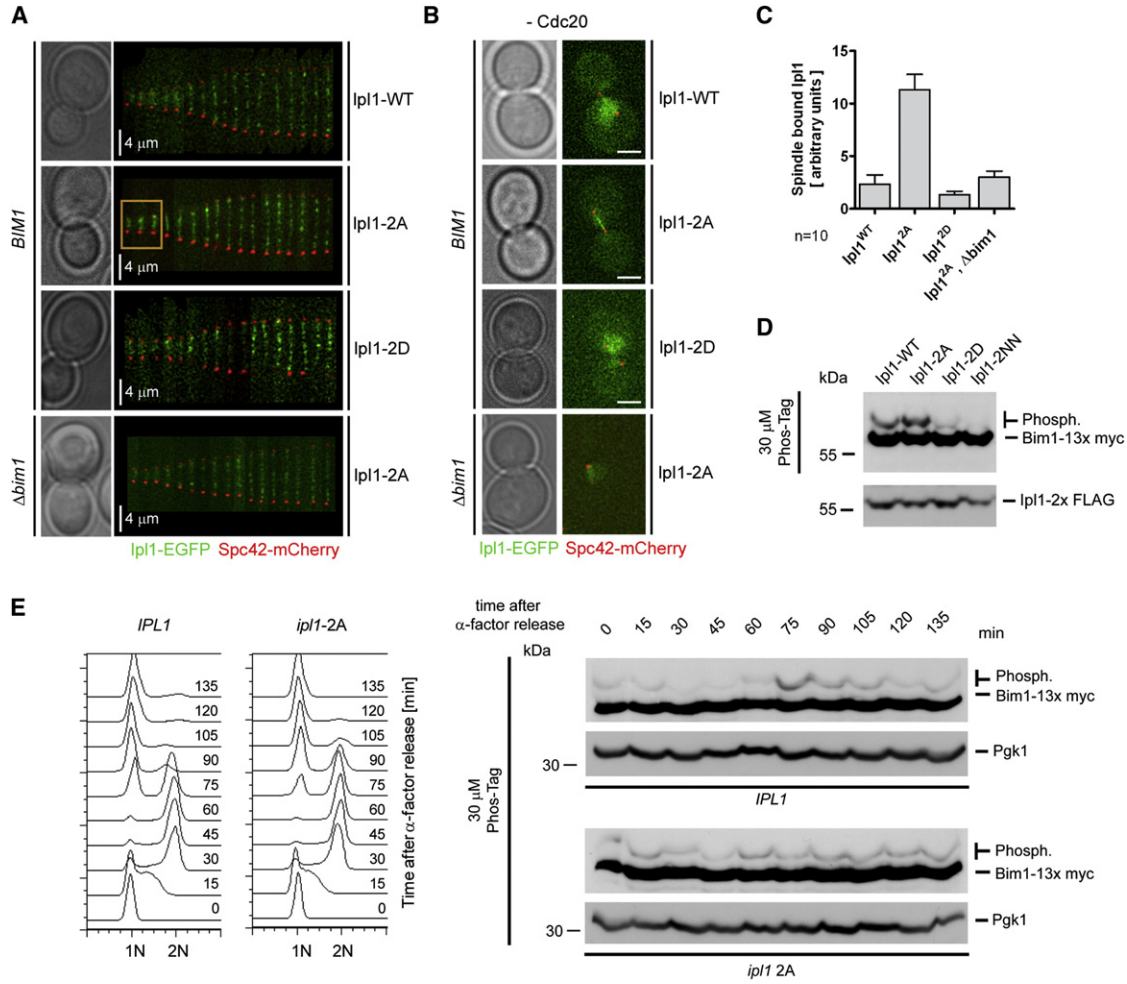

Figure 3. Ipl1 Kinase Compromised in Cdk1 Phosphorylation Is Prematurely Loaded onto the Metaphase Spindle

(A) Dynamic localization pattern of Ipl1-EGFP WT, defective in Cdk1 phosphorylation (*ipl1-2A*) or Bim1 binding (*ipl1-2D*) visualized using Deltavision microscopy. Orange box indicates the fraction of Ipl1-2A prematurely associated with the metaphase spindle.

(B) *ipl1-2A* is prematurely loaded to spindle MTs in metaphase-arrested cells (Cdc20 depletion). Scale bars represent 3 μm.

(C) Quantification of spindle-bound Ipl1 in metaphase cells. Error bars represent SEM.

(D) Elimination of Cdk1 control (*ipl1-2A*) or Ipl1-Bim1 binding (*ipl1-2D* or *ipl1-2×NN*) results in hyperphosphorylation of Bim1 by Ipl1 kinase or elimination of slower-migrating forms of Bim1, respectively. Western blot analysis of Bim1-13xmyc after SDS-PAGE with additional 30 μM Phos-Tag reagent.

(E) Anaphase-specific Bim1 phosphorylation becomes detectable throughout the entire cell cycle in the Cdk1-compromised *ipl1-2A* background. Cells expressing Bim1-13xmyc from α factor arrest/release experiment were analyzed by flow cytometry for DNA content or used in Phos-tag western blot.

See also Figure S3.

precisely coincided with minimal Ipl1-GST-Bim1 interaction (Figure 2D). Consistently, after Securin degradation, Ipl1 became dephosphorylated, leading to a gradual increase in Bim1 binding. Compared to WT, the *ipl1-2A* mutant displayed a reduced phospho-shift in vivo (Figure S2D). The effects of Cdk1 and its opposing phosphatase Cdc14 on Ipl1 phosphorylation were tested with the analog-sensitive *cdc28-as1* and the temperature-sensitive *cdc14-1* allele. Treatment of *cdc28-as1* cells with 1NM-PP1 eliminated the slowly migrating Ipl1 phospho-isoforms and increased the ability of Ipl1 to interact with GST-Bim1. Surprisingly, *cdc28-as1* cells displayed a decreased level of Ipl1 phosphorylation also in the absence of inhibitor, suggesting that this allele is compromised in its basal kinase activity (Figure 2E). Blocking the opposing Cdc14 phosphatase resulted in hyperphosphorylation of Ipl1/Aurora and effectively decreased its binding to Bim1.

Elimination of Cdk1 Control Loads Ipl1 Prematurely onto Spindle MTs

Ipl1 mutants fused to EGFP were integrated into the *IPL1* locus and analyzed by live-cell microscopy. During metaphase, *Ipl1-WT* and *-2D* mutants displayed a diffuse nuclear signal and unambiguously localized to MTs only when the spindle started to elongate (Figure 3A). In contrast, the *ipl1-2A* variant showed a clear metaphase spindle association prior to anaphase onset. In cdc20-depleted cells, the Ipl1 mutant defective in Cdk1 phosphorylation (*ipl1-2A*) displayed relatively strong metaphase spindle localization with only weak nuclear background staining. In comparison, the *Ipl1-WT* and mutant compromised in Bim1 binding showed no obvious MT association (Figure 3B). The premature spindle localization of the *ipl1-2A* mutant depended on the presence of Bim1 (Figure 3C). To test whether Bim1 could be a factor responsible for targeting Ipl1 to the spindle MTs, we performed in vitro MT

cosedimentation experiments with either kinase alone or in the presence of Bim1. Consistent with previous reports [3], we could detect weak MT association of Ipl1 itself; however, its binding to MTs was strongly increased in the presence of Bim1 (Figure S3A).

Cdk1 Prevents the Premature Phosphorylation of Ipl1 Anaphase Substrates

Elimination of Cdk1 regulation allows constitutive interaction between Ipl1 and Bim1, which results in premature loading of the kinase onto the spindle MTs. What is the consequence of this uncontrolled loading on Ipl1 downstream substrates? A well-established anaphase-specific Ipl1 substrate is Bim1 itself [7, 9]. Western blot analysis of myc-tagged Bim1 in cells expressing *ipl1 cdk1* mutants revealed an increased amount of slower-migrating forms of Bim1 when Cdk1 phosphorylation was abolished (*ipl1-2A*) (Figure 3D). Conversely, the phosphorylation of Bim1 was barely detectable in cells expressing Ipl1 impaired in its ability to bind Bim1 (*ipl1-2D* and *-NN*). Because the lack of Cdk1 control leads to overphosphorylation of Bim1 by Ipl1, we next tested the phosphorylation pattern of Bim1 during the cell cycle in the *Ipl1-WT* or *-2A* background. Interestingly, in the WT situation, Bim1 phospho-isoforms are predominantly detectable in the time window 75 min after release, consistent with our previous analysis [9] (Figure 3E). In contrast, cells expressing *ipl1-2A* showed a constant level of Bim1 phosphorylation throughout the entire cell cycle and a slight delay in cytokinesis as judged by the DNA content analysis (compare time points 75 and 90 min). Thus, Cdk1-dependent phosphorylation prevents premature Ipl1-Bim1 association and restricts Ipl1-dependent Bim1 phosphorylation to anaphase. The premature phosphorylation of Bim1 in the *ipl1-2A* did not prevent the association with the metaphase spindle (Figure S3B).

Our experiments suggest that Bim1 promotes targeting of the kinase to MTs in anaphase, whereas Ipl1 phosphorylation of Bim1 inhibits its MT binding [9] and, thus, may negatively regulate Ipl1 function. A number of genetic experiments support this notion: *ipl1-2* or *sli15-3* alleles that display reduced kinase activity at the permissive temperature [25] depend on *BIM1* expression for viability (Figure S3C). Interestingly, Bim1 phosphomutants had opposite effects on the viability of *ipl1-2*. Overexpression of phosphorylation-deficient Bim1 (*bim1-6A*), which shows enhanced association with the anaphase spindle [9], partially rescued the temperature sensitivity of an *ipl1-2* mutant at 34°C, whereas a bim1 phosphomimicking mutant (*bim1-6D*), known to reduce spindle association, further increased the temperature sensitivity of the *ipl1-2* allele (Figure S3C). Thus, Bim1's MT-binding activity is important for the support of Ipl1 function in vivo.

Cdk1 Phosphorylation of the Ipl1-Sli15 Complex Is Critical for Viability

Because Ipl1 and Sli15 are both important targets for Cdk1, we next tested whether the combination of the two alleles defective in Cdk1 phosphorylation would decrease the viability of the *sli15-6A* strain. In genetic crosses we failed to recover viable *ipl1-2A sli15-6A* spores, suggesting that complete elimination of Cdk1 phosphorylation of the Ipl1-Sli15 complex might be lethal. The fact that the *sli15-6A* allele also displayed decreased spore viability in backcrosses to a WT strain, however, complicated genetic analysis. To circumvent this, we applied a conditional system. First, we placed Ipl1 under control of the *GAL* promoter in *sli15-6A* cells and asked whether we could rescue the lethality of Ipl1 repression in glucose-containing medium by providing Ipl1-WT or Cdk1 mutants expressed from the Ipl1 promoter. Under these conditions, Ipl1 WT and Bim1-binding mutants restored the growth of cells on glucose; however, the Cdk1-compromised mutant *ipl1-2A* showed a marked decrease in viability (Figures 4A and 4B). Addition of the MT-destabilizing drug benomyl severely compromised the growth of all Ipl1 mutants, suggesting that the proper regulation of Ipl1-Bim1 binding becomes critical when Sli15 is no longer controlled by Cdk1. Additionally, analysis of cells expressing Ipl1-Sli15 complex defective in Cdk1 phosphorylation revealed an increase of monopolar chromosome segregation and aberrant spindle morphology, suggesting dysfunction of the kinase complex in the process of kinetochore biorientation (Figure 4C).

Conclusions

Our work reveals that Cdk1 initiates a cascade of phosphorylation events in which Ipl1 phosphorylation is temporally separated from Bim1 phosphorylation. In metaphase, when Cdk1 activity is still high, Bim1 is required for the proper formation of the metaphase spindle because *bim1* deletion mutants have a drastically reduced metaphase spindle length [9]. Decrease of Cdk1 activity and release of Cdc14 later allow the formation of a stable Ipl1-Bim1 complex required for efficient localization of Ipl1 to the spindle. The ability of Ipl1 to phosphorylate Bim1 on the anaphase spindle strictly depends on their physical interaction and on the accumulation of Ipl1 on the spindle. This creates a delay in Bim1 phosphorylation, which causes removal of Bim1 from the late anaphase spindle and—in a negative feedback loop—prevents the further targeting of Ipl1. This Cdk1 control of interaction and phosphorylation creates a transient association of the Ipl1 kinase with the mitotic spindle and thus coordinates chromosome-based with MT-based Ipl1 functions (Figure 4D). The autonomous plus-end tracking activity of Bim1 allows differentiation between distinct MT populations within the spindle. At anaphase onset kinetochore MTs shorten, whereas interpolar MTs polymerize and thus contribute to spindle elongation and midzone formation (anaphase B) [26]. Physical interactions have been reported between human Aurora B and EB1, suggesting a possible evolutionary conservation of the complex [27, 28]. The mechanism of interaction, however, may differ because Aurora B lacks clear SxIP motifs. Furthermore, the kinesin-6 motor protein Mklp2, which is absent in yeast, plays a critical role in CPC translocation to the midzone [12, 29]. Interestingly, however, the CPC-Mklp2 interaction is negatively regulated by Cdk1 phosphorylation [30], suggesting that Cdk1 control is an evolutionarily conserved principle of CPC regulation. In support of this, additional CPC subunits have been shown to be under Cdk1 regulation because phosphorylation of fission yeast Bir1/Survivin or human Borealin promotes centromere localization of the CPC by allowing binding to Shugoshin [31].

Supplemental Information

Supplemental Information includes three figures, two tables, and Supplemental Experimental Procedures and can be found with this article online at doi:10.1016/j.cub.2012.03.007.

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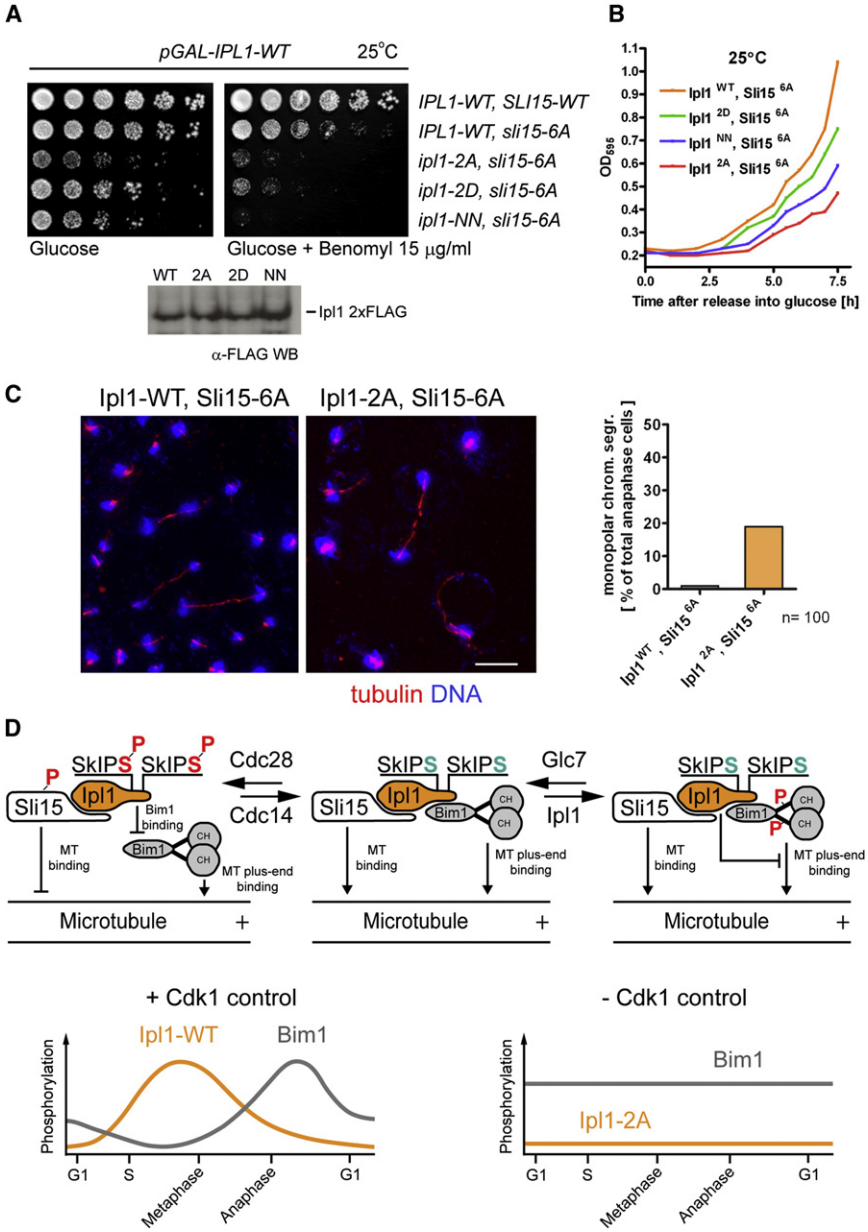

Figure 4. Cdk1 Controls Ipl1-Spindle Association by Regulating Two CPC MT Receptors

(A) Sli15 compromised in Cdk1 phosphorylation (*sli15-6A*) displays synthetic viability defects with Cdk1-defective *ipl1-2A*. Serial dilutions of indicated strains were spotted on rich medium plates with 2% glucose and with additional 15 μg/ml benomyl at 25°C. Western blot analysis indicates that the Ipl1 mutants are expressed at comparable levels.

(B) Indicated strains harboring a conditional *pGAL-Ipl1-WT* were grown in the presence of raffinose/galactose, diluted to optical density (OD) of 0.2 and further grown in medium containing glucose to repress expression of the endogenous Ipl1. Cell proliferation was followed by measuring OD_{595} at the indicated times.

(C) Cells after 7 hr in glucose from experiment in (B) were fixed, and tubulin and DNA content were analyzed by indirect immunofluorescence. Graph shows quantification of anaphase cells with aberrant chromosome segregation.

(D) Model for Cdk1 regulation of the Ipl1-Sli15 complex. During metaphase, Cdk1 phosphorylates Sli15 and inhibits its MT-binding domain, whereas by acting directly on Ipl1, it prevents association with Bim1. These two events contribute to retention of the CPC at the centromere. At the metaphase-anaphase transition, dephosphorylation of Cdk1 sites on Sli15 and Ipl1 by Cdc14 loads the kinase complex onto spindle MTs and allows subsequent Bim1 phosphorylation. Graphs below depict coordination of Ipl1 and Bim1 phosphorylation during the cell cycle as a function of the Cdk1 activity. Cdk1-dependent phosphorylation of Ipl1 regulates association of the kinase with Bim1 and its phosphorylation at the final stages of anaphase. Elimination of the Cdk1 control (*ipl1-2A*) allows unregulated binding of Ipl1 to Bim1 and its constant phosphorylation.

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