Current Biology 24, 1456–1466, July 7, 2014 ©2014 Elsevier Ltd All rights reserved http://dx.doi.org/10.1016/j.cub.2014.05.032

Article

Molecular Mechanisms that Restrict Yeast Centrosome Duplication to One Event per Cell Cycle

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Summary

Background: The spindle pole body (SPB) of budding yeast is the functional equivalent of the mammalian centrosome. Like the centrosome, the SPB duplicates once per cell cycle. The new SPB assembles adjacent to the mother SPB at a substructure called the bridge. The half-bridge, the bridge precursor, is a one-sided extension of the SPB central plaque layered on both sides of the nuclear envelope. Parallel Sfi1 molecules longitudinally span the half-bridge with their N termini embedded in the SPB central plaque, whereas their C termini mark the half-bridge distal end. In early G1, half-bridge elongation by antiparallel C-to-C dimerization of Sfi1 exposes free N-Sfi1 where the new SPB assembles. After SPB duplication, the dimerized Sfi1 is severed to allow spindle formation and SPB reduplication.
Results: We show that Sfi1 C-terminal domain harbors phosphorylation sites for Cdk1 and the polo-like kinase Cdc5. Cdk1 and, to a lesser extent, Cdc5 inhibit SPB duplication as phosphomimetic *sfi1* mutations lead to metaphase cells with a single SPB. In contrast, phosphoinhibitory *sfi1* mutations in Cdk1 sites are lethal because cells fail to sever the bridge after SPB duplication. Moreover, Cdc14 dephosphorylates C-Sfi1 to prepare it for a new round of duplication, and the kinase Mps1 promotes Sfi1 extension in G1.
Conclusions: Positive (Cdc14) and negative (Cdk1 and Cdc5) SPB duplication signals are integrated at the level of the half-bridge component Sfi1. In addition, Mps1 activates Sfi1 duplication. Fluctuating activities of these regulators ensure one SPB duplication event per cell cycle.

Introduction

The yeast spindle pole body (SPB) is the functional equivalent of the mammalian centrosome. Both organize microtubules, and both are duplicated in a cell-cycle-dependent manner only once per cell cycle [1–3]. Misregulation of centrosome duplication in human cells leads to chromosome segregation defects and eventually to cancer [4].

The SPB is a multilayered structure that is embedded in the nuclear envelope (NE) throughout the cell cycle [5]. SPB duplication takes place at the half-bridge, an electron-dense, one-sided extension of the central SPB plaque that is layered on the nuclear and cytoplasmic sides of the NE [5, 6] (Figure 1A). SPB duplication starts in early G1 phase with the elongation of the half-bridge and the formation of the bridge structure, which, at its distal end, serves as an assembly platform for the formation of the new SPB [5]. In late S phase, the two side-by-side SPBs are separated by medial fission of the bridge to allow spindle formation by the two SPBs each bearing a half-bridge. Genetic data suggest that the kinesin-5 motor proteins Cin8 and Kip1, at least partly, drive this separation by creating a force that pulls both SPBs apart [12–14].

The half-bridge is composed of four proteins: the yeast centrin Cdc31 and its binding partner Sfi1, the tail-anchored protein Kar1 that also interacts with Cdc31, and the SUN domain protein Mps3 [15–22] (Figure 1A). Sfi1 plays a major role in half-bridge elongation. Sfi1 is a long filamentous protein that spans the length of the half-bridge on the cytoplasmic side of the NE. The N terminus of Sfi1 is close to the SPB central plaque, whereas the C terminus defines the distal end of the half-bridge [20, 21]. The central part of Sfi1 contains 20 centrin binding sites. Half-bridge extension is probably driven by C-to-C dimerization of Sfi1 molecules (Figure 1A). This leads to free Sfi1 N termini at the distal end of the bridge. It was suggested that in G1, Sfi1 N termini initiate the assembly of a miniature version of the SPB that is known as the "satellite." The satellite proceeds in the formation of the complete SPB after START of the cell cycle [5, 20] (Figure 1A).

SPB duplication is regulated by phosphorylation and dephosphorylation events. Cyclin-dependent kinase (Cdk1) activity either promotes or inhibits SPB duplication depending on the cell-cycle phase in which it acts [23]. Cdk1 in complex with G1 cyclins Cln1–Cln3 stimulates the formation of the new SPB by expanding the satellite into a full SPB [24]. S phase Cdk1 (Clb5 and Clb6) activity promotes separation of the two side-by-side SPBs by inhibiting APC^{Cdh1} in order to protect the motor proteins Cin8 and Kip1 from degradation [12–14, 25, 26]. Later in the cell cycle, mitotic Cdk1 (Clb1–Clb4) inhibits SPB reduplication through a mechanism that is currently unclear [23, 26, 27]. In addition, the kinase Mps1 is essential for a number of SPB duplication steps [28, 29].

Here, we show that SPB duplication in budding yeast is regulated through the phosphorylation and dephosphorylation of the C terminus of Sfi1 by the kinases Cdk1 and polo-like Cdc5 and the phosphatase Cdc14. Cdc14 drives mitotic exit by dephosphorylating a subset of Cdk1 substrates in anaphase [30]. Cdc14 and Mps1 kinase promote C-to-C dimerization of Sfi1 in G1. Phosphorylation of Sfi1 by Cdk1 and Cdc5 prevents this dimerization step in mitosis. After SPB duplication, phosphorylation of Sfi1 by Cdk1 is important for the separation of the two adjacent SPBs. Thus, we describe how phosphoregulation of Sfi1 restricts SPB duplication to one event per cell cycle.

Results

Cdk1 Phosphorylation Regulates Sfi1

The molecular basis by which cell-cycle regulators control the initiation of SPB duplication has yet to be elucidated. A potential target of Cdk1 is the half-bridge component Sfi1. Cells bearing a mutation in the *SFI1* gene that impairs phosphorylation of the Cdk1 phosphosite S855 are unable to grow in a background deficient in spindle assembly checkpoint (SAC;

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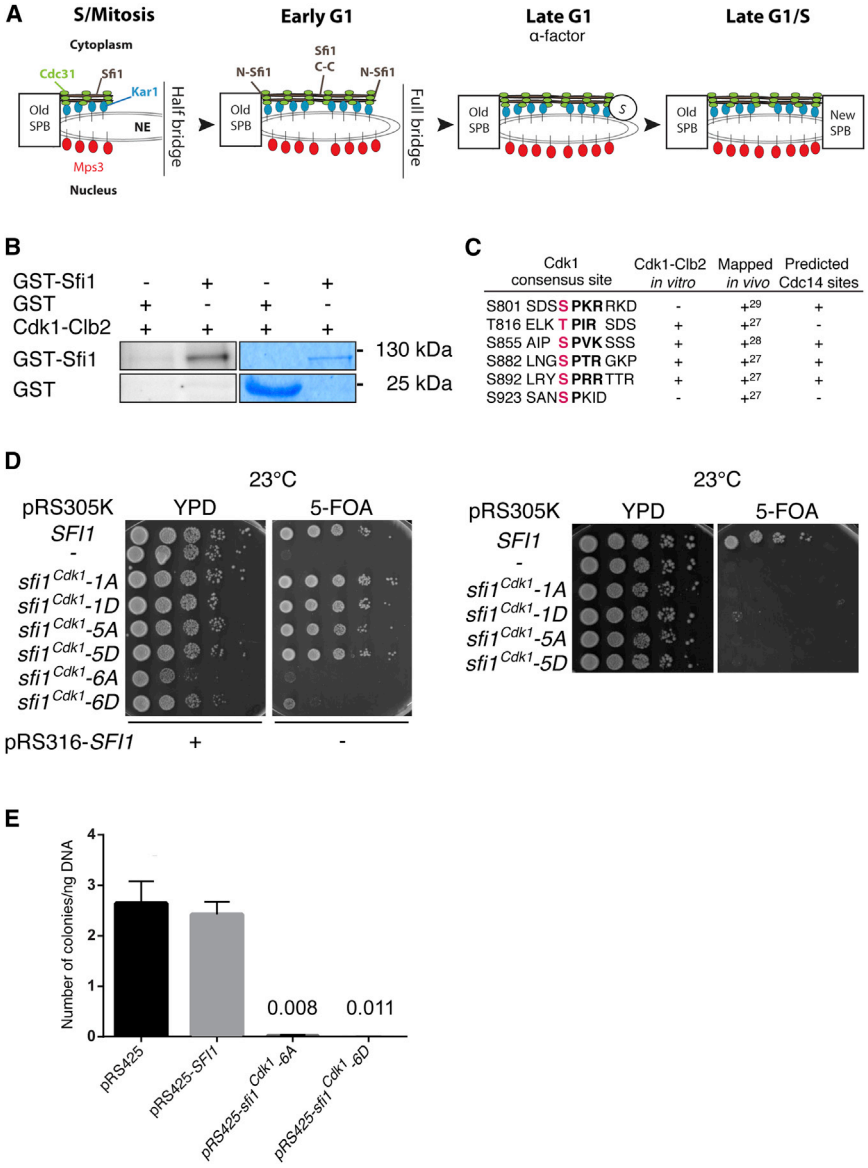

Figure 1. Cdk1 Phosphorylation Regulates Sfi1

(A) The SPB duplication model. Nuclear envelope (NE), N terminus of Sfi1 (N-Sfi1), C-terminal dimerization domain of Sfi1 (Sfi1 C-C), and Satellite (S) are shown here.

(B) In vitro kinase reaction with purified Cdk1-Clb2 and recombinant GST and GST-Sfi1. The kinase reaction was performed in the presence of γ-^{32}P-ATP. The experiment was analyzed by SDS-PAGE and autoradiography.

(C) Mass spectrometry analysis of Sfi1 phosphorylated by Cdk1-Clb2 in vitro. Shown are the identified Cdk1 consensus sites in the C terminus of Sfi1. These sites were also mapped in vivo [7–9]. The plus symbol (+) in the third column indicates Cdk1 sites (SPxK/R) predicted to be dephosphorylated by Cdc14 [10].

(D) Plasmid shuffle experiment to test growth of yeast cells containing *SFI1* alleles. The *SFI1* shuffle strain (*sfi1Δ::HIS3* pRS316-*Sfi1*) (left) and a *SFI1* shuffle strain with *mad2*Δ (right) were transformed with the integration plasmid pRS305K (−), pRS305K-*SFI1* (*SFI1*), or the indicated pRS305K-*sfi1*^{Cdk1}mutant alleles. Cells were incubated on YPD and 5-fluoroorotic acid (5-FOA) plates for 3 days. Drop tests were performed with serial 10-fold dilutions starting with equivalent cell concentrations.

(E) Dominancy of *sfi1*^{Cdk1}-6A and *sfi1*^{Cdk1}-6D alleles was tested by transforming high gene dosage pRS425 [11], pRS425-*SFI1*, pRS425-*sfi1*^{Cdk1}*-6A*, or pRS425-*sfi1*^{Cdk1}*-6D* plasmids in wild-type yeast strain ESM356. Shown is the mean of two independent experiments using the same batch of frozen competent ESM356 cells. Error bars show mean ± SD.

mad2Δ) because of an SPB separation defect [31]. To obtain a more-complete picture of how Cdk1 regulates Sfi1, we purified both proteins and performed an in vitro kinase reaction in the presence of γ-^{32}P-ATP. Sfi1 was phosphorylated by Cdk1-Clb2, whereas glutathione S-transferase (GST) alone was not (Figure 1B). Mass spectrometric analysis of in vitro phosphorylated Sfi1 identified four phosphosites (T816, S855, S882, and S892) in the C terminus of Sfi1. These sites correspond to the Cdk1 consensus S/TPxK/R (Figure 1C) and are phosphorylated in vivo [8, 9]. In addition, the Cdk1 consensus sites S801 and S923 in C-Sfi1 are also known to be phosphorylated in vivo [7, 8] (Figure 1C). Thus, Cdk1 is able to phosphorylate several sites within the C-terminal region of Sfi1.

To confirm that C-Sfi1 does not carry additional Cdk1 sites, we performed an in vitro kinase assay with C-Sfi1 and C-Sfi1^{6A} (serine and threonine residues of the six Cdk1 sites were changed to alanine) as substrates. We also tested whether the S phase cyclin Clb5 and the mitotic cyclin Clb2 have different specificity toward C-Sfi1. We coimmunoprecipitated Cdk1^{as1} in complex with either Clb5-TAP or Clb2-TAP from yeast lysates [32]. *cdk1*^{as1} encodes a mutated allele of *CDK1* that can be inhibited by the ATP analog 1NM-PP1 [33, 34]. The C terminus of Sfi1 was phosphorylated by both Cdk1^{as1}-Clb2 and Cdk1^{as1}-Clb5 (Figure S1A available online). However, Cdk1^{as1}-Clb2 was clearly more efficient in phosphorylating C-Sfi1 than Cdk1^{as1}-Clb5 was. In contrast, Cdk1^{as1}-Clb5 phosphorylated a contaminating peptide more efficiently than Cdk1^{as1}-Clb2. In the presence of the Cdk1^{as1} inhibitor 1NM-PP1, phosphorylation of C-Sfi1 could not be detected (Figure S1A). Importantly, Cdk1 did not phosphorylate the C terminus of Sfi1-6A, indicating the lack of additional Cdk1 phosphorylation sites in C-Sfi1.

We next asked which Cdk1-cyclin complex may regulate C-Sfi1 in vivo. In the yeast two-hybrid system, the mitotic cyclins Clb2, Clb3, and Clb4 showed weak to strong interactions with C-Sfi1, respectively (Figure S1B). The B type cyclin Clb4 is especially interesting because the Cdk1-Clb4 complex already binds in early S to the cytoplasmic side of the SPB where it may also regulate Sfi1 [35, 36].

The Cdk1 sites in C-Sfi1 were mutated to investigate their function. The phosphoacceptor serine and threonine residues of each Cdk1 site were mutated to alanine (phosphoinhibiting) or aspartic acid (phosphomimetic). *sfi1*^{Cdk1}*-1A/1D* (*sfi1-S855*) and *sfi1*^{Cdk1}*-5D* (*sfi1-S801D T816D S882D S892D S923D*) mutant cells grew at all temperatures tested, whereas *sfi1*^{Cdk1}*-5A* (*sfi1-S801A T816A S882A S892A S923A*) cells