Note

Analysis of Ipl1-Mediated Phosphorylation of the Ndc80 Kinetochore Protein in *Saccharomyces cerevisiae*

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ABSTRACT

Phosphorylation of the Ndc80 kinetochore protein by the Ipl1/Aurora B kinase reduces its microtubule binding activity *in vitro*. We found that kinetochore-bound Ndc80 is phosphorylated on Ipl1 sites *in vivo*, but this phosphorylation is not essential. Instead, we show that additional Ipl1 targets contribute to segregation and the spindle checkpoint.
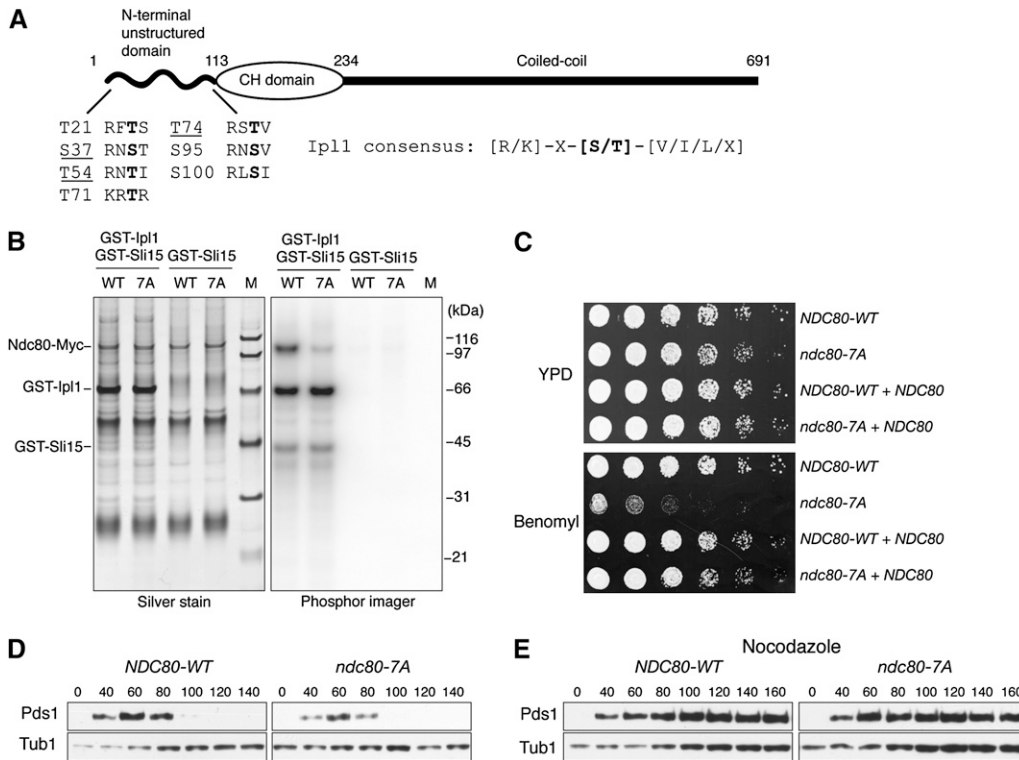
THE faithful transmission of genetic material requires chromosomes to biorient such that sister kinetochores attach to spindle microtubules from opposite poles. In the absence of biorientation, cells activate the spindle checkpoint to halt the cell cycle and give cells time to fix errors. The conserved Ipl1 protein kinase is essential for both error correction and checkpoint activation because it destabilizes aberrant interactions between kinetochores and microtubules, creating unattached kinetochores that trigger the checkpoint (BIGGINS *et al.* 1999; BIGGINS and MURRAY 2001; TANAKA *et al.* 2002; PINSKY *et al.* 2006b). The Ndc80 outer kinetochore protein that directly binds to microtubules has been proposed to be a key target of Ipl1 for biorientation because phosphorylation of its unstructured N terminus reduces its affinity toward microtubules *in vitro* (CHEESEMAN *et al.* 2006; DELUCA *et al.* 2006; WEI *et al.* 2007; CIFERRI *et al.* 2008; GUIMARAES *et al.* 2008; MILLER *et al.* 2008). However, it is not known whether Ndc80 is phosphorylated on kinetochores *in vivo*. In addition, the relative contributions of Ipl1-mediated phosphorylation of Ndc80 to segregation and the spindle checkpoint have not been analyzed.

We recently developed a method to detect phosphorylation on centromere-bound kinetochore proteins *in vivo* by isolating minichromosomes and then performing mass spectrometry (MS) (B. AKIYOSHI, C. R.

NELSON, J. A. RANISH, S. BIGGINS, unpublished data). We applied this method to identify Ipl1 phosphorylation on kinetochores by purifying minichromosomes from conditions where the activity of the Glc7 protein phosphatase 1 (PP1), which opposes Ipl1, was decreased (FRANCISCO *et al.* 1994). We overexpressed the Gip4 regulatory subunit that titrates Glc7 from the nucleus to enrich for cells arrested in mitosis with increased phosphorylation on Ipl1 targets (PINSKY *et al.* 2006a). When we analyzed the minichromosomes purified from this condition by MS, we detected three phosphorylated peptides corresponding to Ipl1 consensus sites in the N terminus of Ndc80 (S37, T54, and T74, Figure 1A and data not shown), providing the first evidence that kinetochore-bound Ndc80 is phosphorylated *in vivo* on its N-terminal domain. In addition, it was previously reported that S100 on Ndc80 is phosphorylated *in vivo* (CHEESEMAN *et al.* 2002). All of these residues map to the Ndc80 unstructured N-terminal domain that is phosphorylated by Ipl1/Aurora *in vitro* and implicated in regulating microtubule attachments to kinetochores in multicellular eukaryotes (CHEESEMAN *et al.* 2006; DELUCA *et al.* 2006; CIFERRI *et al.* 2008; GUIMARAES *et al.* 2008; MILLER *et al.* 2008). We therefore examined the potential relevance of phosphorylation of the budding yeast Ndc80 N-terminal region (1–112 residues) on the Ipl1 consensus sites in this domain by mutating all of them to create the Ndc80-7A mutant (Figure 1A). We confirmed that phosphorylation of the Ndc80-7A protein by Ipl1 is decreased *in vitro* (Figure 1B). The residual phosphorylation of Ndc80-7A is likely due to the presence of other putative Ipl1 sites outside of the N terminus. However, these sites are not conserved in

FIGURE 1.—An N-terminal Ndc80 mutant lacking Ipl1 phosphorylation sites is benomyl sensitive but spindle checkpoint proficient. (A) A schematic of Ndc80 shows that it contains seven putative Ipl1 phosphorylation Ser/Thr sites (shown in boldface type) in the unstructured N terminus. Phosphorylation sites detected by MS are underlined. Minichromosomes were purified from cells expressing Gip4 from the galactose promoter (SBY6247) for 3 hr and subjected to MS analysis (KIM *et al.* 2007 and unpublished data). Yeast strains used in this study are listed in supporting information, Table S1. (B) Phosphorylation of the Ndc80-7A phosphodeficient mutant by Ipl1 is reduced *in vitro*. Ndc80-WT-Myc (WT, SBY7258) and Ndc80-7A-Myc (7A, SBY7259) proteins were immunoprecipitated with anti-Myc antibodies and used as substrates *in vitro* for an Ipl1 kinase assay as previously described (BUVELOT *et al.* 2003). M, molecular weight markers. (C) *Ndc80-7A* mutant cells are viable, but show hypersensitivity to benomyl that is rescued by endogenous *NDC80*. Serial dilutions (fivefold) of *NDC80-WT* and *ndc80-7A* cells in the presence or absence of an endogenous *NDC80* were plated on YPD plates with or without 7.5 μg/ml benomyl (SBY7258, SBY7259, SBY6859, and SBY6860). (D) *Ndc80-7A* cells do not exhibit defects in cell-cycle progression. Cells containing Pds1-Myc and either *NDC80-WT* (SBY7120) or *ndc80-7A* (SBY7123) were released from G1 and lysates were prepared at the indicated time points and monitored for Pds1-Myc by immunoblot as previously described (BIGGINS *et al.* 1999). (E) *Ndc80-7A* cells activate the spindle checkpoint in response to microtubule depolymerization. The experiment in D was repeated by releasing cells into 10 μg/ml nocodazole.

multicellular eukaryotes and do not map to the microtubule binding region of Ndc80, and phosphorylation on these residues *in vivo* has not been reported.

We tested whether Ndc80-7A is functional *in vivo* by testing whether it could complement an *ndc80Δ* mutant. Strikingly, *ndc80-7A* cells were viable and did not exhibit any obvious growth defects at various temperatures (Figure 1C and data not shown), or defects in bipolar spindle assembly (data not shown). These data are consistent with the report that cells are viable when this domain is completely deleted (KEMMLER *et al.* 2009). In addition, there was no delay in cell cycle progression when the levels of the anaphase inhibitor Pds1 were monitored as cells were released from G1 (Figure 1D). However, although Ndc80-7A protein levels are similar to Ndc80 (data not shown), we found that *ndc80-7A* mutant cells exhibited hypersensitivity to the microtubule depolymerizing agent, benomyl (Figure 1C). Spindle checkpoint mutants are benomyl sensitive because the cell cycle continues when microtubules are depolymerized (HOYT *et al.* 1991; LI and MURRAY 1991). However, *ndc80-7A* cells activate the checkpoint and arrest in metaphase with high levels of Pds1 when treated with the microtubule destabilizing drug, noco-

dazole (Figure 1E). Therefore, the *ndc80-7A* mutant cells likely have altered microtubule dynamics that result in a benomyl-sensitive phenotype.

We next examined the importance of individual phosphorylation sites by reversing each site in *ndc80-7A* back to wild type, creating seven different *ndc80-6A* mutants. These mutants exhibited various degrees of sensitivity to benomyl and revealed that S37, S95, and S100 are more important than other residues (Figure 2A). To test whether specific sites are sufficient to lead to benomyl sensitivity, we constructed *ndc80-2A* (S95A S100A), *ndc80-3A* (S37A S95A S100A), and *ndc80-4A* (S37A T54A T71A T74A) mutants. However, none of these mutants were hypersensitive to benomyl (Figure 2B). Taken together, these results demonstrate that the presence of multiple sites is important, but that no single specific site is critical for Ndc80 function. The interaction between Ndc80 and microtubules is primarily electrostatic (the former being positively charged and the latter negatively charged), and the N-terminal unstructured domain is highly positively charged to strengthen the interaction (CHEESEMAN *et al.* 2006; DELUCA *et al.* 2006; WEI *et al.* 2007; CIFERRI *et al.* 2008; GUIMARAES *et al.* 2008; MILLER *et al.* 2008).
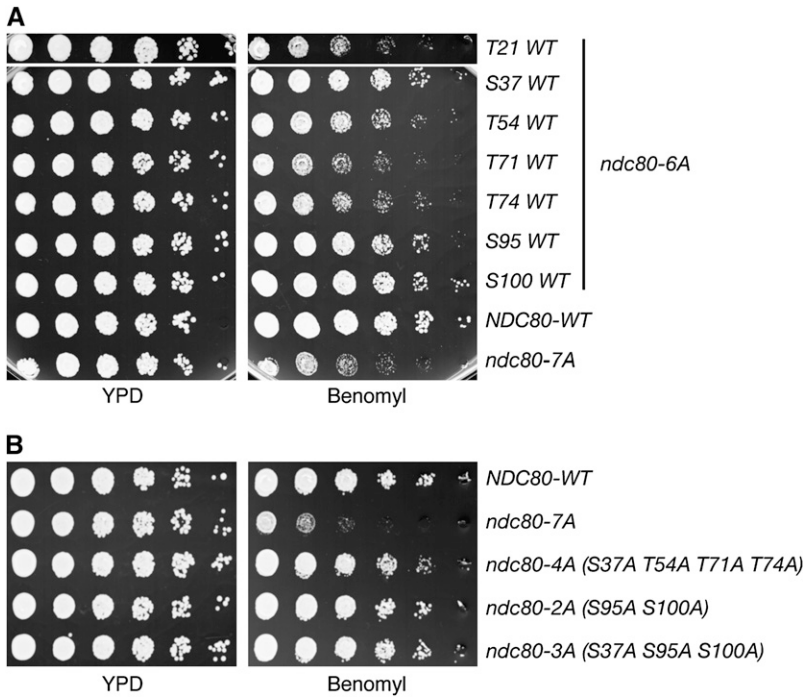
FIGURE 2.—Multiple phosphorylations on Ndc80 are important for proper microtubule dynamics. (A) *Ndc80-6A* mutants exhibit varying degrees of sensitivity to benomyl. Cells containing six mutations were assayed for benomyl sensitivity as in Figure 1C (SBY7257, SBY7255, SBY7252, SBY7328, SBY7329, SBY7326, SBY7327, SBY7258, and SBY7259). (B) *Ndc80-4A*, *ndc80-2A*, and *ndc80-3A* mutants do not exhibit benomyl hypersensitivity, indicating that there is no specific site required for the phenotype (SBY7258, SBY7259, SBY7296, SBY7325, and SBY8597).

Therefore, it is most likely that the overall charge state of the N-terminal domain, but not any specific site, is important for the function.

We previously found that Ipl1 is essential for checkpoint activation when kinetochores are not under tension, possibly due to its role in generating unattached kinet-ochores that signal the checkpoint (BIGGINS and MURRAY 2001; PINSKY *et al.* 2006b). We therefore tested whether the *ndc80-7A* mutant is defective in the tension checkpoint by analyzing Pds1 levels in two conditions that create tension defects (a cohesion defect and a bipolar spindle assembly defect). Cells containing *ndc80-7A* activated
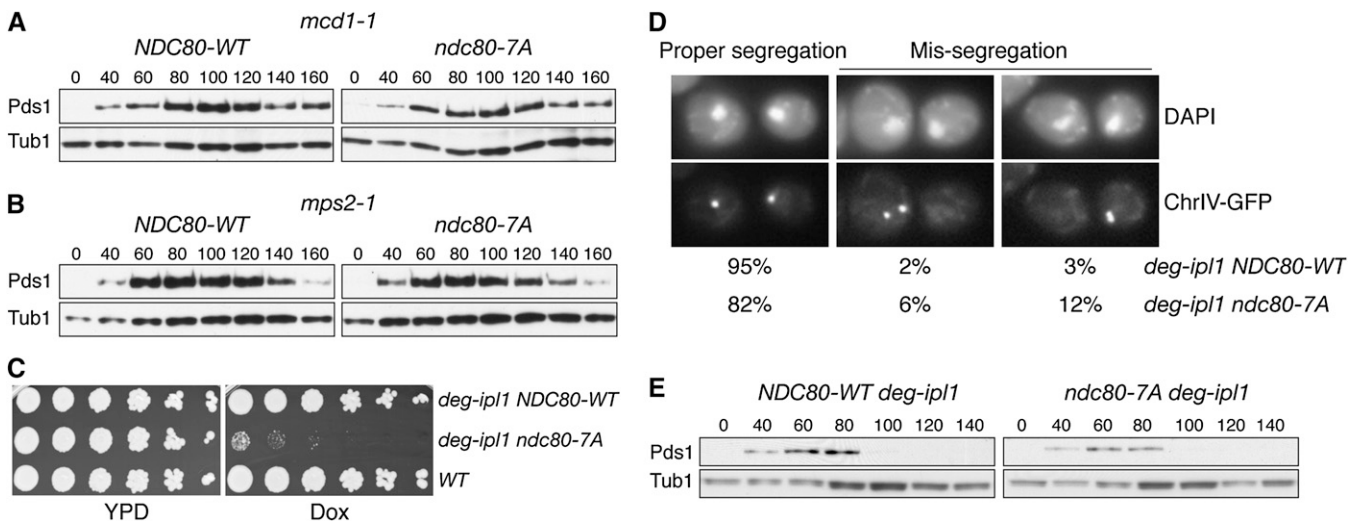

FIGURE 3.—The Ndc80 N-terminal domain is not the sole Ipl1 downstream target for segregation and spindle checkpoint activation. (A and B) *Ndc80-7A* mutant cells do not bypass the checkpoint in response to a defect in cohesion (A; *mcd1-1*) or bipolar spindle assembly (B; *mps2-1*). Cells containing either *NDC80-WT* (SBY7131, SBY7118) or *ndc80-7A* (SBY7133, SBY7121) were arrested in G1, released to 37°, and analyzed as in Figure 1D. (C) *Ndc80-7A* is synthetically lethal with *deg-ipl1*. Serial dilutions (fivefold) of *deg-ipl1 NDC80-WT* (SBY8325), *deg-ipl1 ndc80-7A* (SBY8241), and WT (SBY818) cells were plated onto YPD with or without 25 µg/ml Dox. (D and E) The *ndc80-7A deg-ipl1* mutant exhibits chromosome missegregation but does not activate the spindle checkpoint. Cells containing *deg-ipl1 NDC80-WT* or *deg-ipl1 ndc80-7A* were arrested in G1 for 2 hr and then 25 µg/ml Dox was added for an additional 1.5 hr. Cells were released into media containing Dox and either fixed at 140 min for microscopy (D), as previously described (BIGGINS *et al.* 1999) [examples of proper segregation (left), missegregation to the mother cell (middle), or daughter cell (right) are shown] or monitored for Pds1 levels at the indicated time points (E).

the checkpoint in both conditions (Figure 3, A and B), suggesting that Ipl1 phosphorylates one or more additional targets or sites in Ndc80 to activate the spindle checkpoint.

If Ndc80 contributes to Ipl1 functions *in vivo*, we expected that the *ndc80-7A* mutant would exhibit genetic interactions with *ipl1* mutants. Consistent with this, we found that *ndc80-7A* is synthetically lethal with the *ipl1-321* temperature sensitive mutant (data not shown). It is noteworthy that an *ndc80-1* temperature-sensitive allele does not exhibit lethal interactions with *ipl1-321* (Pinsky *et al.* 2006b), suggesting that *ndc80-7A* is more specifically defective in an Ipl1-related function than the *ndc80-1* mutant. This result also suggested that *ipl1-321* cells retain enough activity for survival when Ndc80 sites can be phosphorylated by Ipl1, but becomes lethal when Ndc80 has reduced capacity to be phosphorylated by Ipl1. Taken together, these data strongly suggest that there are unidentified targets of Ipl1. Interestingly, *ndc80-7A* is also synthetically lethal with the *mps1-1* temperature-sensitive mutant (data not shown). Because Mps1 targets some of the Ipl1 sites of Ndc80 (Kemmler *et al.* 2009), this result may suggest that Ipl1 and Mps1 have overlapping substrate specificity *in vivo*. Alternatively, Mps1 may be required for activating Ipl1 or vice versa (Jelluma *et al.* 2008).

To directly analyze whether Ndc80 phosphorylation contributes to the biorientation function of Ipl1, we created a conditional *ndc80-7A deg-ipl1* double mutant to analyze chromosome segregation. We previously found that although *deg-ipl1* has reduced function, it retains viability even when transcription is repressed by the addition of doxycycline (Dox) (Ng *et al.* 2009). Consistent with this, *deg-ipl1 ndc80-7A* double mutant cells grew in the absence of Dox, but *deg-ipl1* function was sufficiently reduced to be inviable when combined with *ndc80-7A* in the presence of Dox (Figure 3C). This allowed us to analyze segregation in the double mutant cells by releasing them from G1 into media containing Dox and monitoring the fate of a fluorescently marked chromosome (Straight *et al.* 1996). Strikingly, 18% of cells missegregated Chr IV to the same pole instead of opposite poles (Figure 3D), strongly suggesting that the lethality of the double mutant is due to a biorientation defect. If every chromosome exhibited a similar biorientation defect, few cells would remain viable. The chromosome segregation defect in these cells should lead to activation of the spindle checkpoint, so we analyzed Pds1 levels in cells released from G1. However, similar to *ipl1* mutants (Biggins and Murray 2001; Pinsky *et al.* 2006b), the *ndc80-7A deg-ipl1* cells did not activate the checkpoint in response to the segregation defect (Figure 3E). Taken together, these data suggest that N-terminal Ndc80 phosphorylation contributes to both the segregation and checkpoint functions of Ipl1.

In summary, our studies show that multiple Ipl1 sites on the N-terminal domain of Ndc80 are phosphorylated

when it is associated with kinetochores *in vivo*. Strikingly, although this phosphorylation contributes to the role of Ipl1 in both chromosome segregation and spindle checkpoint activation, our data strongly support the idea that additional key targets of Ipl1 must be phosphorylated in combination with Ndc80 to carry out the precise functions of the kinase. In the future, it will be critical to identify the complete complement of these phosphorylation events to fully understand the mechanism by which Ipl1 mediates segregation and checkpoint activation to ensure genomic stability.

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GENETICS

Analysis of Ipl1-Mediated Phosphorylation of the Ndc80 Kinetochore Protein in *Saccharomyces cerevisiae*

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TABLE S1

Yeast strains used in this study. All strains are isogenic with the W303 background. Plasmids are indicated in brackets.

Strain	Genotype
SBY3	*MAT**a** ura3-1 leu2,3-112 his3-11 trp1-1 ade2-1 LYS2 can1-100 bar1Δ*
SBY818	*MAT**a** ura3-1 leu2,3-112 his3-11::pCUP1-GFP12-lacI12:HIS3 trp1-1:256lacO::TRP1 ade2-1 lys2Δ can1-100 bar1Δ PDS1-myc18:LEU2*
SBY6247	*MAT**a** ura3-1::pCMV-LacI-3FLAG::URA3 leu2,3-112 his3-11 trp1-1 ade2-1 can1-100 lys2Δ bar1Δ pGAL-HA3-GIP4 PDS1-myc18:LEU2 [WT CEN3 Minichromosome, TRP1]*
SBY6859	*MAT**a** ura3-1::NDC80-WT-12myc::URA3 leu2,3-112 his3-11 trp1-1 ade2-1 can1-100 bar1Δ FIN1-3FLAG::KAN*
SBY6860	*MAT**a** ura3-1::ndc80-7A-12myc::URA3 leu2,3-112 his3-11 trp1-1 ade2-1 can1-100 bar1Δ FIN1-3FLAG::KAN*
SBY6932	*MAT**a**/α ura3-1/ura3-1 leu2,3-112/leu2,3-112 his3-11/his3-11 trp1-1/trp1-1 ade2-1/ade2-1 LYS2/LYS2 can1-100/can1-100 bar1Δ/bar1Δ NDC80/ndc80ΔHIS3*
SBY7118	*MAT**a** ura3-1::NDC80-WT::URA3 leu2,3-112 his3-11 trp1-1 ade2-1 lys2Δ can1-100 bar1Δ ndc80ΔHIS3 PDS1-myc18:LEU2 mps2-1*
SBY7120	*MAT**a** ura3-1::NDC80-WT::URA3 leu2,3-112 his3-11 trp1-1 ade2-1 lys2Δ can1-100 bar1Δ ndc80ΔHIS3 PDS1-myc18:LEU2*
SBY7121	*MAT**a** ura3-1::ndc80-7A::URA3 leu2,3-112 his3-11 trp1-1 ade2-1 lys2Δ can1-100 bar1Δ ndc80ΔHIS3 PDS1-myc18:LEU2 mps2-1*
SBY7123	*MAT**a** ura3-1::ndc80-7A::URA3 leu2,3-112 his3-11 trp1-1 ade2-1 LYS2 can1-100 bar1Δ ndc80ΔHIS3 PDS1-myc18:LEU2*
SBY7131	*MAT**a** ura3-1::NDC80-WT::URA3 leu2,3-112 his3-11 trp1-1 ade2-1 can1-100 bar1Δ ndc80ΔHIS3 PDS1-myc18:LEU2 mcd1-1*
SBY7133	*MAT**a** ura3-1::ndc80-7A::URA3 leu2,3-112 his3-11 trp1-1 ade2-1 can1-100 bar1Δ ndc80ΔHIS3 PDS1-myc18:LEU2 mcd1-1*
SBY7229	*MAT**a** ura3-1 leu2,3-112 his3-11 trp1-1 ade2-1 can1-100 bar1Δ ndc80ΔHIS3 [NDC80-WT, CEN, URA3 (pSB1284)]*
SBY7252	*MAT**a** ura3-1 leu2,3-112 his3-11 trp1-1 ade2-1 can1-100 bar1Δ ndc80ΔHIS3::ndc80-6A(T54WT)-myc12::TRP1*
SBY7255	*MAT**a** ura3-1 leu2,3-112 his3-11 trp1-1 ade2-1 can1-100 bar1Δ ndc80ΔHIS3::ndc80-6A(S37WT)-myc12::TRP1*
SBY7257	*MAT**a** ura3-1 leu2,3-112 his3-11 trp1-1 ade2-1 can1-100 bar1Δ ndc80ΔHIS3::ndc80-6A(T21WT)-myc12::TRP1*
SBY7258	*MAT**a** ura3-1 leu2,3-112 his3-11 trp1-1 ade2-1 can1-100 bar1Δ ndc80ΔHIS3::NDC80-WT-myc12::TRP1*
SBY7259	*MAT**a** ura3-1 leu2,3-112 his3-11 trp1-1 ade2-1 can1-100 bar1Δ ndc80ΔHIS3::ndc80-7A-myc12::TRP1*
SBY7296	*MAT**a** ura3-1::NDC80-4A(S37A T54A T71A T74A)-myc12::URA3 leu2,3-112 his3-11 trp1-1 ade2-*

	1 can1-100 bar1Δ ndc80ΔHIS3
SBY7325	*MAT**a** ura3-1 leu2,3-112 his3-11 trp1-1 ade2-1 can1-100 bar1Δ ndc80ΔHIS3::ndc80-2A(S95A S100A)-myc12::TRP1*
SBY7326	*MAT**a** ura3-1 leu2,3-112 his3-11 trp1-1 ade2-1 can1-100 bar1Δ ndc80ΔHIS3::ndc80-6A(S95WT)-myc12::TRP1*
SBY7327	*MAT**a** ura3-1 leu2,3-112 his3-11 trp1-1 ade2-1 can1-100 bar1Δ ndc80ΔHIS3::ndc80-6A(S100WT)-myc12::TRP1*
SBY7328	*MAT**a** ura3-1 leu2,3-112 his3-11 trp1-1 ade2-1 can1-100 bar1Δ ndc80ΔHIS3::ndc80-6A(T71WT)-myc12::TRP1*
SBY7329	*MAT**a** ura3-1 leu2,3-112 his3-11 trp1-1 ade2-1 can1-100 bar1Δ ndc80ΔHIS3::ndc80-6A(T74WT)-myc12::TRP1*
SBY8241	*MAT**a** ura3-1 leu2,3-112 his3-11::pCUP1-GFP12-lacI12:HIS3 trp1-1:256lacO::TRP1 ade2-1::7tetOp-UB-R-IPL1::ADE2 LYS2 can1-100 bar1Δ PDS1-myc18:LEU2 ipl1ΔKAN ndc80ΔNAT::ndc80-7A::TRP1*
SBY8325	*MAT**a** ura3-1 leu2,3-112 his3-11::pCUP1-GFP12-lacI12:HIS3 trp1-1:256lacO::TRP1 ade2-1::7tetOp-UB-R-IPL1::ADE2 LYS2 can1-100 bar1Δ PDS1-myc18:LEU2 ipl1ΔKAN ndc80ΔNAT::NDC80-WT::TRP1*
SBY8597	*MAT**a** ura3-1 leu2,3-112 his3-11 trp1-1 ade2-1 can1-100 bar1Δ ndc80ΔHIS3::ndc80-3A(S37A S95A S100A)-myc12::TRP1*

The *mcd1-1* (GUACCI *et al.* 1997), *mps2-1* (WINEY *et al.* 1991), *ipl1-321* (BIGGINS *et al.* 1999), *pGAL-HA-Gip4* (PINSKY *et al.* 2006) and *deg-ipl1* (NG *et al.* 2009) alleles were crossed to make strains for this study. Deletions were made using a PCR-based integration system and confirmed by PCR (LONGTINE *et al.* 1998; GOLDSTEIN and MCCUSKER 1999). To generate pSB1225 (*pNDC80-NDC80-WT-12Myc, URA3*), the *NDC80* gene with the upstream 600 bp was PCR amplified with primers SB1949 and SB1975 that have *Xho*I and *Xma*I site engineered respectively, and cloned into the same sites in pSB167 (BIGGINS *et al.* 1999). pSB1277 (*pNDC80-NDC80-WT-12Myc, TRP1*) was made by digesting pSB1225 with *Xho*I and *Sac*II and cloned into the same sites in pRS304 (SIKORSKI and HIETER 1989). pSB1273 (*pNDC80-NDC80-WT-12Myc, CEN, URA3*) was made by digesting pSB1225 with *Xho*I and *Sac*II, and cloned into the same sites in pRS316 (SIKORSKI and HIETER 1989). pSB1284 (*pNDC80-NDC80-WT, CEN, URA3*) was made by introducing a stop codon just prior to the 12Myc ORF of pSB1273 using a Quickchange site-directed mutagenesis kit (Stratagene). Ipl1 consensus sites in the N-terminal unstructured domain were mutated using Quickchange site-directed mutagenesis (Stratagene). Specific primers for all techniques are available upon request. pSB1225 and its derivatives were digested with *Stu*I and integrated into SBY6932 at the *URA3* locus, and transformants were dissected. pSB1277 and its derivatives were digested with *BstE*II and integrated into the *NDC80* promoter region of SBY7229, and then the plasmid pSB1284 (*pNDC80-NDC80-WT, CEN, URA3*) was selected against using 5-FOA (BOEKE *et al.* 1987).

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