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Glc7/Protein Phosphatase 1 Regulatory Subunits Can Oppose the Ipl1/Aurora Protein Kinase by Redistributing Glc7

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Faithful chromosome segregation depends on the opposing activities of the budding yeast Glc7/PP1 protein phosphatase and Ipl1/Aurora protein kinase. We explored the relationship between Glc7 and Ipl1 and found that the phosphorylation of the Ipl1 substrate, Dam1, was altered by decreased Glc7 activity, whereas Ipl1 levels, localization, and kinase activity were not. These data strongly suggest that Glc7 ensures accurate chromosome segregation by dephosphorylating Ipl1 targets rather than regulating the Ipl1 kinase. To identify potential Glc7 and Ipl1 substrates, we isolated *ipl1-321* dosage suppressors. Seven genes (*SDS22*, *BUD14*, *GIP3*, *GIP4*, *SOL1*, *SOL2*, and *PEX31*) encode newly identified *ipl1* dosage suppressors, and all 10 suppressors encode proteins that physically interact with Glc7. The overexpression of the Gip3 and Gip4 suppressors altered Glc7 localization, indicating they are previously unidentified Glc7 regulatory subunits. In addition, the overexpression of Gip3 and Gip4 from the galactose promoter restored Dam1 phosphorylation in *ipl1-321* mutant cells and caused wild-type cells to arrest in metaphase with unsegregated chromosomes, suggesting that Gip3 and Gip4 overexpression impairs Glc7's mitotic functions. We therefore propose that the overexpression of Glc7 regulatory subunits can titrate Glc7 away from relevant Ipl1 targets and thereby suppress *ipl1-321* cells by restoring the balance of phosphatase/kinase activity.

The accurate partitioning of the genome during mitosis requires the precise regulation of the connection between chromosomes and the mitotic spindle. This fundamental interaction is mediated by the kinetochore, a specialized protein complex that assembles on centromeric DNA and facilitates the capture of dynamic spindle microtubules that arise from opposite poles (for reviews, see references 5, 13, and 17). Bipolar attachments promote accurate chromosome segregation by ensuring that the spindle forces on the replicated chromosomes (sister chromatids) are directed toward opposite sides of the cell. Once all chromosomes make proper bipolar attachments, the cell transitions to anaphase where sister chromatids are pulled to opposite poles. Failure to achieve bipolar attachments results in chromosome missegregation, and this aneuploid state predisposes multicellular organisms to the development of a variety of diseases. To prevent the premature segregation of improperly attached chromosomes, the spindle checkpoint monitors kinetochore-microtubule interactions and delays the metaphase to anaphase transition until bipolar attachments are achieved (for a review, see reference 42).

An important regulator of both kinetochore attachment and the spindle checkpoint is the conserved Ipl1/Aurora B protein kinase, a component of the chromosomal passenger complex that localizes to kinetochores, spindles, and the spindle midzone and midbody (for reviews, see references 25 and 69). Ipl1/Aurora B facilitates proper attachments by destabilizing inappropriate kinetochore-microtubule interactions, such as monopolar attachments in which kinetochores bind microtubules emanating from the same pole (4, 12, 39, 54, 63). Despite the presence of improper attachments that should activate the spindle checkpoint, cells with impaired Ipl1/Aurora B function proceed through the cell cycle (3, 10, 19, 26, 40). Ipl1 is thought to promote proper chromosome segregation, in part, by phosphorylating components of the Dam1/DASH/DDD complex, an essential regulator of kinetochore-microtubule interactions and microtubule function (15, 16, 34, 35, 43, 44, 48, 59, 73).

Ipl1 activity is opposed by Glc7, the sole essential protein phosphatase 1 (PP1) catalytic subunit in budding yeast (21, 22, 32, 58, 76). Glc7 regulates numerous cellular processes including mitosis, meiosis, glycogen and sugar metabolism, transcription, translation, and mRNA processing (for a review, see reference 11). The regulation of these processes is guided by Glc7 interactions with specific regulatory subunits that target the phosphatase to appropriate substrates. Many *glc7* alleles cause cells to arrest in mitosis (1, 6, 29, 46), suggesting that Glc7 substrates must be dephosphorylated to allow cell cycle progression. Furthermore, impairing Glc7 function suppresses the *ipl1* temperature-sensitive growth defect and restores the phosphorylation of the Ipl1 targets Ndc10 and histone H3, indicating that Glc7 antagonizes Ipl1-mediated phosphorylation (21, 22, 32, 58). In addition, genetic interactions between *glc7* mutants and mutants that alter the phosphorylation status of the Ipl1 substrate Dam1 also support this idea (15, 76). Consistent with this, some *glc7* mutants activate the spindle checkpoint and exhibit reduced kinetochore binding to microtubules in vitro (7, 58). Despite these observations, the precise relationship between the kinase and phosphatase is not well understood, and Glc7 regulation of Ipl1 function has not been examined.

Here, we further explore the relationship between Ipl1 and Glc7. We found that Glc7 does not appear to directly modulate

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TABLE 1. Yeast strains used in this study[a]

Strain	Genotype
SBY3	*MAT***a** *ura3-1 leu2,3-112 his3-11 trp1-1 ade2-1 can1-100 bar1*Δ
SBY214	*MAT***a** *ura3-1 leu2,3-112 his3-11*::*pCUP1-GFP12-lacI12*::*HIS3 trp1-1*::*256lacO*::*TRP1 lys2*Δ *ade2-1 can1-100 bar1*Δ
SBY322	*MAT***a** *ura3-1 leu2,3-112 his3-11*::*pCUP1-GFP12-lacI12*::*HIS3 trp1-1*::*256lacO*::*TRP1 lys2*Δ *ade2-1 can1-100 bar1*Δ *ipl1-321*
SBY625	*MAT***a** *ura3-1 leu2,3-112 his3-11 trp1-1 ade2-1 can1-100 bar1*Δ *GLC7-HA3*::*HIS3*
SBY818	*MAT***a** *ura3-1 leu2,3-112 his3-11*::*pCUP1-GFP12-lacI12*::*HIS3 trp1-1*::*256lacO*::*TRP1 lys2*Δ *ade2-1 can1-100 bar1*Δ *PDS1-myc18*::*LEU2*
SBY1063	*MAT***a** *ura3-1 leu2,3-112*::*pGAL-ipl1*(*R343A*)::*LEU2 his3-11*::*pCUP1-GFP12-lacI12*::*HIS3 trp1-1*::*256lacO*::*TRP1 lys2*Δ *ade2-1 can1-100 bar1*Δ *ipl1-321*
SBY1258	*MAT***a** *ura3-1 leu2,3-112 his3-11 trp1-1*::*glc7-10*::*TRP1 ade2-1 can1-100 glc7*::*LEU2 ipl1*Δ*KAN*::*ipl1T260A*::*LEU2*
SBY1264	*MAT***a** *ura3-1 leu2,3-112 his3-11 trp1-1*::*GLC7*::*TRP1 ade2-1 can1-100 glc7*::*LEU2 ipl1*Δ*KAN*::*ipl1T260A*::*LEU2*
SBY1306	*MAT***a** *ura3-1 leu2,3-112 his3-11 trp1-1*::*glc7-10*::*TRP1 ade2-1 can1-100 glc7*::*LEU2 bar1*Δ
SBY1994	*MAT***a** *ura3-1 leu2,3-112 his3-11 trp1-1*::*glc7-10*::*TRP1 ade2-1 can1-100 bar1*Δ *glc7*::*LEU2 ipl1-321*
SBY2055	*MAT***a** *ura3-1 leu2,3-112 his3-11 trp1-1 lys2*Δ *ade2-1 can1-100 bar1*Δ *DAM1-myc9*::*TRP1*
SBY2833	*MAT***a** *ura3-1*::*TUB1-CFP*::*URA3 leu2,3-112 his3-11 trp1-1 ade2-1 can1-100 bar1*Δ *IPL1-GFP3*::*HIS3*
SBY3672	*MAT***a** *ura3-1 leu2,3-112 his3-11 trp1-1 ade2-1 can1-100 bar1*Δ *IPL11-FLAG3*::*KAN*
SBY3675	*MAT***a** *ura3-1 leu2,3-112 his3-11 trp1-1*::*glc7-12*::*TRP1 ade2-1 can1-100 bar1*Δ *glc7*::*LEU2*
SBY4114	*MAT***a** *ura3-1 leu2,3-112 his3-11 trp1-1 ade2-1 can1-100 bar1*Δ *gip3*Δ*KAN*
SBY4175	*MAT***a** *ura3-1 leu2,3-112 his3-11 trp1-1 ade2-1 can1-100 bar1*Δ *gip4*Δ*KAN*
SBY4179	*MAT***a** *ura3-1 leu2,3-112 his3-11 trp1-1 ade2-1 can1-100 bar1*Δ *pGAL-HA3-GIP3*::*HIS3*
SBY4209	*MAT***a** *ura3-1 leu2,3-112 his3-11 trp1-1 ade2-1 can1-100 bar1*Δ *GLC7-HA3*::*HIS3 GIP4-myc13*::*KAN*
SBY4292	*MAT***a** *ura3-1 leu2,3-112 his3-11 trp1-1 ade2-1 lys2*Δ *can1-100 bar1*Δ *pGAL-HA3-GIP4*::*HIS3 PDS1-myc18*::*LEU2*
SBY4541	*MAT***a** *ura3-1 leu2,3-112 his3-11 trp1-1 ade2-1 can1-100 bar1*Δ *ipl1-321-FLAG3*::*KAN*
SBY4764	*MAT***a** *ura3-1 leu2,3-112 his3-11 trp1-1 ade2-1 can1-100 bar1*Δ *DAM1-myc9*::*TRP1 ipl1-321*
SBY4801	*MAT***a** *ura3-1 leu2,3-112 his3-11 trp1-1*::*glc7-10*::*TRP1 ade2-1 can1-100 bar1*Δ *glc7*::*LEU2 ipl1-321 DAM1-myc9*::*TRP1*
SBY4822	*MAT***a** *ura3-1 leu2,3-112 his3-11 trp1-1 ade2-1 can1-100 bar1*Δ *GLC7-HA3*::*HIS3 IPL1-myc13*::*KAN*
SBY4826	*MAT***a** *ura3-1 leu2,3-112 his3-11 trp1-1*::*glc7-10*::*TRP1 ade2-1 can1-100 bar1*Δ *glc7*::*LEU2 DAM1-myc9*::*TRP1*
SBY4892	*MAT***a** *ura3-1 leu2,3-112 his3-11 trp1-1 ade2-1 can1-100 bar1*Δ *GLC7-3GFP*::*HIS3 NIC96-CFP*::*KAN*
SBY4874	*MAT***a** *ura3-1 leu2,3-112 his3-11 trp1-1 ade2-1 can1-100 bar1*Δ *GLC7-HA3*::*HIS3 SOL1-myc13*::*KAN*
SBY4920	*MAT***a** *ura3-1 leu2,3-112 his3-11 trp1-1 ade2-1 can1-100 bar1*Δ *GLC7-HA3*::*HIS3 PEX31-myc13*::*KAN*
SBY4995	*MAT***a** *ura3-1 leu2,3-112 his3-11 trp1-1 ade2-1 can1-100 bar1*Δ *GLC7-3GFP*::*HIS3 NIC96-CFP*::*KAN pGAL-HA3-GIP3*::*HIS3*
SBY4999	*MAT***a** *ura3-1*::*TUB1-CFP*::*URA3 leu2,3-112 his3-11 trp1-1*::*glc7-10*::*TRP1 ade2-1 can1-100 bar1*Δ *glc7*::*LEU2 IPL1-3GFP*::*HIS3*
SBY5004	*MAT***a** *ura3-1 leu2,3-112 his3-11 trp1-1*::*glc7-12*::*TRP1 ade2-1 can1-100 bar1*Δ *glc7*::*LEU2 ipl1-321*
SBY5070	***MAT*a**/α *ura3-1/ura3-1 leu2,3-112/leu2,3-112 his3-11*::*pCUP1-GFP12-lacI12*::*HIS3/his3-11,15 trp1-1*::*GLC7*::*TRP1/trp1-1*::*256lacO*::*TRP1 ade2-1/ade2-1 lys2*Δ*/LSY2 can1-100/can1-100 bar1*Δ*/bar1*Δ *ipl1-321/IPL1 GLC7/glc7*::*LEU2*
SBY5072	*MAT***a**/α *ura3-1/ura3-1 leu2,3-112/leu2,3-112 his3-11*::*pCUP1-GFP12-lacI12*::*HIS3/his3-11,15 trp1-1*::*GLC7*::*TRP1/trp1-1*::*256lacO*::*TRP1 ade2-1/ade2-1 lys2*Δ*/LSY2 can1-100/can1-100 bar1*Δ*/bar1*Δ *ipl1-321/ipl1-321 GLC7/glc7*::*LEU2*
SBY5074	*MAT***a**/α *ura3-1/ura3-1 leu2,3-112/leu2,3-112 his3-11*::*pCUP1-GFP12-lacI12*::*HIS3/his3-11,15 trp1-1*::*glc7-12*::*TRP1 trp1-1*::*256lacO*::*TRP1 ade2-1/ade2-1 lys2*Δ*/LSY2 can1-100/can1-100 bar1*Δ*/bar1*Δ *ipl1-321/ipl1-321 GLC7/glc7*::*LEU2*
SBY5127	*MAT***a** *ura3-1 leu2,3-112 his3-11 trp1-1 ade2-1 can1-100 bar1*Δ *DAM1-myc9*::*TRP1 ipl1-321 pGAL-HA3-GIP3*::*HIS3*
SBY5128	*MAT***a** *ura3-1 leu2,3-112 his3-11 trp1-1 ade2-1 can1-100 bar1*Δ *DAM1-myc9*::*TRP1 ipl1-321 pGAL-HA3-GIP4*::*HIS3*
SBY5032	*MAT***a** *ura3-1 leu2,3-112 his3-11 trp1-1*::*glc7-12*::*TRP1 lys2*Δ *ade2-1 can1-100 bar1*Δ *glc7*::*LEU2 DAM1-myc9*::*TRP1 ipl1-321*
SBY5034	*MAT***a** *ura3-1 leu2,3-112 his3-11 trp1-1*::*glc7-12*::*TRP1 lys2*Δ *ade2-1 can1-100 bar1*Δ *glc7*::*LEU2 DAM1-myc9*::*TRP1*
SBY5274	*MAT***a** *ura3-1 leu2,3-112 his3-11 trp1-1 ade2-1 can1-100 bar1*Δ *gip3*Δ*KAN DAM1-myc9*::*TRP1*
SBY5275	*MAT***a** *ura3-1 leu2,3-112 his3-11 trp1-1 ade2-1 can1-100 bar1*Δ *DAM1-myc9*::*TRP1*
SBY5276	*MAT***a** *ura3-1 leu2,3-112 his3-11 trp1-1 ade2-1 can1-100 bar1*Δ *gip4*Δ*KAN DAM1-myc9*::*TRP*
SBY5277	*MAT***a** *ura3-1 leu2,3-112 his3-11 trp1-1*::*glc7-10*::*TRP1 ade2-1 can1-100 bar1*Δ *glc7*::*LEU2 ipl1-321-3FLAG*::*KAN*
SBY5278	*MAT***a** *ura3-1 leu2,3-112 his3-11 trp1-1*::*glc7-10*::*TRP1 ade2-1 can1-100 bar1*Δ *glc7*::*LEU2 IPL1-3FLAG*::*KAN*
SBY5279	*MAT***a** *ura3-1 leu2,3-112 his3-11*::*pCUP1-GFP12-lacI12*::*HIS3 trp1-1*::*256lacO*::*TRP1 ade2-1 can1-100 bar1*Δ *pGAL-HA3-GIP3*::*HIS3*
SBY5284	*MAT***a** *ura3-1 leu2,3-112 his3-11 trp1-1 ade2-1 can1-100 bar1*Δ *GLC7-3GFP*::*HIS3 gip3*Δ*KAN*
SBY5285	*MAT***a** *ura3-1 leu2,3-112 his3-11 trp1-1 ade2-1 can1-100 bar1*Δ *GLC7-3GFP*::*HIS3*
SBY5287	*MAT***a** *ura3-1 leu2,3-112 his3-11*::*pCUP1-GFP12-lacI12*::*HIS3 trp1-1*::*256lacO*::*TRP1 ade2-1 can1-100 gip3*Δ*KAN bar1*Δ
SBY5288	*MAT***a** *ura3-1 leu2,3-112 his3-11*::*pCUP1-GFP12-lacI12*::*HIS3 trp1-1*::*256lacO*::*TRP1 ade2-1 can1-100 gip4*Δ*KAN bar1*Δ
SBY5294	*MAT***a** *ura3-1 leu2,3-112 his3-11*::*pCUP1-GFP12-lacI12*::*HIS3 trp1-1*::*256lacO*::*TRP1 ade2-1 can1-100 bar1*Δ *pGAL-HA3-GIP4*::*HIS3*

[a] All strains are isogenic with the W303 background and were generated for this study.

Ipl1 and likely opposes the essential functions of Ipl1 by dephosphorylating common substrates. We identified proteins that physically interact with Glc7 as dosage suppressors of an *ipl1* mutant and found that two of these proteins, Gip3 and Gip4, are previously unidentified Glc7 regulatory subunits. Consistent with this, phosphorylation of the essential Ipl1 substrate, Dam1, is restored in *ipl1* mutant cells when Glc7 is relocalized out of the nucleus by Gip3 and Gip4 overexpression. We propose that Glc7 regulatory subunits restore the kinase/phosphatase balance in *ipl1* mutants by titrating Glc7 away from essential mitotic substrates.

MATERIALS AND METHODS

Microbial techniques and yeast strain construction. Media and microbial techniques were essentially as previously described (57). Nocodazole was used at 10 μg/ml. Galactose was added to a final concentration of 4%. Yeast strains are listed in Table 1 and were constructed by standard genetic techniques. The *glc7-10* (58) and *glc7-12* (46) (gifts from Michael Stark, University of Dundee, Dundee, United Kingdom) and *ipl1-321* (4) alleles were crossed to make strains

for this study. Strains containing *TUB1-CFP*::*URA3* were obtained by integrating plasmid pSB375 (a gift from Kerry Bloom, University of North Carolina, Chapel Hill, NC) digested with StuI at the *URA3* locus. *GLC7-GFP3* strains were made by integration of plasmid pSB881 digested with EcoRI at the *GLC7* locus. Insertion of the *pGAL* promoter and the HA3, myc13, and FLAG3 epitope tags and construction of *gip3* and *gip4* deletion strains were made using a PCR-based integration system (45) and were confirmed by PCR. Specific primer sequences are available upon request. All fusion proteins are fully functional.

Plasmid construction. The *GLC7-GFP3* integrating plasmid was made by PCR amplification of the C-terminal 400 bp of *GLC7* from pKC1048 (a gift from John Cannon, University of Missouri, Columbia, MO) using primers SB1047 and SB1048 that have ClaI and BamHI restriction sites engineered, respectively. The resulting PCR product was digested with ClaI and BamHI and ligated into the ClaI and BamHI sites of PB1585 (a gift from David Pellman, Harvard Medical School, Boston, MA) to create pSB881. Glutathione S-transferase (GST)–Dam1 was constructed by PCR amplification of Dam1 using primers SB283 and SB284 that have BamHI sites engineered. The PCR product was digested with BamHI and ligated into the BamHI site of pGEX-2T (Pharmacia) to create pSB449.

***ipl1-321* dosage suppressor screen.** The *ipl1-321* strain (SBY1063) was transformed with a 2μm *URA3*-marked genomic yeast library, plated on selective medium at a permissive temperature (23°C) for 3 days, and then replica printed to the restrictive temperature (35.5°C) for 1 day. Of the 48 temperature-resistant colonies identified, 29 showed temperature resistance that was plasmid dependent and were subjected to plasmid rescue and retransformation. The 26 remaining positive colonies were grouped based on restriction mapping, and representatives from each group were sequenced with primers SB359 and SB360 (sequences available upon request). A total of 12 genomic regions containing the following genes were identified: *IPL1* (four times), *GLC8* (two times), *SCD5* (two times), *SDS22* (one time), *BUD14* (two times), *FUN21*/*GIP4* (two times), *PEX31* (one times), *SOL1* (two times), *SOL2* (one time), *YPL137C*/*GIP3* (seven times), *YOR342C* (one time), and *glc7*Δ*186-312*. *glc7*Δ*186-312*, *GLC8*, and *SCD5* were previously identified as *ipl1-1* dosage suppressors, so their genomic regions were not further dissected. *FUN21*/*GIP4* and *YPL137C*/*GIP3* were confirmed to encode the dosage suppressors by generation of a series of plasmid deletions that were retested for temperature resistance in SBY1063. To determine which genes encoded the remaining dosage suppressors, we obtained strains from the *GST-ORF* collection (a gift from Stan Fields, University of Washington, Seattle, WA) for each of the open reading frames in the above genomic regions. We isolated the *GST-ORF* plasmids, retransformed them into SBY1063, and screened them for temperature resistance. By this method, we identified *SDS22*, *BUD14*, *SOL1*, and *PEX31* as dosage suppressors. Although *SOL2* is 78% identical to *SOL1*, we have not eliminated the possibility that another gene in the genomic region is the dosage suppressor. We have not determined which gene in the *YOR342C* genomic region suppresses *ipl1-321*.

Microscopy. Live microscopy was performed as described previously (9). More than 100 cells were analyzed for all reported experiments.

Protein and immunological techniques. Protein extracts were made and immunoblotted as described previously (47). 9E10 antibodies that recognize the myc tag and 12CA5 antibodies that recognize the hemagglutinin (HA) tag were obtained from Covance and used at a 1:10,000 dilution. GST-Dam1 was purified as previously described (36). To analyze Dam1 phosphorylation, 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis gels with decreased bisacrylamide were used.

For immunoprecipitations, 50-ml cultures of mid-log-phase cells were collected, and lysates were prepared as previously described (9). A total of 450 μl of supernatant was incubated with 5 μl protein G-coated Dynabeads (Dynal Biotech, Inc.) and 2 μl of M2 anti-flag antibody (Sigma) or 5 μl of A-14 anti-myc antibody (Santa Cruz Biotechnology) for 2 h at 4°C. The beads were washed five times with 500 μl lysis buffer, and the immunoprecipitates were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and immunoblotted as above.

Kinase assays were performed as previously described (9), except that 5 μg histone H3 (Roche) or GST-Dam1 was used as a substrate.

RESULTS

Glc7 does not regulate Ipl1 levels or localization. Though the phosphorylation status of several proteins is modulated by the opposing activities of Glc7 and Ipl1 (15, 32, 35, 58), the precise relationship between the yeast phosphatase and kinase has not been studied. Because *glc7* mutants suppress *ipl1* mutants (21, 22, 32), one possibility is that Glc7 directly inhibits Ipl1 activity. We therefore tested whether Glc7 physically interacts with Ipl1. Using strains coexpressing endogenous COOH-terminal fusions of Glc7-HA3 and Ipl1-myc13, we were unable to detect the physical association of Glc7 with Ipl1 (Fig. 1A). In addition, Glc7 did not coprecipitate with the Ipl1 activator, Sli15 (data not shown). These data indicate that Ipl1 and Glc7 do not form a detectable complex in vivo under these conditions.

We next examined whether Glc7 alters Ipl1 protein levels in strains carrying the temperature-sensitive *glc7-10* allele. If Glc7 were a negative regulator of Ipl1 levels, Glc7 mutant cells would express more Ipl1 protein. The *glc7-10* mutant suppresses *ipl1-321* at high temperatures (see Fig. 3B) and is defective in Glc7's known mitotic functions (1, 58). Wild-type and *glc7-10* cells expressing Ipl1-myc13 were arrested in mitosis with nocodazole to eliminate cell cycle variation, shifted to the restrictive temperature (37°C) for 2 h, and monitored for Ipl1 protein levels by α-myc immunoblotting (Fig. 1B). Wild-type and *glc7-10* cells expressed equal amounts of Ipl1, indicating that Glc7 does not regulate Ipl1 levels. We obtained similar results using cells asynchronously shifted to the restrictive temperature (data not shown).

Since the metaphase kinetochore localization of Ipl1 is thought to reflect its role in chromosome segregation, we analyzed Glc7 effects on this localization. We visualized triple green fluorescent protein (GFP) epitope-tagged Ipl1 (Ipl1-GFP3) in wild-type and *glc7-10* cells coexpressing cyan fluorescent protein (CFP)-tagged tubulin to mark spindles (Tub1-CFP) after they were shifted to the restrictive temperature for 2 h (37°C) (Fig. 1C). In wild-type cells, Ipl1-GFP3 localized to kinetochores and microtubules on short (1.5- to 3.0-μm) metaphase spindles, as previously described (9). Ipl1-GFP3 localization was similar in that observed with *glc7-10* cells, indicating that Glc7 does not regulate Ipl1's metaphase localization.

Glc7 does not regulate Ipl1 kinase activity. We next tested whether Glc7 negatively regulates Ipl1 kinase activity as previously described (9). We analyzed the activity of both the wild-type Ipl1 protein and the temperature-sensitive Ipl1-321 protein, which has reduced catalytic activity (4). Wild-type and *glc7-10* cells expressing Ipl1-FLAG3 or Ipl1-321–FLAG3 were arrested in mitosis with nocodazole and shifted to the restrictive temperature (37°C) for 2 h. Ipl1 and Ipl1-321 were immunoprecipitated from cell lysates and used in kinase assays in vitro with the substrates histone H3 and Dam1 (Fig. 2A). There were equivalent amounts of wild-type Ipl1 kinase activity against H3 and Dam1 in both wild-type and *glc7-10* mutant cells, indicating that Glc7 does not regulate bulk Ipl1 activity. Cells asynchronously shifted to the restrictive temperature also contained equal amounts of Ipl1 kinase activity (data not shown). Although the kinase activity of Ipl1-321 was much lower than that of wild-type Ipl1, it was also similar in wild-type and *glc7-10* cells. Because *ipl1-321 glc7-10* cells are viable at the nonpermissive temperature but *ipl1-321* cells are nonviable, it is highly unlikely that the *glc7-10* suppression of *ipl1-321* is due to direct regulation of Ipl1 kinase activity.

As a second test of Ipl1 regulation by Glc7, we analyzed genetic interactions between the *glc7-10* allele and an *ipl1* allele that cannot be phosphorylated on the activating residue. The Aurora kinases are activated by phosphorylation of a threonine residue in the activation loop that corresponds to

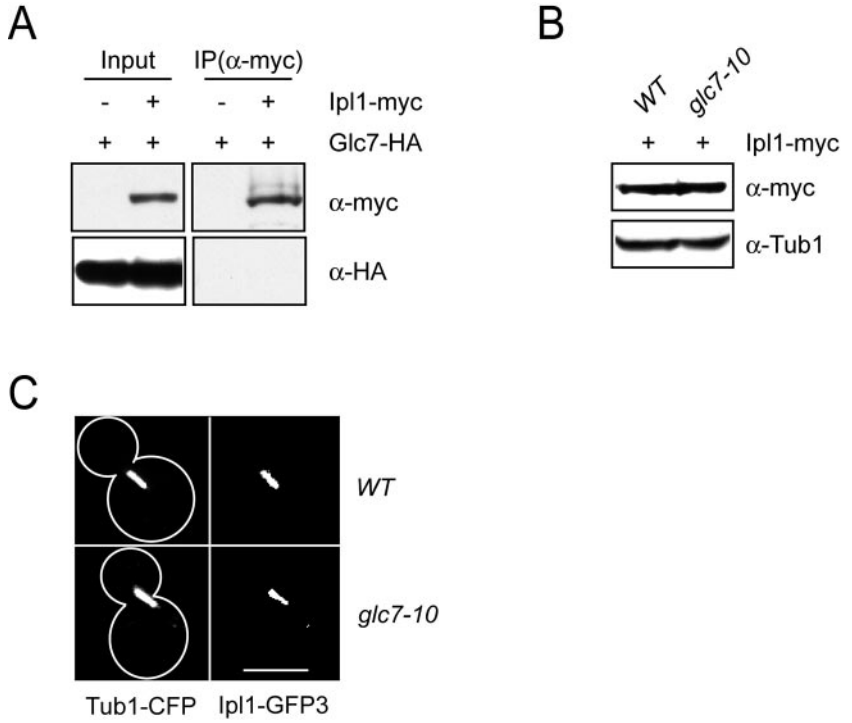

FIG. 1. Glc7 does not regulate Ipl1 levels or localization. (A) Glc7 and Ipl1 do not form a detectable complex. Extracts from cells expressing Glc7-HA3 alone (SBY625) or in combination with Ipl1-myc13 (SBY4822), were immunoprecipitated with anti-myc antibody. Extracts (Input) and immunoprecipitates (IP) were analyzed by anti-HA and anti-myc immunoblotting. (B) Glc7 does not alter Ipl1 protein levels. Wild-type (SBY4767) and *glc7-10* mutant cells (SBY4766) expressing Ipl1-myc13 were arrested in mitosis with nocodazole and shifted to 37°C for 2 h, and the extracts were analyzed by anti-myc and anti-Tub1 immunoblotting as a loading control. (C) Glc7 does not regulate Ipl1's metaphase localization. Wild-type (SBY2833) and *glc7-10* cells (SBY4999) expressing Ipl1-GFP3 and Tub1-CFP were grown at 37°C for 2 h. Scale bar, 5 μm.

threonine 260 (T260) in Ipl1. This residue was phosphorylated in vivo, and a mutation of T260 to alanine in Ipl1 (*ipl1-T260A*) resulted in a temperature-sensitive phenotype (Fig. 2B) (15, 22). If Glc7 dephosphorylated T260, a reduction in Glc7 activity would not alter the temperature sensitivity of the *ipl1-T260A* mutant because it cannot be phosphorylated. In contrast, a reduction in Glc7 activity would be predicted to suppress the temperature sensitivity of the *ipl1-T260A* mutant if Glc7 acts on Ipl1 targets. We found that *glc7-10* did suppress the temperature-sensitive phenotype of the *ipl1-T260A* allele (Fig. 2B), indicating that it is unlikely that Glc7 opposes Ipl1 activity by dephosphorylating the kinase directly. Taken together, these data show that negative regulation of Ipl1 by Glc7 is unlikely to explain the relationship between the kinase and phosphatase.

Glc7 and Ipl1 activity must be precisely balanced. Another possibility is that Ipl1 and Glc7 regulate a common set of substrates, as proposed (21, 22). We therefore determined whether the balance of kinase and phosphatase regulates the phosphorylation of an essential Ipl1 substrate, Dam1 (15). To do this we monitored *ipl1* and *glc7* mutants, as well as *ipl1 glc7* double mutants, for Dam1 gel mobility as previously described (35, 44). In addition to the *glc7-10* allele, we also analyzed *glc7-12*, another mitotic defective allele (46). Wild-type, *ipl1-321*, *glc7-10*, *ipl1-321 glc7-10*, *glc7-12*, and *ipl1-321 glc7-12* cells expressing an endogenous COOH-terminal fusion of Dam1-myc9 were asynchronously shifted to the restrictive temperature (35°C) for 3 h. Dam1 displayed a series of slower-migrat-

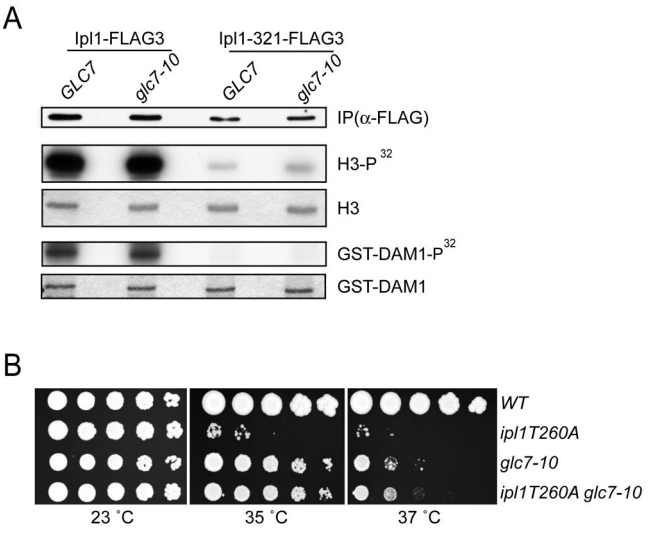

FIG. 2. Glc7 does not regulate Ipl1 kinase activity. (A) Ipl1 kinase activity is normal in *glc7* mutant cells. Wild-type and *glc7-10* mutant cells expressing Ipl1-FLAG3 (SBY3672 and SBY5278) or Ipl1-321–FLAG3 (SBY4541 and SBY5277) were arrested in mitosis with nocodazole and shifted to 37°C for 2 h. Extracts were immunoprecipitated (IP) with α-FLAG antibody, and the IPs were analyzed by α-FLAG immunoblotting and used in kinase assays in vitro with the substrates histone H3 or GST-Dam1. (B) *glc7-10* suppresses *ipl1T260A*. Fivefold serial dilutions of wild-type (SBY2055), *ipl1T260A* (SBY1264), *glc7-10* (SBY1306), and *ipl1T260 glc7-10* (SBY1258) cells were incubated for 3 days at the temperatures indicated.

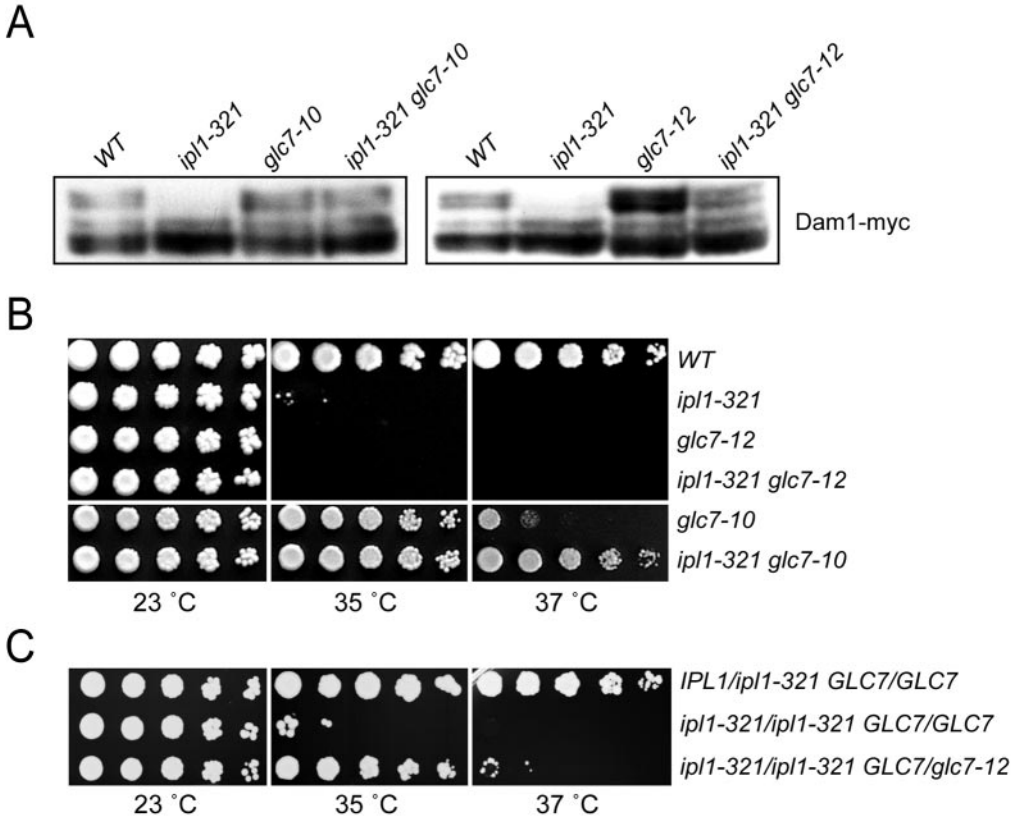

FIG. 3. Ipl1 and Glc7 activities must be precisely balanced. (A) The balance of Ipl1 kinase and Glc7 phosphatase controls Dam1 phosphorylation. Wild-type (SBY2055), *ipl1-321* (SBY4764), *glc7-10* (SBY4826), *ipl1-321 glc7-10* (SBY4801), *glc7-12* (SBY5034), and *ipl1-321 glc7-12* (SBY5032) cells expressing Dam1-myc9 were grown at 35°C for 3 h. Extracts were analyzed by anti-myc immunoblotting for changes in Dam1 gel mobility and showed that the upper Dam1 phosphoforms missing in *ipl1-321* cells were restored in *ipl1-321 glc7-10* and *ipl1-321 glc7-12* mutant cells. (B) *ipl1-321* temperature sensitivity is suppressed by *glc7-10* but not *glc7-12* in haploid cells. Fivefold serial dilutions of wild-type (SBY214), *ipl1-321* (SBY322), *glc7-12* (SBY3675), *glc7-12 ipl1-321* (SBY5004), *glc7-10* (SBY1306), and *ipl1-321 glc7-10* (SBY1994) cells were incubated for 3 days at 23°C and 2 days at 35 and 37°C. (C) The *ipl1-321* temperature sensitivity is suppressed by *glc7-12* in the presence of wild-type *GLC7*. Fivefold serial dilutions of *ipl1-321*/*IPl1 GLC7*/*GLC7* (SBY5070), *ipl1-321*/*ipl1-321 GLC7*/*GLC7* (SBY5072), and *ipl1-321*/*ipl1-321 glc7-12*/*GLC7* (SBY5074) cells were incubated for 3 days at 23°C and 2 days at 35 and 37°C.

ing phosphoforms in wild-type cells that were abolished in *ipl1-321* mutant cells as previously reported (Fig. 3A) (35, 44). Importantly, the Dam1 phosphoforms were more similar to the wild type in both the *glc7-10 ipl1-321* and *glc7-12 ipl1-321* double mutant cells than in the *ipl1-321* cells. This restoration of phosphorylation indicates that the *ipl1-321* allele retains some enzymatic activity at higher temperatures and is consistent with Dam1 phosphorylation being regulated by a balance of Ipl1 kinase and Glc7 phosphatase activity in vivo.

Although the Dam1 phosphoforms appeared to be restored in both *glc7-10 ipl1-321* and *glc7-12 ipl1-321* mutant cells at 35°C, the *glc7-10* allele suppressed the temperature sensitivity of *ipl1-321* at 35°C, while *glc7-12* did not (Fig. 3B). The Dam1 phosphoforms were more intense in *glc7-12* cells than in *glc7-10* cells (Fig. 3A), indicating that *glc7-12* likely retained less residual phosphatase activity at the restrictive temperature. If this were true, a possible explanation for the inability of *glc7-12* to suppress *ipl1-321* is that there is not enough residual phosphatase activity to oppose the remaining Ipl1-321 kinase activity. To test this hypothesis, we analyzed growth in diploids where the balance of Ipl1 and Glc7 could be altered by changing allele copy numbers. Similar to *ipl1-321* haploid cells, *ipl1-321*/*ipl1-321* homozygous diploid cells were temperature sensitive at 35°C and 37°C (Fig. 3C). However, unlike the haploid cells, the *ipl1-321*/*ipl1-321* homozygous mutants were suppressed by a single copy of the *glc7-12* allele. Because *glc7-12* suppressed *ipl1-321* in the presence of a wild-type copy of *GLC7*, it strongly supported our hypothesis that *glc7-12* retains too little phosphatase activity at the restrictive temperature to balance the remaining *ipl1-321* kinase activity. Although the Dam1 phosphoforms appeared to be restored to wild-type levels in *glc7-12 ipl1-321* cells, it may be that the Dam1 gel mobility assay was not sensitive enough to distinguish small differences in the phosphorylation state. Taken together, these data provide further evidence that the Ipl1 kinase and Glc7 phosphatase activities must be precisely balanced.

Ipl1-321 high-copy-number suppressor screen. To identify potential Ipl1 and Glc7 substrates or Ipl1 regulators, we carried out a dosage suppressor screen of the *ipl1-321* temperature-sensitive growth defect at 35.5°C (4). We found 10 genes (Table 2) that suppressed *ipl1-321* when present on a high-copy-number 2μm plasmid (Fig. 4A). Consistent with previous dosage suppressor screens using the temperature-sensitive *ipl1-1* allele, we identified the dominant negative PP1 allele

TABLE 2. *ipl1-321* dosage suppressors

Gene name	Description	Localization[a]	Glc7 interaction[a]	Reference(s)
glc7Δ 186-312	Dominant negative *GLC7* allele	NA	NA	21, 72
GLC8	*GLC7* regulatory subunit	Cytoplasm, nucleus	2H, AP	30, 33, 38, 55, 70
SCD5	Multicopy suppressor of clathrin deficiency	Cell cortex	2H, AP, Co-IP	14, 28, 30, 50, 68, 70
SDS22	*GLC7* regulatory subunit	Nucleus, cytoplasm	2H, AP, Co-IP	23, 27, 31, 33, 38, 53, 55, 75
BUD14	Involved in bud site selection	Bud site and neck	2H, Co-IP	18, 33, 37, 38, 41, 51, 68
YPL137c/GIP3	Uncharacterized open reading frame	Cytoplasm, endoplasmic reticulum	AP	33, 71
FUN21/GIP4	Uncharacterized open reading frame	Cytoplasm	Co-IP[b]	33
SOL1	Regulator of tRNA function	Cytoplasm, nucleus	Co-IP[b]	33, 38
SOL2	Regulator of tRNA function	Cytoplasm	Unknown	33
PEX31	Peroxisomal integral membrane protein	Peroxisomes, cytoplasm	Co-IP[b]	38

[a] NA, not applicable; 2H, two hybrid; AP, affinity precipitation; Co-IP, coimmunoprecipitation.
[b] This work.

glc7Δ186-312, as well as the *GLC8* and *SCD5* genes (22, 66, 72). In addition to these known suppressors, we identified seven novel *ipl1-321* dosage suppressors: *SDS22*, *BUD14*, *YPL137C*, *FUN21*, *SOL1*, *SOL2*, and *PEX31*. At the restrictive temperature of 35°C, all of the dosage suppressors restored *ipl1-321* growth to near-wild-type levels, while at 37°C there were various levels of suppression (Fig. 4A).

Fun21, Sol1, and Pex31 physically interact with Glc7. It was originally proposed that reduced Glc7 activity suppresses Ipl1 mutations by restoring the balance of kinase/phosphatase ac-

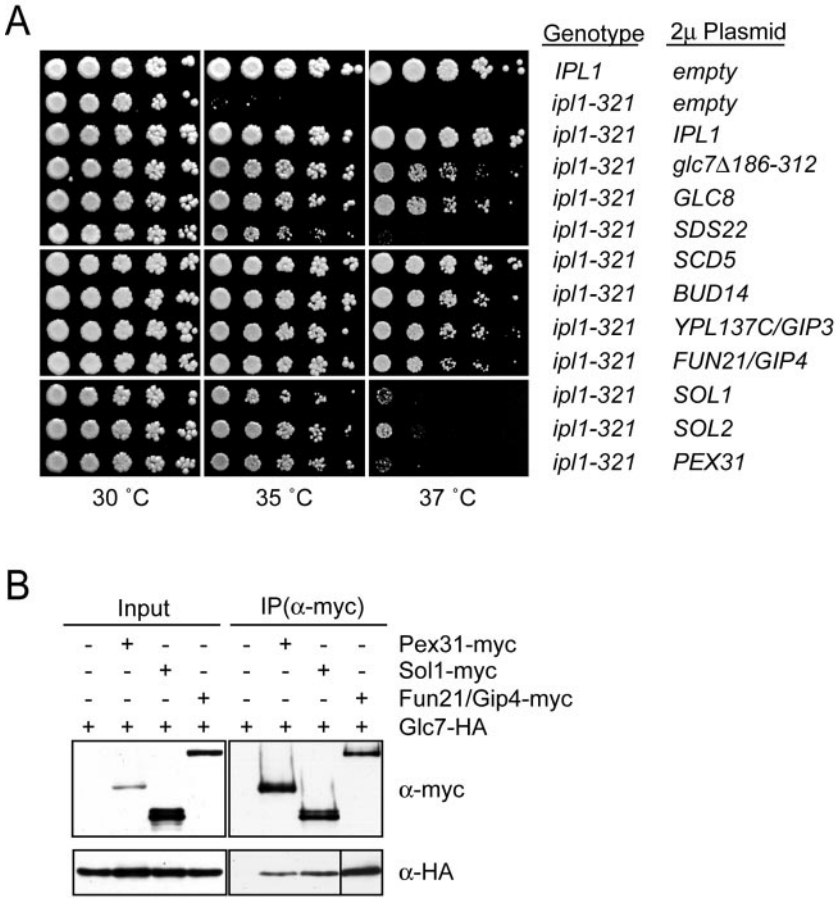

FIG. 4. *ipl1-321* dosage suppressors encode Glc7-interacting proteins. (A) Glc7 regulators are high-copy-number *ipl1-321* suppressors. Fivefold serial dilutions of wild-type cells (SBY214) with an empty 2μm plasmid, *ipl1-321* cells (SBY1063) with an empty 2μm plasmid, or *ipl1-321* cells with 2μm plasmids containing the indicated dosage suppressors were incubated for 2 days at 30, 35, and 37°C. (B) Glc7 physically associates with Pex31, Sol1, and Fun21/Gip4. Extracts from cells expressing Glc7-HA3 alone (SBY625) or in combination with Pex31-myc13 (SBY4920), Sol1-myc13 (SBY4874), and Fun21/Gip4-myc13 (SBY4209) were immunoprecipitated with α-myc antibody. Extracts (Input) and immunoprecipitates (IP) were analyzed by anti-HA and anti-myc immunoblotting. The anti-HA immunoblot of the Fun21/Gip4 IP was exposed for 1/10 of the time used for the Pex31 and Sol1 IPs.

tivity (22). The *ipl1-321* suppressors Glc8, Scd5, Sds22, Bud14, and Ypl137C physically interact with Glc7 (14, 23, 27, 30, 31, 37, 41, 53, 55, 65, 68, 70, 71, 75). We therefore tested whether the remaining dosage suppressors (Pex31, Sol1, and Fun21) also interacted with Glc7. We generated strains coexpressing endogenous COOH-terminal fusions of Glc7-HA3 with Pex31-myc13, Sol1-myc13, and Fun21-myc13 and found that all three proteins immunoprecipitated Glc7 (Fig. 4B). Therefore, like the known *ipl1* dosage suppressors, Pex31, Sol1, and Fun21 physically interacted with Glc7. Since the Ypl137C and Fun21 proteins physically interacted with Glc7, we named the genes that encode them *GIP3* and *GIP4*, respectively, for Glc7-interacting protein (65).

Gip3 and Gip4 do not regulate chromosome segregation. We further characterized the functions of Gip3 and Gip4 to determine how they suppress *ipl1-321* temperature-sensitive cells when expressed from a 2μm plasmid. First, we tested whether the Gip3 and Gip4 proteins regulate chromosome segregation in a manner similar to Ipl1. The *GIP3* and *GIP4* genes were deleted, and the corresponding strains were viable as previously reported (24, 60) and did not exhibit growth defects at higher temperatures (Fig. 5A). To analyze chromosome segregation, wild-type, *gip3*Δ, and *gip4*Δ cells that contained fluorescently marked chromosome IV (ChrIV) were arrested in G_1 and released into the cell cycle. All three strains began budding at 40 min after release and remained synchronous throughout the time course (data not shown). Similar to wild-type cells, the *gip3*Δ and *gip4*Δ mutant cells segregated ChrIV to opposite poles, indicating that Gip3 and Gip4 do not have apparent roles in chromosome segregation (Fig. 5B).

We next determined whether Gip3 and Gip4 affect the phosphorylation status of the Dam1 protein that is regulated by Ipl1 and Glc7. Extracts prepared from asynchronously growing wild-type, *gip3*Δ and *gip4*Δ cells containing Dam1-myc9 were analyzed for Dam1 phosphorylation (Fig. 5C). There was no change in Dam1 phosphorylation in either mutant strain, indicating that Gip3 and Gip4 do not regulate Dam1. Taken together, these data suggest that unlike Ipl1 and Glc7, Gip3 and Gip4 do not have functions related to chromosome segregation.

Because Gip3 and Gip4 physically interact with Glc7, we considered the possibility that they were previously unidentified Glc7 regulatory subunits that control Glc7 localization. Consistent with this hypothesis, both proteins contain the R/K-V/I-X-F motif that targeting subunits use to bind to protein phosphatase 1 (20). We therefore analyzed the localization of a fully functional endogenous COOH-terminal fusion of Glc7 to triple green fluorescent protein (Glc7-GFP3) in wild-type, *gip3*Δ, and *gip4*Δ strains throughout the cell cycle (Fig. 5D and data not shown). In wild-type cells, Glc7-GFP3 localized to the nucleus throughout the cell cycle, as previously reported (8, 77). In addition, Glc7 localized to the presumptive bud site during G_1 phase and then the bud neck and bud cortex during S phase through telophase (8). As previously noted, anaphase and some telophase cells contained two dots of Glc7 at opposite ends of the nucleus (8). This localization was reported to be spindle pole body (SPB) staining because it colocalized with the Nuf2 protein that was originally thought to be an SPB component (52). However, it was subsequently shown that Nuf2 is a kinetochore protein (74), indicating that Glc7 localizes to kinetochores instead of SPBs during anaphase. Because there were no differences in Glc7 localization at any of these cellular sites in the absence of Gip3 (Fig. 5D) and Gip4 (data not shown), these proteins cannot be the sole regulators of Glc7 localization to any of these locations.

Gip3 and Gip4 overexpression is lethal and prevents chromosome segregation. Although we did not detect growth defects when Gip3 and Gip4 were deleted, it was previously reported that Gip3 overexpression is lethal (60). We therefore analyzed the phenotypes of cells expressing Gip3 and Gip4 from the highly inducible galactose promoter. Although wild-type cells grow on both glucose and galactose media, cells expressing *pGAL-GIP4* cannot grow on galactose medium, and cells expressing *pGAL-GIP3* are severely compromised for growth as previously reported (Fig. 6A) (60).

To better understand this growth inhibition, we assessed cell cycle progression when Gip3 and Gip4 were overexpressed. Wild-type, *pGAL-GIP3*, and *pGAL-GIP4* cells were grown in galactose for 4 h and analyzed for cell cycle morphology (Fig. 6B). Prior to induction, the distribution of cells throughout the cell cycle was similar in all three strains. However, after 4 h in galactose medium, there was an increase in large budded cells in the *pGAL-GIP3* and *pGAL-GIP4* strains. The increase was greater in *pGAL-GIP4* strains, consistent with the stronger growth defect observed when this gene was overexpressed. We next analyzed chromosome segregation in the large budded cells after 4 h of galactose induction (Fig. 6C). We monitored both the overall DNA by staining with DAPI (4′,6′-diamidino-2-phenylindole), as well as a single chromosome by using fluorescently tagged ChrIV. In the majority of wild-type cells (79%), the DNA and ChrIV segregated to opposite poles (Fig. 6C and D). However, in large budded cells overexpressing Gip3 and Gip4, neither the total DNA nor ChrIV segregated. Strikingly, these data are consistent with the phenotypes of *glc7* mutant cells that arrest in metaphase (1, 2, 6, 29, 46).

Overexpression of Gip3 and Gip4 titrates Glc7 from the nucleus. Because the overexpression of Gip3 and Gip4 led to a phenotype that resembled a decrease in the mitotic functions of Glc7, we considered the possibility that they decreased the nuclear pool of Glc7. We first analyzed Glc7 levels when Gip3 and Gip4 were overexpressed and found that they were not altered (data not shown). We therefore analyzed Glc7 localization when they were overexpressed. Glc7-GFP3 was localized in wild-type cells and cells overexpressing galactose-inducible Gip3 and Gip4, which are reported to be in the cytoplasm (Fig. 7A; Table 2) (33 and data not shown). Nuclei were visualized by coexpressing the nuclear pore component Nic96 fused to cyan fluorescent protein (Nic96-CFP). In cells overexpressing Gip3 and Gip4, Glc7-GFP3 nuclear localization disappeared, and the phosphatase was predominantly cytoplasmic at all cell cycle stages. However, localization to the bud neck and bud cortex was unaltered. Because Gip3 and Gip4 altered Glc7 localization when overexpressed, they are likely to be previously unidentified regulatory subunits.

The overexpression of Gip3 and Gip4 restores Dam1 phosphorylation in *ipl1* mutants. The observation that Gip3 and Gip4 overexpression reduces the nuclear pool of Glc7 suggests that *ipl1-321* dosage suppression may occur by spatially separating the phosphatase from its relevant nuclear targets opposing Ipl1 function. Although all of the *ipl1-321* dosage suppressors physi-

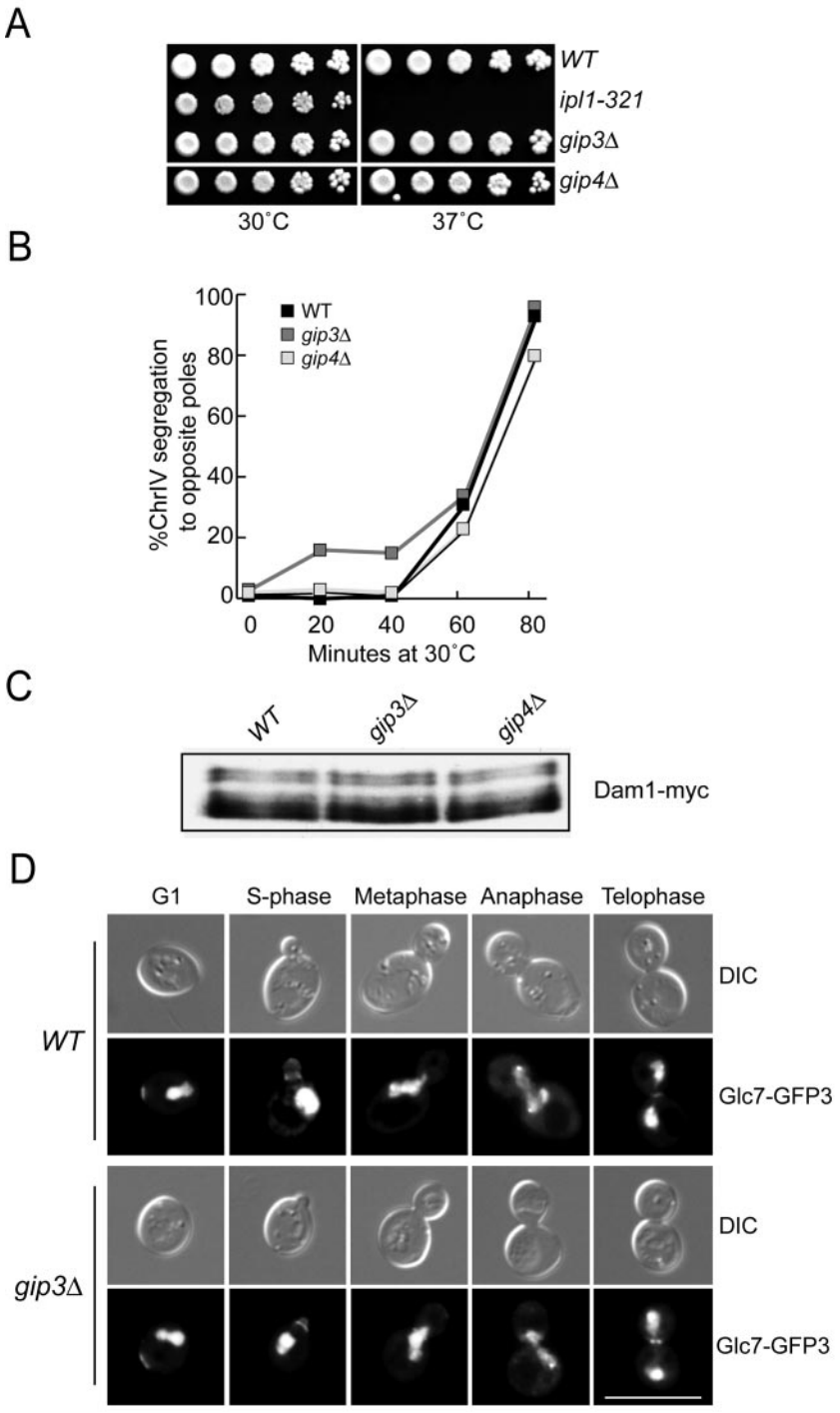

FIG. 5. Gip3 and Gip4 do not regulate chromosome segregation. (A) *gip3*Δ and *gip4*Δ strains grow normally at all temperatures. Serial dilutions of wild-type (SBY3), *ipl1-321* (SBY322), *gip3*Δ (SBY4114), and *gip4*Δ (SBY4175) cells were incubated for 2 days at 30 and 37°C. (B) Gip3 and Gip4 are not required for chromosome segregation. Wild-type (SBY818), *gip3*Δ (SBY5287), and *gip4*Δ (SBY5288) cells were arrested in G_1 and released into the cell cycle. Fluorescently tagged ChrIV was monitored over the time course and segregated to opposite poles in all strains by 80 min after release. (C) Dam1 phosphorylation is normal in *gip3*Δ and *gip4*Δ strains. Wild-type (SBY5275), *gip3*Δ (SBY5274), and *gip4*Δ (SBY5276) strains containing Dam1-myc9 were grown at 23°C. Extracts were analyzed by α-myc immunoblotting for changes in Dam1 gel mobility and show that the Dam1 phosphoforms are not altered in the absence of Gip3 or Gip4. (D) Glc7 localization is normal in the absence of Gip3 and Gip4. Wild-type (SBY5285) and *gip3*Δ (SBY5284) cells containing Glc7-GFP3 were analyzed throughout the cell cycle for Glc7 localization. Scale bar, 10 μm.

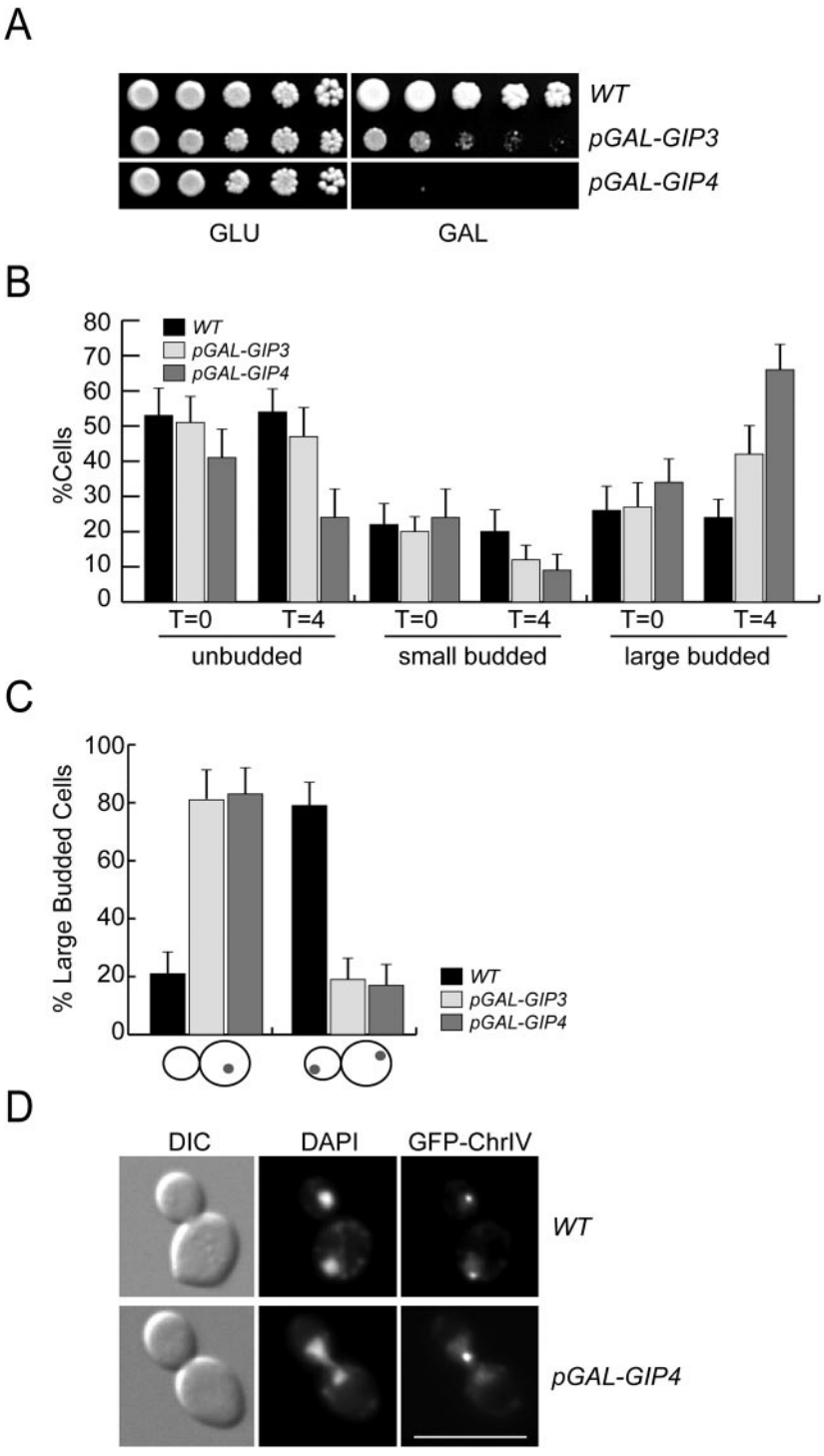

FIG. 6. The overexpression of Gip3 and Gip4 inhibits cell growth and prevents chromosome segregation. (A) Gip3 and Gip4 overexpression from the galactose promoter inhibits cell growth. Serial dilutions of wild-type (SBY3), *pGAL-GIP3* (SBY4179) and *pGAL-GIP4* (SBY4292) cells were plated onto glucose (GLU) and galactose (GAL) media and incubated for 2 days at 30°C. (B) Gip3 and Gip4 overexpression increases the population of large budded cells. Wild-type (SBY818), *pGAL-GIP3* (SBY5279), and *pGAL-GIP4* (SBY5294) cells were induced with galactose for 4 h, and the percentage of cells at each stage of the cell cycle was quantified. Error bars indicate the 95% confidence interval. (C) Gip3 and Gip4 overexpression prevents chromosome segregation. Cells containing fluorescently tagged ChrIV were grown as described in the legend to panel B. Large budded cells were scored for either unsegregated ChrIV (left) or segregation of ChrIV to opposite poles (right). (D) Examples of wild-type cells (SBY818) where ChrIV segregates to opposite poles and cells overexpressing Gip4 (SBY5294) where ChrIV does not segregate. Scale bar, 10 μm.

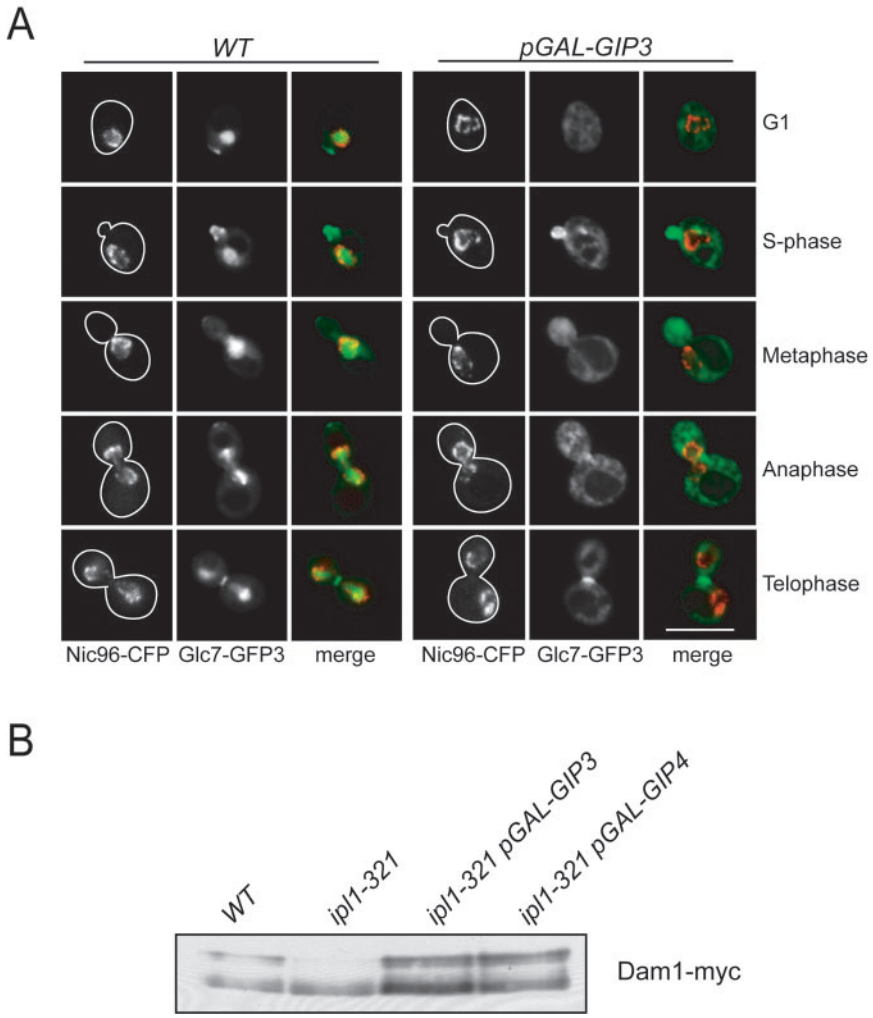

FIG. 7. Gip3 and Gip4 overexpression redistributes Glc7 from the nucleus and alters Dam1 phosphorylation. (A) Overexpression of *GIP3* from the galactose promoter reduces nuclear Glc7. Wild-type (SBY4892) and *pGAL-GIP3* (SBY4995) cells expressing Glc7-GFP3 and Nic96-CFP were grown in galactose for 4 h at 30°C. A representative cell from each stage of the cell cycle is shown, as well as a merge of the CFP and GFP channels. Scale bar, 5 μm. (B) *GIP3* and *GIP4* overexpression restores Dam1 phosphorylation in *ipl1-321* cells. Wild-type (SBY2055), *ipl1-321* (SBY4764), *pGAL-GIP3 ipl1-321* (SBY5127) and *pGAL-GIP4 ipl1-321* (SBY5128) cells containing Dam1-myc9 were grown in galactose at 23°C for 30 min and shifted to 35°C in the presence of galactose for 3 h. Extracts were analyzed by anti-myc immunoblotting for changes in Dam1 gel mobility and show that the upper Dam1 phosphoforms missing in *ipl1-321* cells are restored when Gip3 and Gip4 are overexpressed.

cally interacted with Glc7, they were involved in disparate cellular processes and exhibited different localization patterns (Table 2). We therefore hypothesized that the suppressors may inhibit Glc7's mitotic functions opposing Ipl1 kinase activity by titrating Glc7 away from the relevant Ipl1 substrates. To test whether Gip3 and Gip4 overexpression alters the phosphorylation of important Ipl1 substrates, we analyzed Dam1 phosphorylation. Wild-type, *ipl1-321*, and *ipl1-321* cells expressing galactose-inducible Gip3 and Gip4 were grown under inducing conditions for 30 min, shifted to 35°C for 3 h, and analyzed for Dam1-myc9 phosphorylation. Strikingly, the Dam1 mobility shift that was abolished in *ipl1-321* mutants was restored when either Gip3 or Gip4 was overexpressed (Fig. 7B). Therefore, the overexpression of these proteins likely suppressed *ipl1-321* by titrating Glc7 away from important Ipl1 targets, such as Dam1, thus restoring the balance of kinase and phosphatase activity.

DISCUSSION

The opposing activities of the Ipl1/Aurora protein kinase and Glc7/PP1 protein phosphatase are required for accurate chromosome segregation. Decreased Glc7 activity affected the phosphorylation of the Ipl1 substrate Dam1 but did not alter Ipl1 levels, localization, or bulk kinase activity, supporting the proposal that Glc7 opposes Ipl1 function by regulating the phosphorylation of common targets. We show here that at least two *ipl1* dosage suppressors encode Glc7 interacting proteins that, when overexpressed, likely restore the kinase/phosphatase balance by reducing Glc7 access to relevant substrates.

Ipl1 and Glc7 regulate a common set of substrates. Three simple models have been proposed that could account for the functional interaction between Glc7 and Ipl1. (i) Glc7 negatively regulates Ipl1. (ii) Ipl1 negatively regulates Glc7. (iii) Ipl1 and Glc7 modulate the phosphorylation status of a com-

mon set of substrates (21, 22, 32, 58). Attempts to distinguish between these models have not yet been carried out with budding yeast. Here, we show that Ipl1 activity was not affected by decreased Glc7 activity. Because our kinase assay can only measure bulk Ipl1 activity, the possibility remains that a subset of Ipl1 is directly regulated by Glc7. However, an Ipl1 activation loop mutant that cannot be phosphorylated was still suppressed by a reduction in Glc7 activity, making it highly unlikely that Glc7 regulates Ipl1 activity through dephosphorylation of this residue. To date, the only other site where phosphorylation has been detected on Ipl1 in vivo is S76 (15). Although the S76 site is predicted to be a CDK phosphorylation site and is phosphorylated by Cdc28 in vitro (67), mutation of S76 to alanine does not result in any growth defects when integrated into the genome (data not shown). Therefore, even if S76 was regulated by Glc7, it is unlikely to explain the nature of the essential interaction between Ipl1 and Glc7. We cannot exclude the possibility that phosphorylation on other unidentified Ipl1 sites is regulated by Glc7. However, our data contrast with results from cultured vertebrate cells and *Xenopus* chromatin, where incubation with PP1 inhibitors resulted in elevated Aurora B kinase activity (49, 62). We also did not observe a change in the levels or localization of Ipl1 in mitotic cells with reduced Glc7 function, as has been described for Aurora B in meiotic *Caenorhabditis elegans* cells treated with PP1 RNA interference (56). It is possible that these results reveal true differences in Ipl1 and Aurora B regulation among organisms, although they may also represent a lack of inhibitor specificity or the limitations of our Ipl1 kinase and localization assays. In addition, we were unable to detect a physical interaction between Glc7 and Ipl1 when expressed at endogenous levels. However, Aurora B interacted with each of the three PP1 isoforms (α, δ, and γ) when they were co-overexpressed in cultured cells (62). It is not clear if the potential association of Glc7 with Ipl1 escaped our detection due to a weak, transient, or cell cycle stage-specific interaction, whether the overexpression studies promoted an interaction that is not present under normal conditions, or whether the interactions between Ipl1 and Glc7 were also organism specific. Consistent with our results indicating that Glc7 likely does not directly regulate Ipl1, the kinetochore-associated PP1γ isoform localizes to a domain distinct from Aurora B in cultured cells (64). We have not eliminated the possibility that Ipl1 negatively regulates Glc7 or its mitotic regulatory subunit(s), though Ipl1 does not phosphorylate Glc7 in vitro (data not shown) and Glc7 is not phosphorylated in vivo in budding yeast (61).

It was previously shown that Ipl1/Aurora B and Glc7/PP1 regulate the phosphorylation of the histone H3 and Ndc10 proteins (32, 58). Although these studies did not differentiate between the models described above, these results and genetic studies (15, 76) are consistent with a role for the kinase and the phosphatase working in parallel to control the phosphorylation level of a common set of substrates. Similarly, we found that impairing Glc7 function restores the phosphorylation of the Dam1 protein in *ipl1* mutant cells, consistent with previously reported genetic interactions (15). Taken together, these results suggest that in the budding yeast, Ipl1 and Glc7 act on common targets to promote proper chromosome segregation.

A genetic screen for ipl1 dosage suppressors identifies Glc7 regulatory subunits. Protein phosphatase 1 catalytic subunits, such as Glc7, control numerous cellular processes through their interaction with specialized regulatory subunits that target the phosphatase to appropriate substrates (for a review, see reference 11). We show here that the *ipl1* temperature-sensitive growth defect is suppressed by the increased dosage of genes encoding Glc7-interacting proteins (Table 2). These genes include previously described *ipl1* dosage suppressors *GLC8* and *SCD5*, as well as *SDS22*, *BUD14*, and *GIP3*, newly identified dosage suppressors that encode known Glc7-interacting proteins (14, 23, 27, 30, 31, 37, 41, 53, 55, 65, 68, 70, 71, 75). In addition, we identified *GIP4*, *SOL1*, *SOL2*, and *PEX31* as *ipl1* dosage suppressors and showed that these genes also encode proteins that physically interact with Glc7. Given the exquisite sensitivity of *ipl1* mutant cell growth to the dosage of genes encoding Glc7 interacting proteins, the careful evaluation of changes in the levels of Glc7 interactors should be considered for any *ipl1* suppressor.

Because the Gip3 and Gip4 proteins physically interact with Glc7 and cause its relocalization when overexpressed, we propose that they are previously unidentified Glc7 regulatory subunits. Although we did not detect changes in Glc7 localization when Gip3 and Gip4 were deleted, this may have been due to redundant functions with other Glc7 regulatory subunits. Though *gip3*Δ strains are viable, a *gip3*Δ strain is inviable when combined with a deletion of the open reading frame *YOR227W* (60), suggesting that these two genes act in parallel pathways to regulate a common, essential function. The protein product of the *YOR227W* gene has been affinity purified with Glc7 (30), consistent with the possibility that it is also a previously unidentified regulatory subunit that could have an overlapping function with Gip3. Because we were not able to detect any defects in chromosome segregation or Dam1 phosphorylation in the absence of Gip3 and Gip4, it is unlikely that these proteins participate in the essential functions of Ipl1. Future characterization of the functions of these genes should therefore reveal additional cellular roles for the Glc7 phosphatase.

Overexpression of Glc7 regulatory subunits can restore the kinase/phosphatase balance by relocalizing Glc7. It is likely that the mechanism of *ipl1* mutant dosage suppression involves the redistribution of Glc7 away from the targets relevant to Ipl1's essential functions. Consistent with this idea, cells overexpressing *GIP3* and *GIP4* have reduced Glc7 in the nucleus, and most of the other dosage suppressors encode cytoplasmic or membrane-bound proteins that would be predicted to mislocalize Glc7 away from nuclear Ipl1 targets when overexpressed (Table 2). In contrast, increased levels of a mitotic Glc7 regulatory subunit important for directing the phosphatase to Ipl1 substrates should exacerbate the *ipl1* temperature sensitivity by further skewing the kinase/phosphatase balance toward a lack of phosphorylation. It is therefore unlikely that any of the dosage suppressors encode this Glc7 mitotic regulator. Although previous work suggested that Sds22 was the Glc7 mitotic regulator (53), we isolated *SDS22* as an *ipl1* dosage suppressor. Because s*ds22* mutants also suppress the *ipl1* temperature sensitivity and result in Glc7 mislocalization, the proposal that Sds22 acts as a Glc7 chaperone is more consistent with our observations (53). The hypothesis that the Glc7 regulatory subunits titrate Glc7 away from essential Ipl1 targets is supported by two observations. First, the phosphorylation of an essential Ipl1 substrate, Dam1, was restored in

ipl1-321 cells when Gip3 and Gip4 were overexpressed. Second, the overexpression of Gip3 and Gip4 from the galactose promoter caused lethality and prevented chromosome segregation in wild-type cells, phenotypes consistent with a reduction in the mitotic functions of Glc7 (1, 2, 6, 29, 46). Because these genes were not lethal when expressed on 2μm plasmids, it is likely that the levels of expression from the 2μm plasmid were lower than from the strong galactose promoter. We propose that the other dosage suppressors act in a manner similar to Gip3 and Gip4 overexpression and reduce the effective mitotic functions of Glc7. Although Sds22 is a nuclear protein, it could titrate Glc7 away from essential Ipl1 targets that presumably localize to kinetochores or kinetochore microtubules.

Our data indicate that yeast cells must carefully balance the levels of the numerous Glc7 regulatory subunits. In addition, our results emphasize the importance of maintaining the balance between the kinase and phosphatase to ensure accurate chromosome segregation. In the future, it will be critical to isolate the Glc7 mitotic regulators to elucidate the mechanisms that control Glc7 activity toward Ipl1 substrates.

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