Ypi1, a Positive Regulator of Nuclear Protein Phosphatase Type 1 Activity in *Saccharomyces cerevisiae*

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The catalytic subunit of protein phosphatase type 1 (PP1) has an essential role in mitosis, acting in opposition to the Ipl1/Aurora B protein kinase to ensure proper kinetochore-microtubule interactions. However, the regulatory subunit(s) that completes the PP1 holoenzyme that functions in this capacity is not known. We show here that the budding yeast Ypi1 protein is a nuclear protein that functions with PP1 (Glc7) in this mitotic role. Depletion of cellular Ypi1 induces mitotic arrest due to activation of the spindle checkpoint. Ypi1 depletion is accompanied by a reduction of nuclear PP1 and by loss of nuclear Sds22, a Glc7 binding partner that is found in a ternary complex with Ypi1 and Glc7. Expression of a Ypi1 variant that binds weakly to PP1 also activates the spindle checkpoint and suppresses the temperature sensitivity of an *ipl1-2* mutant. These results, together with genetic interactions among *YPI1*, *GLC7*, and *SDS22* mutants, indicate that Ypi1 and Sds22 are positive regulators of the nuclear Glc7 activity that is required for mitosis.

INTRODUCTION

Bipolar attachment of spindle microtubules to chromosome kinetochores is a prerequisite for the equal segregation of chromosomes to daughter cells and is therefore a critical step in mitosis. The general mechanism leading to the bipolar attachment of chromosomes appears to be a two-step process (reviewed in Gadde and Heald, 2004; Hauf and Watanabe, 2004). Dynamic spindle microtubules first capture kinetochores in a relatively inexact process. Microtubule-kinetochore interactions are then regulated by a mechanism that strengthens appropriate or weakens inappropriate contacts. The spindle checkpoint prevents entry into anaphase until all chromosomes are bioriented (reviewed in Lew and Burke, 2003; Pinsky and Biggins, 2005; Tanaka *et al.*, 2005).

The evolutionarily conserved Ipl1/Aurora B protein kinase plays a major role in promoting biorientation of kinetochores by promoting the turnover of kinetochore-microtubule contacts in response to tension (Tanaka *et al.*, 2002; Hauf *et al.*, 2003; Cimini *et al.*, 2006; Pinsky *et al.*, 2006b). Presumably, phosphorylation of one or more kinetochore proteins by Ipl1/Aurora B reduces the interaction between microtubules and kinetochores. Candidate substrates include components of inner kinetochore complexes (Westermann *et al.*, 2003), the DAM/DASH complex (Kang *et al.*, 2001; Cheeseman

et al., 2002; Li *et al.*, 2002; Shang *et al.*, 2003; Pinsky *et al.*, 2006b), and Ndc80/Hec1 (Cheeseman *et al.*, 2006; DeLuca *et al.*, 2006). Together, these results indicate that phosphorylation of kinetochore proteins by Ipl1/Aurora B serves to increase microtubule-kinetochore dynamics and allow for proper biorientation of kinetochore microtubules. The corollary of this hypothesis is that phosphorylated substrates should become dephosphorylated when kinetochores become bioriented. A large body of evidence suggests that type 1 protein phosphatase (PP1) carries out this dephosphorylation. The injection of anti-PP1 antibodies into mammalian cells induces metaphase arrest (Fernandez *et al.*, 1992), and mammalian PP1 α and PP1 γ isoforms are located at kinetochores (Trinkle-Mulcahy *et al.*, 2003, 2006). PP1 mutations in *Drosophila*, yeast, and fungi can induce mitotic arrest (Doonan and Morris, 1989; Ohkura *et al.*, 1989; Axton *et al.*, 1990; Hisamoto *et al.*, 1994; Black *et al.*, 1995; MacKelvie *et al.*, 1995; Baker *et al.*, 1997; Bloecher and Tatchell, 1999; Andrews and Stark, 2000). In mutants in the yeast PP1 catalytic subunit, *glc7-129* and *glc7-10*, this arrest requires a functional spindle checkpoint (Bloecher and Tatchell, 1999; Sassoon *et al.*, 1999). Extracts from *glc7-10* mutants also have low levels of kinetochore-microtubule binding activity (Sassoon *et al.*, 1999). Furthermore, *GLC7* mutations can partially suppress the temperature sensitivity of *ipl1* mutants (Francisco *et al.*, 1994; Hsu *et al.*, 2000; Pinsky *et al.*, 2006a) and restore the normal phosphorylation levels for several Ipl1 substrates (Hsu *et al.*, 2000; Pinsky *et al.*, 2006a). Collectively, these results suggest that Glc7 either regulates Ipl1 activity or acts to dephosphorylate Ipl1 substrates. Although this question has not been definitively answered, Pinsky *et al.* (2006a) have found no evidence that Glc7 activity influences Ipl1 levels, localization, or activity, suggesting that Glc7 acts directly on Ipl1 substrates.

In contrast to the convincing biochemical evidence that Ipl1/Aurora B phosphorylates kinetochore components and modulates microtubule-kinetochore interactions, the evi-

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Table 1. Yeast strains

Strain	Genotype	Source or Reference
KT1112	<i>MATa leu2 ura3-52 his3</i>	Stuart <i>et al.</i> (1994)
KT1113	<i>MATα leu2 ura3-52 his3</i>	Stuart <i>et al.</i> (1994)
KT1963	<i>MATa leu2 ura3-52 his3 ipl1-2</i>	This study
KT1968	<i>MATa leu2 ura3-52 his3 ipl1-2 glc7-127</i>	Hsu <i>et al.</i> (2000)
KT2422	<i>MATa leu2 ura3-52 his3 GLC7-mYFP::SpHIS5</i>	This study
KT2432	<i>MATa leu2 ura3-52 his3 GLC7-mYFP::SpHIS5 kanMX6:P_{GAL1}-3HA-YPI1</i>	This study
KT2453	<i>MATa leu2 ura3-52 his3 GLC7-tdimer2::SpHIS5</i>	This study
KT2487	<i>MATa leu2 ura3-52 his3 GLC7-mCherry::SpHIS5</i>	This study
KT2572	<i>MATa leu2 ura3-52 his3 GLC7-mYFP::SpHIS5 YPI1-GFP:kanMX6</i>	This study
KT2668	<i>MATa leu2 ura3-52 his3::GFP-lacI:HIS3 trp1::(lacO)₂₅₆:TRP1 kanMX6:P_{GAL1}-3HA-YPI1</i>	This study
KT2729	<i>MATa leu2 ura3-52 his3 GLC7-tdimer2::SpHIS5 SDS22-mYFP:SpHIS5</i>	This study
KT2739	<i>MATa leu2 ura3-52 his3 GLC7-tdimer2::SpHIS5 kanMX6:P_{GAL1}-3HA-YPI1</i>	This study
KT2767	<i>MATa leu2 ura3-52 his3 GLC7-mYFP::SpHIS5 POM34-mCherry:SpHIS5 kanMX6:P_{GAL1}-3HA-YPI1</i>	This study
KT2771	<i>MATa leu2 his3 ura3::GFP-PPZ1:URA3</i>	This study
KT2779	<i>MATa leu2 ura3-52 his3 kanMX6:P_{GAL1}-3HA-YPI1</i>	This study
KT2780	<i>MATa leu2 ura3-52 his3 GLC7-mCherry::SpHIS5 kanMX6:P_{GAL1}-3HA-YPI1</i>	This study
KT2732	<i>MATa leu2 ura3-52 his3 GLC7-mCherry::SpHIS5 SDS22-mYFP:SpHIS5</i>	This study
LKD3	<i>MATa leu2 his3 ura3::GFP-PPZ1:URA3</i>	This study
LKD4	<i>MATa leu2 his3 ura3::GFP-PPZ1:URA3 kanMX6:P_{GAL1}-3HA-YPI1</i>	This study
JB256	<i>MATa/α leu2/leu2 ura3-52/ura3-52 his3/his3 ypi1::kanMX6/+</i>	This study
JB275-1A	<i>MATa leu2 ura3-52 his3 ypi1::kanMX6 pJB27</i>	This study
JB276-8B	<i>MATα leu2 ura3-52 his3 ypi1::kanMX6 pJB43</i>	This study
JB278-16B	<i>MATa leu2 ura3-52 his3 ypi1::kanMX6 GLC7-mYFP::SpHIS5 pJB43</i>	This study
JB279-2C	<i>MATα leu2 ura3-52 his3 ypi1::kanMX6 ipl1-2 pJB43</i>	This study
JB280-5B	<i>MATa leu2 ura3-52 his3 ypi1::kanMX6 ipl1-2 pJB27</i>	This study
JB282-1B	<i>MATα leu2 ura3-52 his3 YPI1-GFP:kanMX6</i>	This study
JB284-2C	<i>MATa leu2 his3 trp1-1 ura3::GFP-TUB1:URA3 kanMX6:P_{GAL1}-3HA-YPI1</i>	This study
JB289-1A	<i>MATa leu2 his3 ura3::GFP-TUB1:URA3</i>	This study
JB289-1C	<i>MATa leu2 his3 ura3::GFP-TUB1:URA3 mad1::HIS3MX6</i>	This study
JB289-4B	<i>MATa leu2 his3 ura3::GFP-TUB1:URA3 mad1::HIS3MX6 kanMX6:P_{GAL1}-3HA-YPI1</i>	This study
JB290-1B	<i>MATa leu2 ura3-52 his3 glc7-127</i>	This study
JB290-1D	<i>MATa leu2 ura3-52 his3 YPI1-GFP:kanMX6 glc7-127</i>	This study
JB295-1A	<i>MATa leu2 ura3-52 his3 ipl1-2 YPI1-GFP:kanMX6</i>	This study
JB297-2A	<i>MATa leu2 ura3-52 his3 glc7-109</i>	This study
JB297-1A	<i>MATa leu2 ura3-52 his3 YPI1-GFP:kanMX6 glc7-109</i>	This study
JB298-5B	<i>MATa leu2 ura3-52 his3 glc7-132</i>	This study
JB298-7A	<i>MATa leu2 ura3-52 his3 YPI1-GFP:kanMX6 glc7-132</i>	This study
JB300-3A	<i>MATα leu2 ura3-52 his3 ypi1::kanMX6 GLC7-mYFP::SpHIS5 pJB27</i>	This study
JB308-7A	<i>MATa leu2 his3 ypi1::kanMX6 ura3::GFP-TUB1:URA3 pJB43</i>	This study
JB309-8B	<i>MATa leu2 his3 ypi1::kanMX6 ura3::GFP-TUB1:URA3 pJB27</i>	This study
JB320-1B	<i>MATα leu2 ura3-52 his3 SDS22-mYFP:SpHIS5</i>	This study
JB328-12C	<i>MATa leu2 ura3-52 his3 SDS22-mYFP:SpHIS5 POM34-mCherry:SpHIS5</i>	This study
JB331-3A	<i>MATa leu2 ura3-52 his3 SDS22-mYFP:SpHIS5 POM34-mCherry:SpHIS5 kanMX6:P_{GAL1}-3HA-YPI1</i>	This study
JB338-3A	<i>MATα leu2 ura3-52 his3::GFP-lacI:HIS3 trp1::(lacO)₂₅₆:TRP1</i>	This study
JB338-13B	<i>MATa leu2 ura3-52 his3::GFP-lacI:HIS3 trp1::(lacO)₂₅₆:TRP1 mad1::HIS3MX6 kanMX6:P_{GAL1}-3HA-YPI1</i>	This study
JB338-14D	<i>MATα leu2 ura3-52 his3::GFP-lacI:HIS3 trp1::(lacO)₂₅₆:TRP1 mad1::HIS3MX6</i>	This study
JB377-6C	<i>MATα leu2 ura3-52 his3 ypi1::kanMX6 SDS22-mYFP:SpHIS5 POM34-mCherry:SpHIS5 pJB27</i>	This study
JB378-7D	<i>MATa leu2 ura3-52 his3 ypi1::kanMX6 SDS22-mYFP:SpHIS5 POM34-mCherry:SpHIS5 pJB43</i>	This study
JB385-1A	<i>MATα leu2 ura3-52 his3 trp1-1 YPI1-13Myc:kanMX6</i>	This study
JB402-10D	<i>MATα leu2 ura3-52 his3 YPI1-GFP:kanMX6 SDS22-mYFP:SpHIS5</i>	This study
JB403-5C	<i>MATa ura3-52 his3 trp1-1 sds22::TRP1 leu2::sds22-6^{ts}:LEU2 YPI1-13Myc:kanMX6</i>	This study
JB409-1C	<i>MATa leu2 ura3-52 his3 YPI1-13Myc:kanMX6 glc7-129</i>	This study
JB412-1B	<i>MATα leu2 ura3-52 his3 GLC7-13Myc:kanMX6</i>	This study

dence that PP1/Glc7 dephosphorylates these substrates is less convincing. Although mammalian PP1 colocalizes with kinetochores (Trinkle-Mulcahy *et al.*, 2003, 2006), yeast Glc7, while abundant in the nucleus, has not been identified as a component of kinetochore complexes. Glc7 does colocalize with the kinetochore protein Nuf2 (Bloecher and Tatchell, 2000), but this protein is largely associated with spindle pole bodies when Glc7 and Nuf2 colocalize in anaphase (Janke *et al.*, 2001). Another unknown is the nature of the Glc7 holoenzyme that would be acting in opposition to Ipl1. In general, substrate specificity of PP1 enzymes is regulated by a large collection of targeting and/or regulatory subunits

that direct the phosphatase to specific substrates and may regulate activity differentially toward different substrates (reviewed in Cohen, 2002; Ceulemans and Bollen, 2004). Although targeting subunits are essential for many PP1/Glc7-dependent processes, the targeting subunit or subunits involved in its cell cycle activities are still subject to debate. One potential nuclear subunit is Sds22, a leucine-rich repeat protein that was first identified as a multicopy suppressor of the *Schizosaccharomyces pombe* *dis2-11* PP1 mutant (Ohkura and Yanagida, 1991), and subsequently was implicated with regulating Glc7 in *Saccharomyces cerevisiae* (Hisamoto *et al.*, 1995; MacKelvie *et al.*, 1995). Temperature-sensitive *sds22*

Table 2. Primers

Name	Sequence (5'–3')
JB81-F	ACGCGTCGACCTCCGGTACCCGATTGAGGCA
JB82-R	GCTCTAGATTTGTTTATATCTATATATCA
JB90-F	CCAGGAGTTGCGAGCTAAGTCTTCAATTAAGTCTATAAGGCGGATCCCCGGGTAAATTA
JB91-R	TTGCTGCTTCATCGAATATTTTGGCTTTCGTTGTACAAAGCCGAATTCGAGCTCGTTTAAAC
JB94-F	AGGCACAATGTAAGAGCTGAAGAAAAATGTGATTG
JB95-R	CAATCACATTTTCTTCAGCTCTTACATTGTGCCT
JB99-F	TTATTCTGAATACAGGCGAAAACAGCAGGAAAAGAAGGACCGGATCCCCGGGTAAATTA
JB101-R	GCTGCTTCATCGAATATTTTGGCTTTCGTTGTACAAAGCCGAATTCGAGCTCGTTTAAAC
JB102-F	CCTACAAGGCACAATGCAAGAGCTGAAGAAAAATG
JB103-R	CATTTTCTTCAGCTCTTGCATTGTGCCTTGTAGG
JB105-F	GCTCTAGAGATGAGTGGAATCAAATGGCT
JB106-R	ACGCGTCGACGCGATCCTTCTTTCTGCT
JB107-F	CAGAATCTGAGAATGAGAAGGATCTTGACTTTAACGAACGCGGATCCCCGGGTAAATTA
JB108-F	CCAATGCTTATGAAATCCAACAGATTATCTGAATACAGCGGATCCCCGGGTAAATTA
JB109-F	TCCATCCCTACAGAAGATTGATGCGACATATATAAGAGGCGGTGACGGTGCTGGTTTA
JB110-R	GTTTGTGTGTATATAAAAAAAAAATCATTTCTTGATCAAGATCGATGAATTCGAGCTCG
JB111-F	AATATGCATATATGATGAATCAGTCCCCAAGGGGAAAAATAGGTGACGGTGCTGGTTTA
JB112-R	TATATAGCTATGGAAGTATTAATGTTTTTGTCTGTTTTTGTCTGTTTTCGATGAATTCGAGCTCG
JB119-F	TTATTCTGAATACAGGCGAAAACAGCAGGAAAAGAAGGACGGTGACGGTGCTGGTTTA
LUG1-F	CTGCCAGGAGTTGCGAGCTAAGTCTTCAATTAAGTCTATAAGGGAATTCGAGCTCGTTTAAAC
LUG2-R	TCTGTTGTGTTCTGATCCCATAGCCATTGATTTCCTACTGCAGTGCAGCAGCGTAATCTG
LD1-F	CCTACAAGGCACAATGCAAGATGGGAAGAAAAAT
LD2-R	ATTTTCTTCCCATCTTGCAATTGTGCCTTGTAGG
GSH1-F	AAAAAGTCTACCAAGGCAAGCTGGGGGTAGAAAAGAAAAACGGATCCCCGGGTAAATTA
GSH1-R	TATTTTCTTTTAACTTTGATTTAGGACGTGAATCTATGAATTCGAGCTCGTTTAAAC
CAT138F	AAAAAGTCTACCAAGGCAAGCTGGGGGTAGAAAAGAAAAAGGTGACGGTGCTGGTTTA

mutants do not undergo cell cycle arrest at the nonpermissive temperature but exhibit high levels of chromosome loss and suppress the temperature sensitivity of the *ipl1-2* mutant (Peggie *et al.*, 2002). Recently, Yeast Phosphatase Inhibitor 1 (Ypi1), a Glc7-interacting protein (Ho *et al.*, 2002; Garcia-Gimeno *et al.*, 2003; Hazbun *et al.*, 2003; Krogan *et al.*, 2006), so named because it inhibits Glc7 catalytic activity in vitro (Garcia-Gimeno *et al.*, 2003), was shown to associate with Glc7 and Sds22, forming a ternary complex that enhances the ability of Ypi1 to inhibit Glc7 activity in vitro (Pedelini *et al.*, 2007). Increased expression of either Sds22 or Ypi1 was shown to suppress the temperature sensitivity of *ipl1-132* mutants (Pedelini *et al.*, 2007), consistent with the

possibility that Ypi1 acts as an inhibitor in vivo. However, depletion of Ypi1 resulted in mitotic arrest, which is more consistent with Ypi1 acting as a positive regulator of Glc7. Here we report our analysis of the relationship between Ypi1 and Glc7. Our results indicate that Ypi1 acts with Sds22 as an important positive regulator of the nuclear Glc7 activity that opposes the Ipl1/Aurora B kinase.

MATERIALS AND METHODS

Yeast Strains, Media, and General Methods

The yeast strains used in this work are listed in Table 1 and are congenic to JC482 (Cannon and Tatchell, 1987). Yeast strains were grown at 30°C on YPD medium (2% Bacto peptone, 1% yeast extract, and 2% glucose) or synthetic complete (SC) medium unless stated otherwise. Strains were sporulated at 24°C on medium containing 2% Bacto peptone, 1% yeast extract, and 2% potassium acetate. SC medium and media lacking specific amino acids were made as described by Sherman *et al.* (1986). Benomyl (15 µg/ml) plates (used to score *mad1Δ* strains) were made as described in Straight *et al.* (1997). Yeast transformation, manipulation of *Escherichia coli*, and the preparation of bacterial growth media were performed as described previously (Maniatis *et al.*, 1989; Gietz *et al.*, 1992). The expression of *YPI1* in *P_{GAL1}-3HA-YPI1* strains was induced by growing the strains in YPGal (2% Bacto peptone, 1% yeast extract, and 2% galactose) medium overnight at 30°C. Cultures were then diluted back into YPGal and grown at 30°C to midlog phase (density of $\sim 1-5 \times 10^7$ cells per ml). To repress expression of *YPI1*, these cells were then pelleted, washed, and resuspended in YPD medium to a final density of 1×10^4 cells per ml. Cell densities were determined using a Coulter Counter (Beckman Coulter, Miami, FL). Drop growth assays were done as described previously (Williams-Hart *et al.*, 2002). Loss of cell viability was determined as described by Wang and Burke (1995).

Gene deletion, *P_{GAL1}-3HA*, 13Myc, and fluorescent protein fusion cassettes were generated using PCR. Each amplified cassette was transformed into a KT1112xKT1113 diploid strain, drug-resistant or amino acid-prototrophic transformants were sporulated, and haploid meiotic segregants were isolated by tetrad analysis. Primers are listed in Table 2. To create the *ypi1Δ* strain, the deletion cassette was amplified with primers JB90-F and JB91-R, using pFA6a-kanMX6 (Longtine *et al.*, 1998) as a template. To create the *P_{GAL1}-3HA-YPI1* allele, the cassette was amplified with primers LUG-1F and LUG-2R using pFA6a-kanMX6-*P_{GAL1}-3HA* (Longtine *et al.*, 1998) as a template. The 13Myc-integration cassettes were amplified with primers JB99-F and JB101-R for *YPI1-13Myc*, primers JB107-F and JB101-R for *ypi1-Δ114-13Myc*, primers JB108-F and JB101-R for *ypi1-Δ147-13Myc*, primers GSH1-F and GSH1-R for

Table 3. Plasmids

Name	Description	Source
pJB25	<i>YPI1</i> in pGEM-T	This study
pJB27	pRS315 <i>CEN LEU2 YPI1</i>	This study
pJB29	pRS425 2 µ <i>LEU2 YPI1</i>	This study
pJB33	pRS316 <i>CEN URA3 YPI1</i>	This study
pJB43	pRS315 <i>CEN LEU2 ypi1^{W53A}</i>	This study
pJB45	pRS306 <i>URA3 YPI1</i>	This study
pJB47	pRS306 <i>URA3 ypi1^{W53A}</i>	This study
pJB49	pRS315 <i>CEN LEU2 ypi1^{V51A/W53A}</i>	This study
pLD1	pRS315 <i>CEN LEU2 ypi1^{V51A}</i>	This study
pLD3	pRS306 <i>URA3 ypi1^{V51A}</i>	This study
pRS425	<i>LEU2</i> 2 µ vector	Christianson <i>et al.</i> (1992)
pRS315	<i>LEU2 CEN</i> vector	Sikorksi and Hieter (1989)
pRS316	<i>URA3 CEN</i> vector	Sikorksi and Hieter (1989)
pRS306	<i>URA3</i> vector	Sikorksi and Hieter (1989)

Figure 1. Depletion of Ypi1 results in G2 cell cycle delay and activation of the spindle assembly checkpoint. (A) Immunoblot analysis with anti-HA antibody of 3HA-Ypi1 in extracts from WT (KT2422) and *P_{GAL1}-3HA-YPI1* (KT2432) strains, after shifting cells from YPGal to YPD medium as described in *Materials and Methods*. Pgk1 serves as a loading control. (B) Growth curves of the WT (JB289-1A), *P_{GAL1}-3HA-YPI1* (JB284-2C), *mad1Δ* (JB289-1C), and *P_{GAL1}-3HA-YPI1 mad1Δ* (JB289-4B) strains after the shift to glucose. (C) Flow cytometry analysis of DNA content for the strains in B at 12, 16, and 20 h in YPD. (D) Tub1-GFP fluorescence in WT (JB289-1A) and *P_{GAL1}-3HA-YPI1* (JB284-2C) cells after 16 h in YPD medium. Bar, 5 μ m. (E) GFP-lacI fluorescence in the WT (JB338-3A), *P_{GAL1}-3HA-YPI1* (KT2668), *mad1Δ* (JB338-14D), and *P_{GAL1}-3HA-YPI1 mad1Δ* (JB338-13B) strains at 12 h in YPD. DIC and GFP images are shown. White arrow, a large-budded cell with no spot; white arrowhead, a large-budded cell with no spot in the mother and two spots in the daughter cell.

GLC7-13Myc and using pFA6a-13Myc-His3MX6 and pFA6a-13Myc-kanMX6 (Longtine *et al.*, 1998) as templates. The *YPI1*-GFP cassette was amplified with primers JB99-F and JB101-R, using pLK3 (Kozubowski *et al.*, 2003) as a template. To create *YPI1-EmCitrine* the cassette was amplified using primers JB119-F and JB101-R with pKT211 (Sheff and Thorn, 2004) as a template. The *SDS22-EmCitrine* cassette was amplified with primers JB109-F and JB110-R, using pKT211 as a template. To create *POM34-mCherry*, the cassette was amplified with primers JB111-F and JB112-R using pKT355 (pFA6a-L-mCherry-HIS3MX6; K. Thorne, personal communication) as a template. To create *GLC7-EmCitrine*, *GLC7-mCherry*, and *GLC7-timer2*, the cassettes were amplified with primers CAT138-F and GSH1-R using pKT211, pKT146 (Sheff and Thorn, 2004), and pKT355, respectively, as templates. For simplicity, the *EmCitrine* and *mCherry* fusions are referred to as *mYFP* and *mRFP*, respectively, throughout this article. All deletions, truncations, and integrations were confirmed by genomic PCR.

Cells were processed for flow cytometry as described (Baker *et al.*, 1997) with the following modifications. Cells were treated with RNase A overnight at 37°C, harvested, resuspended in phosphate-buffered saline at a cell density of 1×10^7 cells/ml, and stained with propidium iodide (Sigma, St. Louis, MO) at a final concentration of 50 μ g/ml. Flow cytometry was performed at the LSUHSC Flow Cytometry Facility.

Plasmid Construction and Site-directed Mutagenesis

Plasmids used in this study are listed in Table 3. Standard techniques were used for DNA manipulation (Maniatis *et al.*, 1989). Restriction and modification enzymes were used as recommended by the manufacturers (Promega, Madison, WI; New England Biolabs, Beverly, MA; Roche, Mannheim, Germany). The *YPI1* gene (ORF plus 500 bp upstream and 100 bp downstream sequence) was cloned by PCR using primers JB81-F and JB82-R into pGEM-T (using the pGEM-T Easy Vector Systems kit, Promega) to generate pJB25. The entire sequence was confirmed at the Arizona State University DNA facility (Tempe, AZ). The gene was subcloned from pJB25 as a Sall-XbaI fragment into pRS315, pRS316, and pRS306 vectors to create pJB27, pJB33, and pJB45, respectively. The ability of the plasmid-borne *YPI1* to complement the lethality of the *yip1Δ* mutant was tested in a *yip1Δ* heterozygous diploid strain (JB256). *YPI1* was also subcloned as a Sall-SacII fragment into pRS425 to create pJB29. The *YPI1* ORF was cloned using a similar strategy and primers

JB105-F and JB106-R to create pJB25. The sequence was verified as mentioned above. Mutations in *YPI1* were created with the QuikChange kit (Stratagene, La Jolla, CA), using pJB27 as a template (unless mentioned otherwise). Primers were designed according to manufacturer's instructions. Primers JB94-F and JB95-R were used to create *yip1^{W53A}* (pJB43). Primers JB102-F and JB103-R were used to generate *yip1^{V51A}* in pJB43 to create *yip1^{V51A/W53A}* (pJB49). Primers LD1-F and LD2-R were used to create *yip1^{V51A}* (pLD1). For integration, *YPI1*, *yip1^{V51A}*, and *yip1^{W53A}* were subcloned as Sall-XbaI fragments into pRS306 to create pJB45, pLD3, and, pJB47, respectively. These were digested within *URA3* with StuI and transformed into the *yip1Δ* heterozygous diploid (JB256). Transformants were sporulated and segregants analyzed by tetrad analysis. Alternatively, the *LEU2* bearing plasmids were introduced into a *yip1Δ* haploid containing pJB33 (pRS316-*YPI1*, *URA3*), and transformants were selected on SC-Ura-Leu. The *Ura⁺* *Leu⁺* transformants were then grown on 5-fluoro-orotic acid (5-FOA) media to select for viable cells that had lost the *URA3* plasmid but contained the *LEU2* plasmid.

Immunoblot Analysis

To assess tagged protein levels, total protein was prepared from cultures by lysis in trichloroacetic acid (Davis *et al.*, 1993; Stuart *et al.*, 1994). Immunoblot analysis was performed as described (Stuart *et al.*, 1994) using anti-Myc 9E10 ascites antibody or BD Living Colors A.v. monoclonal anti-green fluorescent protein (GFP; JL-8) antibody with subsequent detection using the Enhanced Chemiluminescence System (ECL or ECL Plus, Amersham Biosciences, Piscataway, NJ). Protein levels were quantitated from immunoblots using Image Quant software (Molecular Dynamics, Sunnyvale, CA), with phosphoglycerate kinase (Pgk1) as a loading control.

Microscopy

For live cell imaging, cells were placed onto a pad of 2% agarose in SC or SCGal medium, as indicated, and imaged for GFP, yellow fluorescent protein (YFP), and red fluorescent protein (RFP) as previously described (Kozubowski *et al.*, 2003). DIC and fluorescence images in a series of Z-axis planes (0.5 μ m apart) were acquired using Slidebook software (Intelligent Imaging Innovations, Denver, CO). Indirect immunofluorescence was performed as described previously (Kozubowski *et al.*, 2003). Cells were stained

with anti-Myc 9E10 ascites mAb followed by goat anti-mouse TRITC-conjugated secondary antibody (Sigma). Quantitative analysis of the levels of GFP fluorescence was performed as described (Kozubowski *et al.*, 2003). For quantitative analysis of spindle length in large-budded cells, the diameters of the mother and bud/daughter cells were measured along the mother-daughter axis using Slidebook software. Cells with bud/daughter cell at least 75% the size of the mother cell were considered large-budded cells for this analysis.

RESULTS

Ypi1 Depletion Induces a Delay in G2/M and Activation of the Spindle Assembly Checkpoint

To determine the essential role(s) of Ypi1 *in vivo*, we placed the endogenous *YPI1* gene under the control of a galactose-regulated promoter using the pFA6a-kanMX6:*P_{GAL1}*-3HA cassette (Longtine *et al.*, 1998) as described in *Materials and Methods*. The strain grows normally on galactose (inducing) medium (YPGal) but fails to thrive on glucose (repressing) medium (YPD). To study the consequence(s) of Ypi1 depletion, we grew *P_{GAL1}*-3HA-*YPI1* and congenic *YPI1* strains to log phase in YPGal medium and assayed cell number, viability, morphology, and DNA content after transfer to YPD medium. Immunoblot analysis revealed that 3HA-Ypi1 levels were largely depleted after 2 h in YPD (Figure 1A), but the cells continued to divide at a normal rate for at least 8 h, after which time they grew at progressively slower rates (Figure 1B). The delay in growth was accompanied by an increase in the population of budded cells with 2C DNA content (Figure 1C). By 16 h in YPD, the majority (60%) of the *P_{GAL1}*-3HA-*YPI1* cells were delayed in the G2 phase of the cell cycle with large buds and short spindles (88%), assayed using a GFP-Tub1 fusion to visualize spindle microtubules (Figure 1D, Supplementary Figure S1, and Table 4). These results are similar to those reported for depletion of Ypi1 by placing *YPI1* under the control of the *tetO₇* promoter (Pedelini *et al.*, 2007).

The G2 arrest of some *GLC7* mutants is due to activation of the spindle assembly checkpoint (Bloecher and Tatchell, 1999; Sassoon *et al.*, 1999). To determine if the cell cycle arrest that accompanies Ypi1 depletion is also dependent on this checkpoint, we assayed the cell cycle distribution in a *P_{GAL1}*-3HA-*YPI1* strain that also contained a deletion of the *MAD1* gene (Mitotic Arrest Deficient), an essential component of the checkpoint (Hardwick and Murray, 1995). As shown in Figure 1B, *mad1Δ* had no effect on cell proliferation, but largely suppressed the cell cycle arrest induced by Ypi1 depletion (Figure 1C). The lack of G2 arrest was consistent with an observed increase in the frequency of long anaphase spindles in *P_{GAL1}*-3HA-*YPI1* *mad1Δ* cells after Ypi1 depletion (Table 4).

The release of cell cycle arrest in *GLC7* mutants by inactivation of the spindle assembly checkpoint results in an increase in chromosome loss (Bloecher and Tatchell, 1999; Sassoon *et al.*, 1999). We tested whether depletion of Ypi1 in the *mad1Δ* mutants also results in chromosome segregation defects by assaying the mitotic segregation of chromosome IV that had been tagged with a tandem array of Lac operators (*lacO* array). Expression of GFP-lacI in these cells allows direct visualization of the tagged chromosome (Straight *et al.*, 1997). The *lacO* array is normally observed as a single nuclear spot until anaphase, at which point the spot divides into two, and the two spots move to opposite poles of the anaphase spindle. As shown in Figure 1E and Table 5, depletion of Ypi1 in an otherwise wild-type background results in the accumulation of budded cells with one spot (62% at 12 h in YPD), as expected for cells delayed in G2. In contrast, no such increase occurs after Ypi1 depletion in the *mad1Δ* strain (25% at 12 h in YPD). We noted a high per-

Table 4. Quantitative analysis of spindle length in large-budded cells after Ypi1 depletion

Strain	% Cells with large buds	% Large-budded cells with spindle length		
		0.5–2 μm	>2 μm	Undetermined ^a
<i>WT^b</i>				
0 h	32	33	62	4
8 h	31	20	80	0
12 h	34	18	82	0
16 h	36	28	71	3
20 h	42	35	60	5
<i>P_{GAL1}-YPI1</i>				
0 h	30	17	79	4
8 h	30	54	44	2
12 h	44	87	10	3
16 h	60	88	5	7
20 h	34	77	13	11
<i>P_{GAL1}-YPI1 mad1Δ</i>				
0 h	28	30	63	7
8 h	23	28	67	6
12 h	32	56	42	2
16 h	37	32	55	14
20 h	26	26	71	11
<i>mad1Δ</i>				
0 h	27	46	50	4
8 h	38	15	84	1
12 h	40	37	63	0
16 h	42	25	71	3
20 h	41	49	47	5

^a Large-budded cells without a clearly defined spindle were included in this category.

^b The following strains were used: WT, JB289-1A; *pGAL*-*YPI1*, JB284-2C; *pGAL*-*YPI1* *mad1Δ*, JB289-4B; and *mad1Δ*, JB289-1C.

centage of cells with either too many or no spots (35% at 16 h in YPD; Figure 1E, arrow). Interestingly, the percentage of abnormal cells (cells with no spots and unbudded cells with two spots) was also higher (21%) in the *P_{GAL1}*-3HA-*YPI1* *mad1Δ* strain compared with the single mutants when grown on YPGal medium (Table 5), suggesting that the high level of 3HA-Ypi1 in these cells might also perturb chromosome stability. We also noted that relatively few *P_{GAL1}*-*YPI1* cells growing in YPGal medium have short spindles (Table 4), further suggesting that Ypi1 function is not normal in the *P_{GAL1}*-*YPI1* strains. This could be due to either altered levels of Ypi1 or the presence of the HA epitope tag.

We monitored cell viability in Ypi1-depleted cultures by plating serial dilutions of the cultures grown in YPD onto YPGal medium. Ypi1 depletion results in a ~10–20-fold decrease in cell viability 24 h after the shift to YPD medium but surprisingly, viability is not reduced further in the *mad1Δ* mutant (data not shown), as is the case for *glc7-129* *mad1Δ* strains (Bloecher and Tatchell, 1999). These results suggest that the cell death induced by Ypi1 depletion is caused by a *YPI1*-dependent process(es) separate from or in addition to spindle microtubule attachment.

Ypi1 Is Required for the Nuclear Localization of Glc7 and Sds22

Ypi1 forms a complex with Glc7 *in vivo* and *in vitro* (Hazbun *et al.*, 2003; Pedelini *et al.*, 2007). We therefore assayed the cellular distribution of Glc7 and Sds22 in the

Table 5. Quantitative analysis of chromosome segregation after Ypi1 depletion

No. of of loci	Normal				Abnormal

	1	1	2	2	0	2	>2
<i>YPI1</i> ^a							
0 h	53 ^b	28	1	17	0	0	0
8 h	35	37	3	25	0	0	0
12 h	40	37	2	19	<1	2	0
16 h	41	37	3	18	1	0	0
<i>pGAL-YPI1</i>							
0 h	54	28	<1	18	<1	0	0
8 h	41	41	1	16	0	0	0
12 h	23	62	3	5	<1	5	0
16 h	19	55	11	2	14	8	<1
<i>pGAL-YPI1 mad1Δ</i>							
0 h	39	24	1	14	15	6	0
8 h	40	26	2	17	12	3	0
12 h	40	25	7	12	14	<1	1
16 h	28	20	3	3	34	9	1
<i>mad1Δ</i>							
0 h	78	10	0	9	0	3	0
8 h	38	27	2	27	3	2	1
12 h	41	28	5	21	2	1	1
16 h	40	31	3	17	3	2	3

^a The following strains were used: *YPI1*, JB338-3A; *pGAL-YPI1*, KT2668, *pGAL-YPI1 mad1Δ*, JB338-13B; and *mad1Δ*, JB338-14D.

^b Numbers represent the percentage of cells in each category for a particular strain at the denoted time in YPD medium. n = 100–200 cells for each strain at each time point.

P_{GAL1}-3HA-YPI1 strain to determine if Ypi1 depletion alters the subcellular location of either protein. In both cases, we used fusions to the monomeric form of the codon-optimized YFP variant, EmCitrine (hereafter referred to as mYFP) (Griesbeck *et al.*, 2001). In a separate study (Tatchell, unpublished data), genomic *GLC7*-fluorescent protein (FP) fusions were constructed using a variety of different FP variants, and growth rates of the resulting strains were assayed at different temperatures. Although all fusions suppressed the lethality of a *GLC7* deletion, many variants conferred slow growth. In contrast, the *GLC7-mYFP* strain exhibited a growth rate similar to that of the wild-type strain at temperatures ranging from 11 to 37°C. Therefore, we used this FP fusion for our studies of Glc7 and Sds22. As observed previously for the GFP-Glc7 fusion (Bloecher and Tatchell, 2000), Glc7-mYFP accumulates in the cytoplasm, nucleus, and at the bud neck (Figure 2B). In cells from late log and early stationary phase cultures, Glc7-mYFP accumulates to highest levels in the nucleolus, as reported previously (Bloecher and Tatchell, 2000). Sds22-3Myc was found by indirect immunofluorescence to localize largely to the nucleus (Peggie *et al.*, 2002). However, direct visualization of Sds22-mYFP fluorescence in live cells revealed a more equal distribution between the nucleus and cytoplasm. Although nuclear fluorescence levels were higher than those in the cytoplasm, in some cells, the nuclear and cytoplasmic levels were nearly equal, making it difficult to unambiguously identify the nucleus. Expression of a RFP fusion to Pom34, a nuclear pore protein (Pom34-mCherry) allowed us to delineate the nuclear periphery in these cells. Total fluorescence was much lower in an Sds22-mYFP strain than in the Glc7-mYFP strain. This apparent difference in expression was confirmed by immunoblot analysis (Figure 2A), from which we estimate that Sds22-mYFP is expressed at 10–20-

fold lower levels than is Glc7, a greater difference than the approximately fourfold previously estimated from detection of TAP-tagged fusion proteins (Ghaemmaghami *et al.*, 2003).

Ypi1 protein levels were depleted in the *GLC7-mYFP* and *SDS22-mYFP* strains containing the *P_{GAL1}-3HA-YPI1* promoter fusion. As was previously reported for depletion of Ypi1 protein using a *tetO₂-YPI1* strain (Pedelini *et al.*, 2007), we observed that nuclear Glc7 levels were reduced after transfer from YPGal to YPD. As shown in Figure 2B, nuclear Glc7-mYFP fluorescence was greatly reduced after 8 h in YPD, and after 12 h Glc7-mYFP fluorescence was equally distributed between the nucleus and cytoplasm. We quantitated the levels of Glc7-mYFP fluorescence in the nucleus and in the cytoplasm after Ypi1 depletion and plotted the ratio of nuclear to cytoplasmic fluorescence in Figure 2C. The nuclear/cytoplasmic ratio was the same for WT and *P_{GAL1}-3HA-YPI1* cells on YPGal and through 4 h on YPD, after which time the nuclear/cytoplasmic ratio dropped in the *P_{GAL1}-3HA-YPI1* cells (Figure 2C, filled symbols). We also observed bright fluorescent foci (Figure 2B, arrows) in the Ypi1-depleted cells, as noted previously (Pedelini *et al.*, 2007). The spots were occasionally found in *P_{GAL1}-3HA-YPI1* cells growing in YPGal medium but were prominent after 4 h in YPD. The number, size, and brightness of these punctae varied widely from cell to cell, suggesting they do not represent a well-defined organelle or structure. The spots are similar to the bright punctae of GFP-Glc7 that accumulate in *sds22-6* mutant cells at the nonpermissive temperature (Peggie *et al.*, 2002) and are reminiscent of aggresomes that result from the accumulation of unfolded proteins in mammalian cells (Johnston *et al.*, 1998). Immunoblot analysis for Glc7-mYFP in total cell extracts revealed that Ypi1 depletion had little influence on the total amount of Glc7 (Figure 2D). Thus, Ypi1 is important for maintaining

Figure 2. Ypi1 is required for the nuclear localization of Glc7. (A) Immunoblot analysis of extracts from WT (KT1112), *GLC7-mYFP* (KT2422), and *SDS22-mYFP* (JB320-1B) strains with anti-GFP antibody. Pgk1 serves as a loading control. (B) Subcellular distribution of Glc7-mYFP in WT (KT2771) and *P_{GAL1}-3HA-YPI1* (KT2767) strains at the indicated time after shifting to YPD. Cells were examined by fluorescence microscopy with a GFP filter set for Glc7-mYFP and a rhodamine filter set for Pom34-mRFP. Bar, 5 μ m. (C) Quantitative analysis of the ratio of nuclear to cytoplasmic levels of Glc7-mYFP in the WT (KT2422) (□ and ○) and *P_{GAL1}-3HA-YPI1* (KT2432; ■ and ●) cells shown in B. Squares and circles indicate data from two separate experiments. To accurately compare the changes in fluorescence levels between experiments we determined the nuclear/cytoplasmic fluorescence ratio rather than the absolute signal, which can fluctuate with changes in Hg-Arc intensity. More than 50 cells were examined for each strain at each time point. Bright fluorescent punctae were excluded from the analysis. (D) Immunoblot analysis with anti-GFP antibody of Glc7-mYFP in extracts from the strains in B. Left and right panels, lane 1, *GLC7-mYFP* strain (KT2422); lane 2, *GLC7-mYFP P_{GAL1}-3HA-YPI1* strain (KT2432); and lane 3, WT strain (KT1112). Pgk1 serves as a loading control.

the normal cellular distribution of Glc7 in the cell but it does not influence Glc7 levels.

Ypi1 depletion also results in loss of Sds22-mYFP from the nucleus (Figure 3A) but in contrast to Glc7-mYFP, which becomes equally distributed between the nucleus and the cytoplasm, Sds22-mYFP becomes largely excluded from the nucleus. After 6 h in YPD, nuclear Sds22-mYFP fluorescence was generally lower than in the cytoplasm. By 8–10 h, Sds22 was largely excluded from the nucleus in a majority of cells (Figure 3A, arrows). However, fluorescent punctae were not observed in these cells, even after long periods in YPD medium. As for Glc7, Ypi1 depletion did not result in a reduction of Sds22-mYFP protein levels. In fact, by immunoblot, total endogenous Sds22 levels increased after Ypi1 depletion (Figure 3B).

The reduction in the levels of nuclear Glc7 and Sds22 induced by Ypi1 depletion suggests that Ypi1 could act to

facilitate nuclear import of Glc7. Consistent with the possibility that high nuclear Glc7 levels are necessary for normal cell cycling, Pinsky *et al.* (2006a) observed that overexpression of the cytoplasmic Glc7 targeting subunits Gip3 and Gip4 results in nuclear depletion of Glc7 and concomitant G2/M arrest. However, it is possible that the reduction in nuclear Glc7 in the *YPI1* mutants is only indirectly responsible for the cell cycle arrest. This possibility is supported by the observation that two Glc7 FP fusions that contain tandem copies of RFP variants, tdimer2 and tdTomato, fail to accumulate to high levels in the nucleus. As shown in Figure 4A, Glc7-tdimer2 is targeted to the bud neck but does not accumulate in the nucleus to levels above those in the cytoplasm. The subcellular distribution of Sds22-mYFP in this strain is similar to that observed in wild-type cells. A similar pattern of fluorescence is observed for Glc7-tdTomato (data not shown). In contrast, Glc7-mCherry, a fusion to a mono-

Figure 3. Ypi1 is required for the nuclear localization of Sds22. (A) Subcellular distribution of Sds22-mYFP in WT (JB328-12C) and *P_{GAL1}-3HA-YPI1* (JB331-3A) strains at the indicated times in YPD. Pom34-mRFP serves as a nuclear periphery marker. White arrows indicate loss of Sds22 from the nucleus. Cells were examined by fluorescence microscopy with a YFP filter set for Sds22-mYFP and a rhodamine filter set for Pom34-mRFP. Bar, 5 μ m. (B) Immunoblot analysis of Sds22-mYFP in extracts from the strains in A. Left and right panels, lane 1, *SDS22-mYFP* strain (JB328-12C); lane 2, *SDS22-mYFP P_{GAL1}-3HA-YPI1* strain (JB331-3A). The Sds22 protein level at the 16-h time point is inconsistently low.

Figure 4. Variation in nuclear Glc7 levels between Glc7-RFP variants. (A) Yeast strains KT2729 (GLC7-tdimer2 *SDS22*-mYFP) and KT2732 (GLC7-mCherry *SDS22*-mYFP) were grown to exponential phase in YPD medium, and cells were examined by DIC and fluorescence microscopy. Bar, 5 μ m. (B) Cultures of strain KT2729 (GLC7 P_{GAL1} -YPI1), KT2432 (GLC7-mYFP P_{GAL1} -YPI1), KT2780 (GLC7-mCherry P_{GAL1} -YPI1), KT2739 (GLC7-tdimer2 P_{GAL1} -YPI1), KT1112 (GLC7 YPI1), KT2422 (GLC7-mYFP), KT2487 (GLC7-mCherry), and KT2453 (GLC7-tdimer2) were serially diluted onto YPD (glucose) and YPGal (galactose) plates and incubated at 30°C for 48 h before imaging.

meric RFP variant, has a subcellular distribution similar to that of other Glc7-FP fusions (Figure 4A). Nevertheless, GLC7-tdimer2 and GLC7-mCherry strains grow at similar rates in a wild-type genetic background or with P_{GAL1} -YPI1 (Figure 4B). We also note that GLC7-mCherry and GLC7-tdimer2 strains containing P_{GAL1} -3HA-YPI1 arrest more rapidly on glucose medium than the congenic GLC7-mYFP or GLC7 strains (Figure 4B). A complete understanding of this genetic interaction will require a more thorough characterization but the results indicate that the cell cycle arrest induced by Ypi1 depletion is not simply due to the depletion of Glc7 from the nucleus.

Ypi1 also interacts with the Ppz1 phosphatase in yeast two-hybrid (Venturi *et al.*, 2000) and GST-pulldown assays (Garcia-Gimeno *et al.*, 2003) and can inhibit Ppz1 phosphatase activity in vitro (Garcia-Gimeno *et al.*, 2003). These results suggest that Ypi1 could also act on the Ppz1 phosphatase in vivo. We therefore examined the subcellular location of a GFP-Ppz1 fusion protein after Ypi1 depletion. GFP was inserted near the N-terminus of the protein, after the N-myristoylation site, and the fusion gene was integrated ectopically at the *URA3* locus. GFP-Ppz1 fluorescence appeared to be on internal membranes (Supplementary Figure S2), consistent with myristoylation of the protein (Clotet *et al.*, 1996). After 8 h in YPD medium, the GFP-PPZ1 P_{GAL1} -3HA-YPI1 cells displayed the same cell cycle delay previously observed for other P_{GAL1} -3HA-YPI1 strains. However,

the fluorescence pattern did not change even after 20 h after Ypi1 depletion (Supplementary Figure S2). Although these results do not rule out the possibility that Ypi1 influences Ppz1 activity, they do indicate that Ypi1 is not necessary for the normal subcellular distribution of Ppz1.

Ypi1 Is a Nuclear Protein

The mammalian PP1 inhibitor-3 (Inh3) localizes to nucleoli and centrosomes within nuclei (Huang *et al.*, 2005). Pedelini *et al.* (2007) observed that a Ypi1-GFP fusion protein was also largely nuclear. However, we were unable to observe GFP fluorescence in our yeast strains containing a chromosomally integrated Ypi1-GFP fusion (our unpublished data), as previously reported (Huh *et al.*, 2003). This YPI1-GFP allele is partially defective for Ypi1 activity, as indicated by the strong genetic interactions between YPI1-GFP and different alleles of GLC7 and *SDS22* (see below). The YPI1-mYFP allele shows less severe genetic interactions with GLC7 and *SDS22* alleles than did the YPI1-GFP fusion, but it still displays some defects. Little or no fluorescence was detected for the mYFP fusion, and very little protein was detected by immunoblot analysis (our unpublished data), suggesting that these fusions are not stable. In contrast, a YPI1-13Myc allele is functional (see Table 6). Our studies of Ypi1 localization were therefore conducted with this fusion protein. Indirect immunofluorescence with anti-Myc antibody revealed that Ypi1 is located predominantly in the nucleus (Figure 5A), consistent with the previous report (Pedelini *et al.*, 2007). Ypi1-13Myc is present in the nucleus of all cells regardless of bud size, indicating that there is no cell cycle dependence for its nuclear localization. Immunoblot analysis revealed that the levels of Ypi1-13Myc in total cell extracts are lower than a comparable Glc7-13Myc fusion (Figure 5B).

The dependence of nuclear Glc7 and Sds22 on Ypi1 led us to determine if the nuclear localization of Ypi1 is dependent on normal Glc7 and/or Sds22 activity. Glc7 dependence was assayed by localizing Ypi1 in a *glc7-129* mutant, which delays at G2/M in the cell cycle due to activation of the mitotic checkpoint (Bloecher and Tatchell, 1999) and exhibits genetic interactions with YPI1 mutants (see below). Ypi1-13Myc levels appear to be elevated in the nuclei of *glc7-129* mutant cells (Figure 5A), but total levels of Ypi1-13Myc appear to be unaltered, as judged by immunoblot analysis of whole cell extracts (Supplementary Figure S3). We also tested other GLC7 mutants with reported cell cycle defects (*glc7-127*, *glc7-F256A*) and in all cases nuclear Ypi1 levels are the same or elevated relative to the wild-type strain (data not shown). These results indicate that defects in Glc7 activity do not result in depletion of Ypi1 from the nucleus.

The dependence of Ypi1 localization on Sds22 was assayed in the temperature-sensitive *sds22-6* mutant (Peggie *et al.*, 2002). Nuclear levels of Ypi1-13Myc appear reduced in the *sds22-6* mutant at the permissive temperature (24°C) relative to the wild type (Figure 5C), but transiently increase after shifting cells to the nonpermissive temperature (37°C) for 1 h. Two and 3 h after the temperature shift, the nuclear levels appear similar to those observed at the permissive temperature. However, nuclear Ypi1 levels appear to increase in the wild-type strain after 1 h at 37°C, suggesting that the increase in nuclear localization of Ypi1 upon temperature upshift is not related to the *SDS22* mutation. Immunoblot analysis revealed that total levels of Ypi1-13Myc protein are similar in the WT and *sds22-6* strains (Figure 5D). Together, these results indicate that the ability of Ypi1 to localize to the nucleus may be influenced by Sds22 activity.

Table 6. Genetic interactions between *GLC7*, *SDS22*, and *YPI1* alleles

	<i>YPI1</i> -GFP	<i>YPI1</i> -mYFP	<i>YPI1</i> -13Myc	<i>sds22-6</i>	<i>SDS22</i> -mYFP
<i>glc7-129</i>	Lethal ^a	± at 24° – at 30°C, 37°C	+	Lethal	Lethal
<i>glc7-F256A</i>	Lethal	± at 30°C – at 37°C	+ at 30°C ++/– at 37°C	± at 30°	+ at 30°C – at 37°C
<i>glc7-127</i>	±	+	+	±	+
<i>glc7-132</i>	+ at 30°C ± at 37°C	+	+	+	+
<i>glc7-109</i>	+ at 30°C ^b ± at 37°C	+	+	+	+
<i>sds22-6</i>	± at 30°C – at 37°	+ at 30°C ++/– at 37°C	+		
<i>SDS22</i> -mYFP	±	+	+		

^a The following descriptors are used for the growth of double mutants: lethal, double mutants did not grown into colonies at 24 or 30°C; +, normal growth; ±, slow growth, at all temperatures if not stipulated; –, no growth at a specific temperature; ++/–, slight but perceptible slower growth of the double mutant.

^b Low glycogen accumulation at all temperatures, as assayed by staining colonies with iodine vapor.

The Glc7-binding Motif in *Ypi1* Is Necessary for Activity In Vivo

A mutation in the consensus VXF Glc7 binding motif (VRW in *Ypi1*), *ypi1*^{W53A}, reduces Glc7 binding and blocks the ability of *Ypi1* to inhibit Glc7 activity in vitro (Garcia-Gimeno *et al.*, 2003), but the biological activity of this mutant has not been reported. To address this, we assayed the ability of *ypi1*^{W53A}, *ypi1*^{V51A}, and *ypi1*^{V51A/W53A} alleles to complement the lethality of a *YPI1* deletion. The mutants were expressed from the native promoter either on a *CEN* vector (low-copy) or by ectopic integration at the *URA3* locus as outlined in *Materials and Methods*. The *ypi1*^{W53A} allele expressed from a low copy *CEN* plasmid is able to complement the lethality of *ypi1Δ*. However, the doubling time of the resulting strain is 180 min, twice that of the wild-type strain. *ypi1*^{W53A} is lethal when integrated into the chromosome (Figure 6A), suggesting that the increased dosage of the gene on the *CEN* plasmid provides sufficient activity for viability. The *ypi1*^{V51A/W53A} mutant expressed from the *CEN* vector is unable to complement the lethality due to *ypi1Δ* (Figure 6A). These results indicate that a direct interaction between *Ypi1* and Glc7 is likely to be essential for the activity of *Ypi1*.

The viability of *ypi1*^{W53A} strains allowed us to test genetically whether the essential function of *Ypi1* is to regulate Glc7. If the phenotype of *ypi1*^{W53A} strains resembles that caused by *Ypi1* depletion, then it is likely that the major cellular role of *Ypi1* is to regulate Glc7 or some other protein that binds to the VXF motif. Our results support this hypothesis. Nuclear Glc7-mYFP levels are reduced and bright fluorescent spots are apparent in *ypi1*^{W53A} mutant cells (Figure 6B). Sds22-mYFP is largely excluded from the nucleus (Figure 6C, arrows) in *ypi1*^{W53A} mutants. Quantitative analysis revealed that in 85% of WT cells, Sds22-mYFP fluorescence is greater in the nucleus than in the cytoplasm (*n* = 116 cells). In contrast, for the *ypi1*^{W53A} mutant, only 8% of the cells show higher levels of fluorescence in the nucleus (*n* = 131 cells).

As observed for *Ypi1*-depleted cultures, *ypi1*^{W53A} cultures contain many large-budded cells with short and misaligned spindles (Figure 6E). In some cells, the spindle is located fully in the bud. Flow cytometry analysis of these cells showed a distribution with broad DNA content centered at 2C (Figure 6D). We were unable to recover *mad1Δ ypi1*^{W53A} double mutants, suggesting that the spindle assembly check-

point is essential for the viability of the *ypi1*^{W53A} mutant. Using the *GFP-LacI lacO* array system, we observed extensive evidence for chromosome instability. *ypi1*^{W53A} mutant cells contain multiple chromosome IV spots (data not shown). In conclusion, the similar phenotypes of *ypi1*^{W53A} and *P_{GAL1}-3HA-YPI1* argue that the essential function of *Ypi1* is to associate with and regulate Glc7.

YPI1 Mutations Suppress the Temperature Sensitivity of the *ipl1-2* Mutant

If *Ypi1* regulates the nuclear activity of Glc7, we predict that *YPI1* mutations should suppress the temperature-sensitive growth of mutants in *IPL1*, as has been shown for *GLC7* and *SDS22* mutants (Hsu *et al.*, 2000; Peggie *et al.*, 2002; Pinsky *et al.*, 2006a). Indeed, *ypi1*^{W53A} restores growth of an *ipl1-2^{ts}* mutant at 34°C, similar to the *glc7-127* control (Figure 6F).

As discussed above, the inability to detect *Ypi1*-GFP by fluorescence microscopy or by immunoblot (data not shown) suggests that *YPI1*-GFP may not be fully functional. Consistent with this idea, nuclear levels of Glc7-mYFP and Sds22-mYFP are reduced in *YPI1*-GFP cells (Supplementary Figure S4). Interestingly, Glc7-mYFP is retained at the neck of small and medium budded cells and quantitative analysis of fluorescence revealed a significant increase in Glc7-mYFP at the bud neck (Supplementary Figure S4). Although *YPI1*-GFP strains do not show an obvious growth defect, this allele partially suppresses the temperature-sensitive growth of the *ipl1-2* mutant (Supplementary Figure S4) and shows genetic interactions with *GLC7* and *SDS22* mutations (see below). These results further support the hypothesis that *Ypi1* is an activator of nuclear Glc7.

Genetic Interactions between *GLC7*, *SDS22*, and *YPI1*

The dependence on *Ypi1* activity for the nuclear accumulation of Glc7 and Sds22 is consistent with biochemical evidence that the three proteins form a ternary complex (Hazbun *et al.*, 2003; Pedelini *et al.*, 2007). If this complex is essential for Glc7 function in vivo, then we would predict strong genetic interactions between mutants in the three genes. Thus, the phenotype of a partial loss of function mutation in *Ypi1* should be exacerbated by a mutation in one of its binding partners. The nature of the genetic interactions may also inform us concerning the precise roles of *Ypi1* and Sds22. For example, the ability of *Ypi1* and Sds22

Figure 5. Ypi1 is a nuclear protein. (A) Indirect immunofluorescence with anti-Myc antibody of *YPI1-13Myc* (JB385-1A) and *YPI1-13Myc glc7-129* (JB409-1C) strains. An untagged WT strain (KT1112) serves as the negative control. (B) Immunoblot analysis with anti-Myc antibody of extracts from the *YPI1-13Myc* (JB385-1A) and *GLC7-13Myc* (JB412-1B) strains grown to midlog phase in YPD at 30°C. (C) Indirect immunofluorescence of Ypi1-13Myc in WT (JB385-1A) and *sds22-6* (JB403-5C) strains at the permissive (24°C) and nonpermissive (37°C) temperatures. (D) Immunoblot analysis of Ypi1-13Myc with anti-Myc antibody of extracts from the strains described in C.

to inhibit Glc7 activity in vitro (Garcia-Gimeno *et al.*, 2003; Pedelini *et al.*, 2007) could be interpreted to indicate that the complex has an inhibitory role in vivo. To explore these interactions, we assayed the phenotypes of a collection of *GLC7* mutants that also contained mutations in either *YPI1* or *SDS22*. We tested alleles of *GLC7* that confer cell cycle-related defects (*glc7-129*, *glc7-127*, and *glc7-F256A*; Hsu *et al.*, 2000), and alleles that confer no cell cycle defect (*glc7-132* and *glc7-109*). *glc7-132* mutants grow slowly and fail to accumulate glycogen but show no obvious cell cycle defect (Baker *et al.*, 1997), whereas *glc7-109* mutants are hypersensitive to many cations and accumulate high levels of glycogen (Williams-Hart *et al.*, 2002). We crossed these mutants to *ypi1-GFP* and *sds22-6* mutant strains, and the double mutants were recovered as haploid meiotic spore clones.

An example of our genetic analysis is provided in Figure 7 and the work is summarized in Table 6. One of the most

striking observations is the incompatibility between *GLC7* mutations that confer cell cycle defects with *YPI1-GFP* and *sds22-6*. In some cases the double mutants are inviable. This was true for *glc7-129* with *YPI1-GFP* or *sds22-6*. Similar genetic interactions were observed for *glc7-127* and *glc7-F256A*. Because these three *GLC7* alleles have recessive defects, the lethality or detrimental effects in combination with recessive *YPI1* and *SDS22* mutations is consistent with a positive effect of Ypi1 and Sds22 on the cell cycle function(s) of Glc7. Interestingly, the dominant hyperglycogen trait of *glc7-109* is suppressed by *YPI1-GFP* (Figure 7B) and *sds22-6* (data not shown), again pointing to a positive or activating role for Ypi1 and Sds22. The known roles for Glc7 in glycogen metabolism are the dephosphorylation of glycogen synthase and phosphorylase (François *et al.*, 1992; Hardy *et al.*, 1994; Wilson *et al.*, 2005). Because glycogen is synthesized in the cytoplasm, the influence of *YPI1* on glycogen levels suggests that Ypi1 activity may not be restricted to the nucleus. We never observed a growth condition in which either *YPI1-GFP* or *sds22-6* actually suppress a recessive trait conferred by a *GLC7* mutant. These results together with those described above indicate that Ypi1 and Sds22 have positive effect(s) on Glc7 function in vivo.

DISCUSSION

Ypi1, Sds22, and Glc7 exist as a ternary complex in cell extracts (Hazbun *et al.*, 2003; Pedelini *et al.*, 2007) and two-hybrid studies indicate that Ypi1 may have separable binding sites for Glc7 and Sds22 within its N- and C-terminus, respectively (Pedelini *et al.*, 2007). *YPI1* and *SDS22* are evolutionarily conserved and essential for cell viability, suggesting that this ternary complex could play a key role in the regulation of PP1/Glc7. What is the nature of this regulation? Ypi1 was originally identified as an in vitro inhibitor of Glc7 phosphatase activity toward *p*-nitrophenylphosphate and protein substrates of Glc7 (Garcia-Gimeno *et al.*, 2003; He and Moore, 2005). More recently, Sds22, in a complex with Ypi1, was shown to be a more effective Glc7 inhibitor (Pedelini *et al.*, 2007). Consistent with the possibility that Sds22 and Ypi1 inhibit Glc7 in vivo, overexpression of Ypi1 reduces glycogen levels and overexpression of Sds22 and Ypi1 suppress the temperature-sensitive growth defect of the *ipl1-321* mutant (Garcia-Gimeno *et al.*, 2003; Pedelini *et al.*, 2007). The most closely related mammalian protein, Inhibitor-3, is also an effective inhibitor of PP1 activity in vitro (Zhang *et al.*, 1998). These data support the hypothesis that the Ypi1/Sds22/Glc7 ternary complex has little phosphatase activity in vitro, but they do not directly address its physiological role. To address this, we investigated the physiological consequences of reducing Ypi1 activity by either depleting cells of Ypi1 or in cells expressing *YPI1* mutants. Our results strongly support the hypothesis that Ypi1 has a positive regulatory or modulatory role on Glc7 in cells.

The first line of evidence to suggest that Ypi1 acts as a positive Glc7 regulator is the cell cycle arrest at G2/M brought about by depletion of Ypi1 using the *GAL1* promoter as described here or with the *tetO* promoter as previously reported (Pedelini *et al.*, 2007). We show here that the arrest is dependent on the spindle assembly checkpoint because mutational inactivation of the checkpoint with a *MAD1* mutation reverses the arrest. This phenotype is similar to that of several *GLC7* mutants that activate the mitotic checkpoint (Bloecher and Tatchell, 1999; Sassoon *et al.*, 1999). Furthermore, *ypi1-W53A* mutant cells also accumulate in G2/M with short spindles and are inviable in the absence of the spindle checkpoint. The *ypi1-W53A* protein is partially

Figure 6. Interaction with Ypi1 via the VXW motif is necessary for nuclear targeting and activity of Glc7 in vivo. (A) Diagram of Ypi1 variants and their phenotypes, expressed either from a *CEN* plasmid or by ectopic integration into the genome. (B) Subcellular distribution of Glc7-mYFP in a *ypi1Δ* strain carrying either pYPI1 (JB300-3A) or *ypi1*^{W53A} (JB278-16B). Bar, 5 μm. (C) Subcellular distribution of Sds22-mYFP in a *ypi1Δ* strain carrying either pYPI1 (JB377-6C) or *ypi1*^{W53A} (JB378-7D). White arrows indicate nuclei. Bar, 5 μm. (D) Flow cytometry analysis of *ypi1Δ* mutant cultures containing either pYPI1 (JB300-3A) or *ypi1*^{W53A} (JB278-16B). Transformants were grown in synthetic medium to midlog phase and processed as described in Materials and Methods. (E) GFP-Tub1 fluorescence in WT (JB309-8B) and *ypi1*^{W53A} (JB308-7A) cells. Merged DIC and fluorescence images are shown. (F) Serial dilutions of strains were imaged after incubation for 2 d on YPD at the indicated temperatures. The strains used are WT (JB275-1A), *ipl1-2* (JB280-5B), *ypi1*^{W53A} (JB276-8B), *ipl1-2 ypi1*^{W53A} (JB279-2C), and *glc7-127 ipl1-2* (KT1968).

defective in Glc7 binding (Garcia-Gimeno *et al.*, 2003), supporting the hypothesis that the major role of Ypi1 is to regulate Glc7 activity. Furthermore, *ypi1*^{W53A} and *YPI1*-GFP alleles both suppress partially the temperature sensitivity of *ipl1-2* mutants, as do recessive mutations in *SDS22* and *GLC7* (Francisco and Chan, 1994; Francisco *et al.*, 1994; Sassoon *et al.*, 1999; Hsu *et al.*, 2000; Pegg *et al.*, 2002; Zhang *et al.*, 2005). The results of our genetic analysis further support the conclusion that Ypi1 is a positive regulator of Glc7. *GLC7* mutants with cell cycle defects (*glc7-129*, *glc7-127*, and *glc7-F256A*) are inviable in combination with *ypi1*-W53A and *YPI1*-GFP, whereas *GLC7* mutants with major defects unrelated to the cell cycle are only marginally influenced by *ypi1*^{W53A} and *YPI1*-GFP. It is important to stress that in no case did we see any alleviation or partial suppression of *GLC7*-dependent defects by *YPI1* mutations, as might be expected if Ypi1 has an inhibitory role in vivo. Together, the genetics and cell biological data point to a positive role of Ypi1 in regulating Glc7 activity.

What is the biochemical role of the Glc7-Sds22-Ypi1 complex? Ypi1 could act as a targeting subunit for Glc7 toward nuclear substrates. The physiologically relevant PP1 holoenzyme toward many substrates consists of the conserved PP1/Glc7 catalytic subunit and a VXF-containing targeting subunit (reviewed in Cohen, 2002; Ceulemans and Bollen, 2004). The targeting subunit acts as a specificity factor by either direct association with the substrate and/or by increasing the catalytic activity toward the specific substrate and decreasing it toward others. Ypi1 is a candidate for a nuclear targeting subunit because it binds to PP1 via a VXF motif and because loss of Ypi1 activity results in a phenotype similar to that of *GLC7* mutants. However, at this time there is no evidence that Ypi1 binds to potential nuclear substrates of Glc7, such as Dam1 or Ndc80.

Rather than targeting Glc7 to relevant nuclear substrates, Ypi1 and Sds22 could perform another essential modulatory role. Ypi1 could help target Glc7 and Sds22 to the nucleus. Although mammalian PP1 isoforms and Glc7 are abundant

Figure 7. Genetic interactions between *GLC7* and *YPI1*. (A and B) Images of serial dilutions of the strains after incubation for 2 d on YPD at designated temperatures. The strains used are *YPI1-GFP* (JB282-1B), *glc7-127* (JB290-1B), *YPI1-GFP glc7-127* (JB290-1D), *glc7-132* (JB298-5B), *YPI1-GFP glc7-132* (JB298-7A), WT (KT1112), *glc7-109* (JB297-2A), *YPI1-GFP glc7-109* (JB297-1A). The last image in B is that of strains grown on synthetic complete medium that were stained with iodine to assay glycogen levels. Dark color indicates high glycogen levels.

in the nucleus, the mechanism of their nuclear targeting is poorly understood. Many PP1/Glc7 isoforms contain polybasic sequences at their COOH termini, suggesting that they could be imported by the classic karyopherin-dependent system, but mutational analysis of these sequences indicates that they are not essential for nuclear targeting (Bloecher and Tatchell, 2000; Lesage *et al.*, 2004). However, variants of Glc7 and PP1 that contain mutations in the RVXF-binding channel are less abundant in the nucleus (Wu and Tatchell, 2001; Lesage *et al.*, 2004), suggesting that an RVXF-containing protein is responsible for cotransporting PP1/Glc7 into the nucleus. Overexpression of the nuclear PP1 targeting subunits NIPP1 and PNUTS facilitates nuclear accumulation of PP1_γ-F257A, one variant in the RVXF-binding channel (Lesage *et al.*, 2004), and conversely, overexpression of cytoplasmic Glc7-binding proteins results in decreased levels of nuclear Glc7 (Pinsky *et al.*, 2006a). However, these studies involving protein overexpression do not necessarily inform us on the mechanism of nuclear transport. Ypi1 has a potential NLS near its C-terminus (¹²⁰ERRHRK¹²⁵) and a precise deletion of this sequence results in a slow growth phenotype similar to the *ypl1*^{W53A} mutant (our unpublished observations), but further experiments must be done before we can definitively assign the sequence as an NLS. Nevertheless, it is possible that Glc7 and Sds22 could be imported into the nucleus by piggybacking on Ypi1.

Consistent with a role for Ypi1 in the nuclear targeting of Glc7 and Sds22, nuclear Glc7 levels fall and Sds22 becomes largely excluded from the nucleus after Ypi1 depletion. Our immunoblot analyses of total Ypi1, Sds22, and Glc7 proteins indicate that Glc7 is more abundant than Sds22 and Ypi1, suggesting that a ternary Glc7-Sds22-Ypi1 complex may contain most of the cellular Ypi1 and Sds22 but only a small portion of the total Glc7. This possibility would explain why Ypi1 depletion results in the exclusion of Sds22 from the nucleus but only a reduction in nuclear Glc7 levels.

Ypi1 and Sds22 could also have a chaperone-like role on Glc7 that only indirectly influences phosphatase activity toward nuclear substrates. Precedent for this possibility comes from studies on mammalian inhibitor 2 (I-2) and the related yeast protein, Glc8. I-2 was originally identified as a PP1 inhibitor (Brandt *et al.*, 1975; Huang and Glinnsman,

1976) but was subsequently found to have positive or chaperone-like activity toward PP1 (Alessi *et al.*, 1993; Park *et al.*, 1994). Glc8 also inhibits Glc7 activity in vitro (Tung *et al.*, 1995), but the phenotype of *glc8* mutants is consistent with a positive role (Cannon *et al.*, 1994; Tung *et al.*, 1995; Nigavekar *et al.*, 2002; Tan *et al.*, 2003). As observed for *YPI1*, overexpression of *GLC8* and a *glc8* null mutation can partially suppress the temperature sensitivity of an *ipl1-2* mutation (Tung *et al.*, 1995). However, there are clear differences between Glc8 and Ypi1. *YPI1* is an essential gene, whereas *glc8* null mutants have little or no growth defect. This could be interpreted to suggest that Glc8 modulates predominantly cytoplasmic Glc7, whereas Ypi1 is specialized toward nuclear Glc7 activity. However, the activities of Glc8 and Ypi1 may not be completely separate, because both influence glycogen metabolism, and mutations in both genes partially suppress *ipl1-2*. This possibility is also consistent with our recent observation that *glc8Δ YPI1-GFP* mutants are very slow growing (unpublished observations). We also find it interesting that the levels of Glc7 at the bud neck actually increase in the *YPI1-GFP* mutant (Figure 6B). This suggests that the Ypi1 activity that is required for nuclear Glc7 activity antagonizes other forms of Glc7 or Glc7 holoenzymes, such as that which binds to the bud neck.

In summary, we have presented evidence that Ypi1 forms an essential positive regulatory complex with Sds22 that largely governs Glc7 activity in the nucleus. Although the precise role of this complex is unknown, the fact that *YPI1*, *SDS22*, and *GLC7* are all essential and evolutionarily conserved suggests that understanding its role in *S. cerevisiae* could be relevant to studies of PP1 in metazoans.

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