



Published in final edited form as:

Curr Biol. 2009 July 28; 19(14): 1182–1187. doi:10.1016/j.cub.2009.06.043.

Protein Phosphatase I Regulates Exit from the Spindle Checkpoint in Budding Yeast

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Summary

Accurate chromosome segregation depends on sister kinetochores coming under tension when they make bioriented attachments to microtubules from opposite poles. The spindle checkpoint halts the cell cycle in response to defects in generating proper attachments or tension on kinetochores [1,2], although the precise signal that triggers the checkpoint is unclear because tension and attachment are coupled [3]. The target of the checkpoint is the Cdc20 protein that initiates the anaphase promoting complex (APC)-dependent degradation of the anaphase inhibitor Pds1/securin [4]. Although the molecular details of spindle checkpoint activation are still being elucidated, phosphorylation by at least four kinases is a crucial requirement [5]. However, less is known about the mechanisms that silence the checkpoint once kinetochores biorient. Here, we show that the catalytic subunit of the budding yeast protein phosphatase I, Glc7, regulates exit from the checkpoint. Glc7 overexpression prevents spindle checkpoint activation in response to both tension and attachment defects. Although *glc7* mutant cells are able to efficiently release from a non-checkpoint-mediated metaphase arrest, they are uniquely sensitive to transient spindle checkpoint activation due to a failure in spindle checkpoint exit. We therefore propose that PPI activity silences the checkpoint by reversing key phosphorylation events.

Results and Discussion

Glc7 overexpression leads to chromosome missegregation

We hypothesized that exit from the spindle checkpoint would require phosphatase activity to reverse the phosphorylation that occurs upon checkpoint activation. Because the budding yeast spindle checkpoint response to defects in kinetochore tension requires Ipl1/Aurora protein kinase activity [6], we tested whether overexpression of the Glc7 protein that opposes Ipl1 function [7] would prevent cells from engaging the checkpoint. First, we analyzed the phenotype of cells overexpressing Glc7 by releasing WT and *pGAL-GLC7* cells from G1 into galactose media to induce Glc7 overexpression. We monitored cell cycle progression by scoring the budding index and found that both strains began budding at similar times, but cells overexpressing Glc7 accumulated with large-buds (Fig 1A). To determine whether these cells were delayed in metaphase or anaphase, we monitored the levels of the anaphase inhibitor Pds1 and the mitotic cyclin Clb2 in a similar experiment.

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Although there was little difference in the kinetics of Pds1 accumulation and destruction between *WT* and *pGAL-GLC7* cells (Fig 1B), Clb2 levels rose with similar timing in both strains but remained high in the cells that overexpressed Glc7 (Fig 1C). Clb2 is stabilized when cells do not properly position the mitotic spindle via Bub2-mediated inhibition of the mitotic exit network (for reviews, see [8,9]), so we analyzed Clb2 levels in *bub2 pGAL-GLC7* cells. We found that Clb2 remained high in these cells (Fig S1), indicating that Glc7 overexpression prevents mitotic exit in a manner that is downstream of Bub2 activity and the spindle position checkpoint. Although the role of Glc7 in mitotic exit is not clear, future research may reveal a previously unknown function for PP1 in regulating mitotic exit.

Because cells overexpressing Glc7 entered anaphase with normal timing, we tested whether chromosomes segregated properly. *WT* and *pGAL-GLC7* cells containing fluorescently marked ChrIV [10] were released from G1 into galactose and analyzed for segregation at anaphase (Fig 1D). Glc7 overexpression caused ~60% of the cells to segregate ChrIV to the same pole instead of opposite poles. This phenotype is similar to *ipl1-321* mutant cells that segregate sisters to the same pole due to a defect in kinetochore biorientation [11], although *ipl1-321* mutant cells show a bias in segregation toward the bud that is not observed when Glc7 is overexpressed [12].

Glc7 overexpression bypasses the tension and attachment checkpoints

Our finding that Glc7 overexpression causes chromosome missegregation without stabilizing Pds1 suggested that it might prevent spindle checkpoint activation. Ipl1 activity is required for the checkpoint when kinetochores are not under tension [6], so we tested whether Glc7 overexpression also bypasses the tension checkpoint. We created a tension defect using a temperature sensitive mutation in the Mcd1/Sccl protein that joins sister chromatids [13,14]. Although kinetochores can bind to microtubules in these cells, the spindle checkpoint is activated because tension cannot be generated on unlinked sister chromatids [15,16]. We arrested *WT* and *mcd1-1* cells containing *pGAL-GLC7* or a control vector in G1 and then released them into galactose media to induce Glc7 at the non-permissive temperature. Spindle checkpoint activation was monitored by analyzing Pds1. As expected, Pds1 levels cycled similarly in wild-type cells regardless of whether Glc7 was overexpressed (Fig 1E). However, although Pds1 was stabilized in the *mcd1-1* mutant, it was destroyed when Glc7 was overexpressed in *mcd1-1* cells. Therefore, high levels of the Glc7 phosphatase prevent spindle checkpoint activation in response to tension defects, consistent with a role in reversing Ipl1 phosphorylation.

In budding yeast, mutants in the *IPL1* gene can engage the spindle checkpoint as long as there are unattached kinetochores [6,17]. We therefore tested whether Glc7 overexpression could bypass the spindle checkpoint in response to unattached kinetochores that were created by the addition of the microtubule depolymerizing drug nocodazole. Wild-type cells with or without *pGAL-GLC7* were arrested in G1 and then released into galactose media containing nocodazole. As a control, we also analyzed cells containing a catalytically dead *pGAL-GLC7-H65K* mutant [18]. We confirmed that microtubules were efficiently depolymerized in the strains by analyzing GFP-Tub1 by microscopy (data not shown). However, although Pds1 was stabilized in control cells containing the vector or catalytically inactive Glc7-H65K, it cycled in cells overexpressing wildtype Glc7 (Fig 1F). Therefore, in contrast to *ipl1* mutants, Glc7 overexpression acts similarly to spindle checkpoint mutants that prevent checkpoint arrest in the presence of tension or attachment defects. These data are consistent with the observation that Ipl1/Aurora kinase activity is required for the spindle checkpoint in response to attachment defects in some other organisms [19-21]. One possibility is that Ipl1/Aurora is required for all spindle checkpoint responses in budding yeast, but its role in the response to attachment defects has not been detected because it is necessary to use conditional mutants that retain residual function [22]. An alternative, and

not mutually exclusive possibility, is that Glc7 overexpression reverses the phosphorylation of kinases in addition to Ipl1/Aurora, consistent with the requirement for multiple kinases in spindle checkpoint activation [5].

Glc7 is required for exit from Mps1-induced spindle checkpoint

Our observation that Glc7 overexpression prevented activation of the spindle checkpoint suggested that endogenous levels of Glc7 may be required to exit from a spindle checkpoint arrest. Although it was previously reported that *glc7* mutants stabilize Pds1 in a checkpoint-dependent manner [23,24], it was not clear whether this reflects a role for Glc7 in turning off the spindle checkpoint and/or reversing Ipl1-mediated phosphorylation of kinetochore proteins to stabilize bioriented attachments [7,23-26]. Therefore, to separate the role of Glc7 in kinetochore-microtubule attachments from a potential role in silencing the spindle checkpoint, we utilized cells overexpressing the Mps1 protein kinase. Mps1 overexpression causes constitutive activation of the spindle checkpoint in the absence of detectable spindle or kinetochore defects and it does not depend on kinetochores for checkpoint activation [27,28]. This allowed us to assay the role of Glc7 in spindle checkpoint recovery after normal bioriented kinetochore attachments were achieved. We arrested *pGAL-MPS1 GLC7* and *pGAL-MPS1 glc7-10* cells in galactose media at the permissive temperature to allow cells to establish bioriented attachments and a spindle checkpoint arrest. The cells were then shifted to the non-permissive temperature to inactivate *glc7-10* and released into glucose media to repress Mps1. While Mps1 and Pds1 were degraded within 20 min of release from the spindle checkpoint arrest in *GLC7* cells, Pds1 degradation was delayed up to 40 minutes in *glc7-10* cells despite normal kinetics of Mps1 repression (Fig 2A). To ensure that the cells had achieved normal bioriented attachments, we analyzed the segregation of a fluorescently marked chromosome and found that it segregated to opposite poles in both the *GLC7* and *glc7-10* cells that entered anaphase (Fig 2B). As additional confirmation that chromosome segregation was normal, we also assayed the viability of the cells as they were released from the checkpoint arrest and found no difference between the strains (Fig 2C). Therefore, cells crippled for Glc7 activity appear to be defective in exiting a spindle checkpoint arrest after they have made bioriented attachments.

Defects in PP1 sensitize cells to increased Mps1 kinase activity

While our data suggested a role for Glc7 in recovery from checkpoint activation, we had to perform the experiment at a non-permissive temperature where *glc7-10* mutants are known to make defective kinetochore-microtubule attachments [24,29]. Although we allowed cells to establish bioriented attachments prior to shifting cells to the high temperature, we could not eliminate the possibility that the stabilization of Pds1 was due to transient activation of the checkpoint because of an undetectable kinetochore defect. Indeed, *glc7-10* mutant cells activate the spindle checkpoint and stabilize the Pds1 protein at the non-permissive temperature ((Fig S2) and [24]). To address this concern, we looked for a semi-permissive temperature where *glc7-10* mutant cells no longer triggered the spindle checkpoint. At 30 degrees, we found that there was a 20 min delay in both Pds1 accumulation and destruction in *glc7-10* cells relative to *WT* (Fig 3A). When we monitored the budding index, we confirmed that *glc7-10* cells take ~20 min longer than *WT* cells to enter the cell cycle when released from a pheromone arrest at 30 degrees (Fig S3). When this delay in cell cycle entry is taken into account, Pds1 levels cycle similarly in *WT* and *glc7-10* cells at 30 degrees.

The establishment of a temperature where the *glc7-10* mutant no longer activates the spindle checkpoint due to defective kinetochores allowed us to more carefully address the requirement for Glc7 activity in exit from the checkpoint. Although high levels of Mps1 expression are lethal due to constitutive activation of the spindle checkpoint, lower levels delay the cell cycle creating a mild growth defect [28]. We reasoned that defects in a

phosphatase required to exit the checkpoint would sensitize cells to low levels of Mps1 expression. We therefore analyzed the growth of cells containing an integrated *pGAL-MPS1* gene in the presence and absence of the *glc7-10* temperature sensitive mutation at 30 degrees. As expected, the growth of the *pGAL-MPS1* strain was normal on glucose media but showed a modest defect on galactose plates (Fig 3B). The growth inhibition depended on spindle checkpoint activity because it was suppressed when the *MAD2* checkpoint gene was disrupted. Strikingly, although the growth of *pGAL-MPS1 glc7-10* cells was almost completely inhibited in the presence of galactose, viability was restored in the absence of *MAD2*. These data strongly suggest that the growth defect is related to continued activation of the spindle checkpoint.

To determine whether there is a sustained spindle checkpoint response in *pGAL-MPS1 glc7-10* cells, we analyzed Pds1 levels. *pGAL-MPS1* and *pGAL-MPS1 glc7-10* strains were arrested in G1 in raffinose media to prevent Mps1 expression and then released into the cell cycle in the presence of galactose at 30 degrees. Glucose was added one hour after release to repress Mps1 because continued expression prevented otherwise wild-type cells from degrading Pds1 for at least 6 hours (data not shown). When Mps1 was only expressed for one hour, Pds1 levels started to decline in *GLC7* cells within 150 min (Fig 3C). However, Pds1 levels remained high in *glc7-10* mutants for the duration of the time course, and this was due to spindle checkpoint activation because Pds1 levels cycled normally when *MAD2* was disrupted in the same strains. The sustained checkpoint response in this experiment is more dramatic than when cells overexpressing Mps1 were released at the higher temperature, suggesting that the checkpoint silencing function of the Glc7-10 protein may be more efficiently inactivated earlier in the cell cycle, or that more of the Mps1 kinase acts unopposed when cells are released from G1 instead of mitosis. Alternatively, another phosphatase may be active during the prolonged mitotic arrest in the previous experiment, therefore diminishing the requirement for Glc7.

The delay in Pds1 destruction that occurs when Mps1 is expressed in *glc7-10* mutants at a semi-permissive temperature strongly suggests that Glc7 is required to turn off the spindle checkpoint. Because *glc7-10* mutant cells do not stabilize Pds1 without exogenous checkpoint activation at 30 degrees, these cells most likely make normal kinetochore-microtubule attachments at this temperature. To ensure that kinetochore attachments were normal, we analyzed the segregation of a pair of fluorescently marked sister chromatids. To monitor segregation in the *pGAL-MPS1 GLC7* and *pGAL-MPS1 glc7-10* strains, we also deleted *MAD2* to prevent checkpoint activation. This experiment provides a sensitive way to determine if there are any segregation defects in *glc7-10* cells at 30 degrees because the cells progress into anaphase with similar kinetics and no mitotic delay. Strikingly, greater than 96% of *GLC7 mad2* and *glc7-10 mad2* cells segregated chromosomes to opposite poles in anaphase after Mps1 was expressed for an hour (Fig 3D). Taken together, these data strongly argue that *glc7-10* cells segregate chromosomes normally at 30 degrees, yet are defective in recovery from a transient spindle checkpoint arrest.

Glc7 is specifically required to exit from the spindle checkpoint

In a complimentary test for the role of Glc7 in exit from the spindle checkpoint that did not involve increasing Mps1 levels, we analyzed recovery from a nocodazole arrest. *WT* and *glc7-10* cells were arrested in nocodazole at 23 degrees for 2 hours and then shifted to 30 degrees for 1 hour to decrease *glc7-10* activity. The cells were then washed into media without nocodazole at 30 degrees and Pds1 levels were analyzed. While Pds1 levels begin to decline in *GLC7* cells 40 min after release, they remained stable for up to 70 min in *glc7-10* cells (Fig 4A). Therefore, Glc7 is required for exit from a nocodazole-mediated spindle checkpoint arrest, although the effect is less dramatic than exit from a Gal-Mps1 arrest.

To determine whether the role of Glc7 is specific to reversing the spindle checkpoint, we asked whether Glc7 is required to exit from a metaphase arrest where the spindle checkpoint is not active. To do this, we depleted the APC activator Cdc20 and analyzed Pds1 levels as Cdc20 protein was restored. *GLC7* and *glc7-10* cells containing *CDC20* under control of the methionine promoter (*pMET-CDC20*) were arrested in metaphase at 23 degrees by the addition of methionine for 2 hours, and then shifted to 30 degrees for 1 hour to reduce *glc7-10* activity. The cells were then washed into media lacking methionine to induce Cdc20 at 30 degrees. In *GLC7* cells, Pds1 was destroyed within 20 min, which corresponded to the time that Cdc20 protein started to accumulate (Fig 4B). The kinetics of Pds1 destruction and Cdc20 accumulation was similar in *glc7-10* cells, indicating that *glc7-10* cells recover normally from a metaphase arrest that does not involve exit from the spindle checkpoint.

Taken together, our data strongly suggest that the budding yeast Glc7 protein phosphatase I regulates exit from the spindle checkpoint, consistent with the need to reverse the phosphorylation required to activate the checkpoint. In *S. pombe*, PP1 is also required to exit the spindle checkpoint [21], so this may be a conserved mechanism for checkpoint inactivation. Although it remains possible that Glc7 also directly regulates the APC, the overexpression of Glc7 led to the stabilization of Clb2 which would not be expected if the APC were globally hyperactivated. Consistent with this, *glc7* mutants showed no delay when released from a checkpoint-independent mitotic arrest.

Although PP1 is known to reverse Aurora-mediated phosphorylation [7,25,30-33], it is not clear whether the role for Glc7 in exiting the checkpoint is strictly related to reversing Ipl1 phosphorylation. Because Glc7 overexpression bypasses the arrest caused by unattached kinetochores but *ipl1* mutants do not, we favor the possibility that Glc7 reverses the phosphorylation of additional checkpoint kinases. The sensitivity of slight increases in Mps1 dosage to decreased Glc7 activity is consistent with the possibility that Glc7 counteracts Mps1 phosphorylation. This is also supported by our finding that the defect in recovery from a checkpoint arrest induced by Mps1 overexpression is more severe than a nocodazole arrest when Glc7 is crippled at a semi-permissive temperature. Although we have not detected altered Ipl1 kinase activity in *glc7* mutants [25], Glc7 may also directly regulate the activity of Mps1 and other checkpoint kinases. Taken together, our data suggest that the most likely role for Glc7 is to reverse checkpoint phosphorylation, possibly through multiple mechanisms that will need to be elucidated in the future. It will also be critical to identify and characterize the Glc7 regulatory subunit that directs it to dephosphorylate checkpoint targets to better understand the process of checkpoint silencing.

Experimental Procedures

Microbial Techniques, yeast strains and plasmids

Media and microbial techniques were essentially as described [34]. All experiments in which cells were released from a G1 arrest were carried out by α -factor arrest and release as described where pheromone was added back when cells had entered the cell cycle [6]. All sugars were used at a final concentration of 2%. Yeast strains are listed in Supplementary Table 1 and were constructed by standard genetic techniques or by PCR-based integration [35]. Specific primer sequences are available on request. Deletions and epitope tags were confirmed by PCR. Plasmids pKC991 (pSB344) and pKC1048 (pSB345) that contain *pGAL-GLC7* were the generous gifts of John Cannon (U of Missouri). The catalytically dead *pGAL-GLC7-H65K* plasmid was generated by site directed mutagenesis of plasmid pKC1048 using primers SB1064 and SB1065 to generate pSB1589. The *glc7-10* (PAY700-4) and *GLC7* (PAY704-1) yeast strains that were used to generate strains for this study were the kind gifts of Michael Stark (U of Dundee).

Microscopy, Protein and immunological techniques

Analysis of budding index, sister chromatids and GFP-Tub1 in fixed cells was performed as described [11]. At least 100 cells were analyzed for all reported experiments. Protein extracts were made and immunoblotted as described [36]. 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. Protein loading was confirmed in all experiments by anti-tubulin immunoblotting (data not shown).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We are very grateful to Damien D'Amours, John Cannon, Michael Stark and Dave Toczyski for generously sharing reagents. We thank Kevin Hardwick for discussing data prior to publication and helpful comments on the manuscript. We also thank the Biggins lab and Chitra Kotwaliwale for critical reading of the manuscript. This work was supported by a Paul Allen Foundation fellowship to B. A. P. and a National Institutes of Health Grant to S. B. (R01-GM64386). S. B. is a Scholar of the Leukemia and Lymphoma Society.

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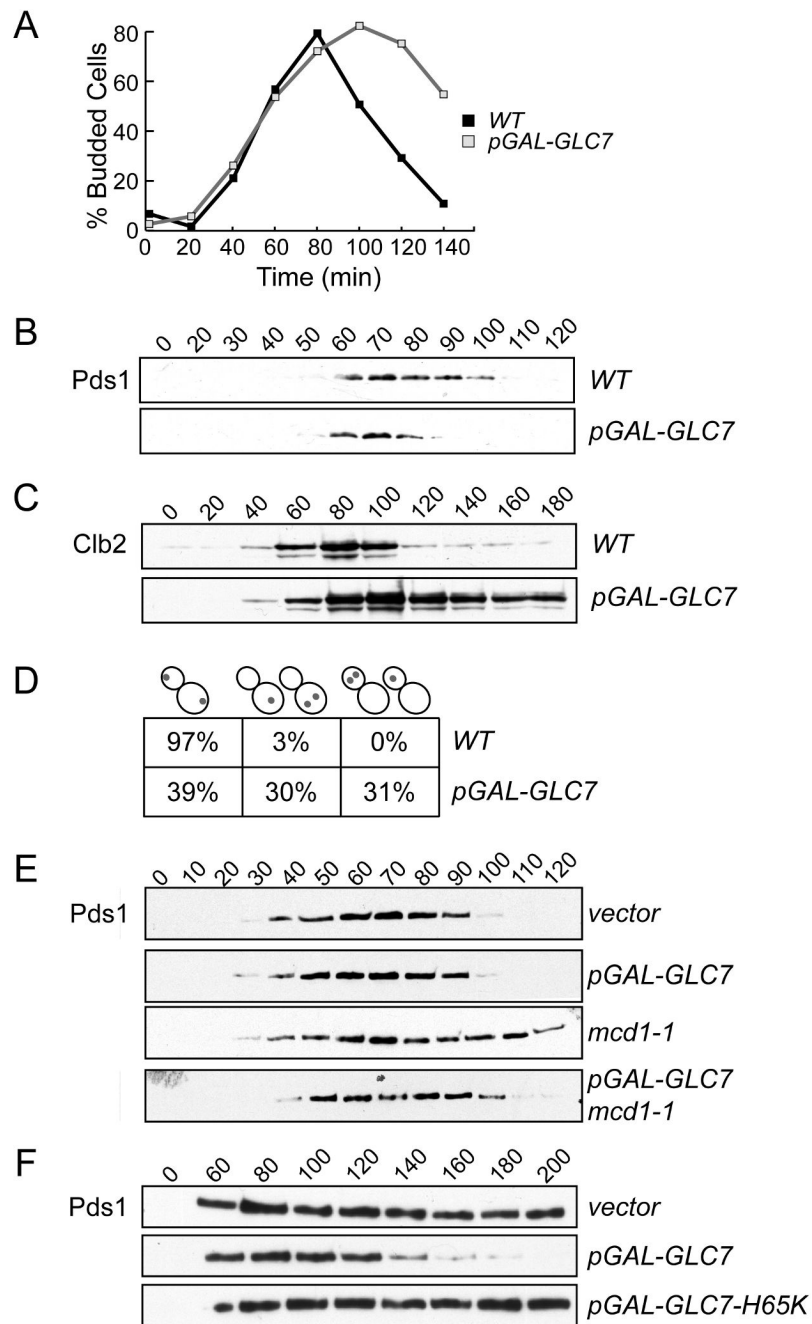
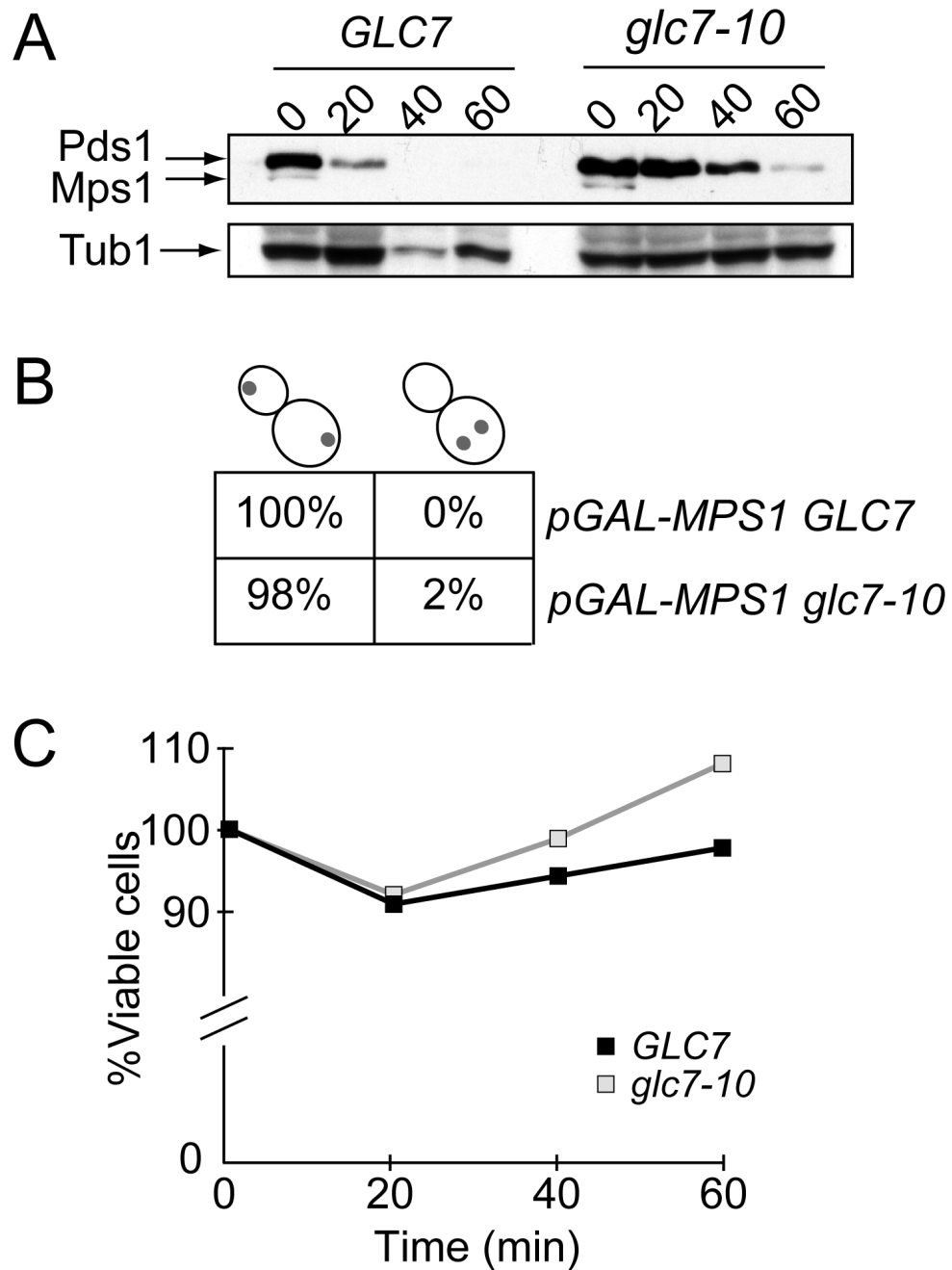


Figure 1.

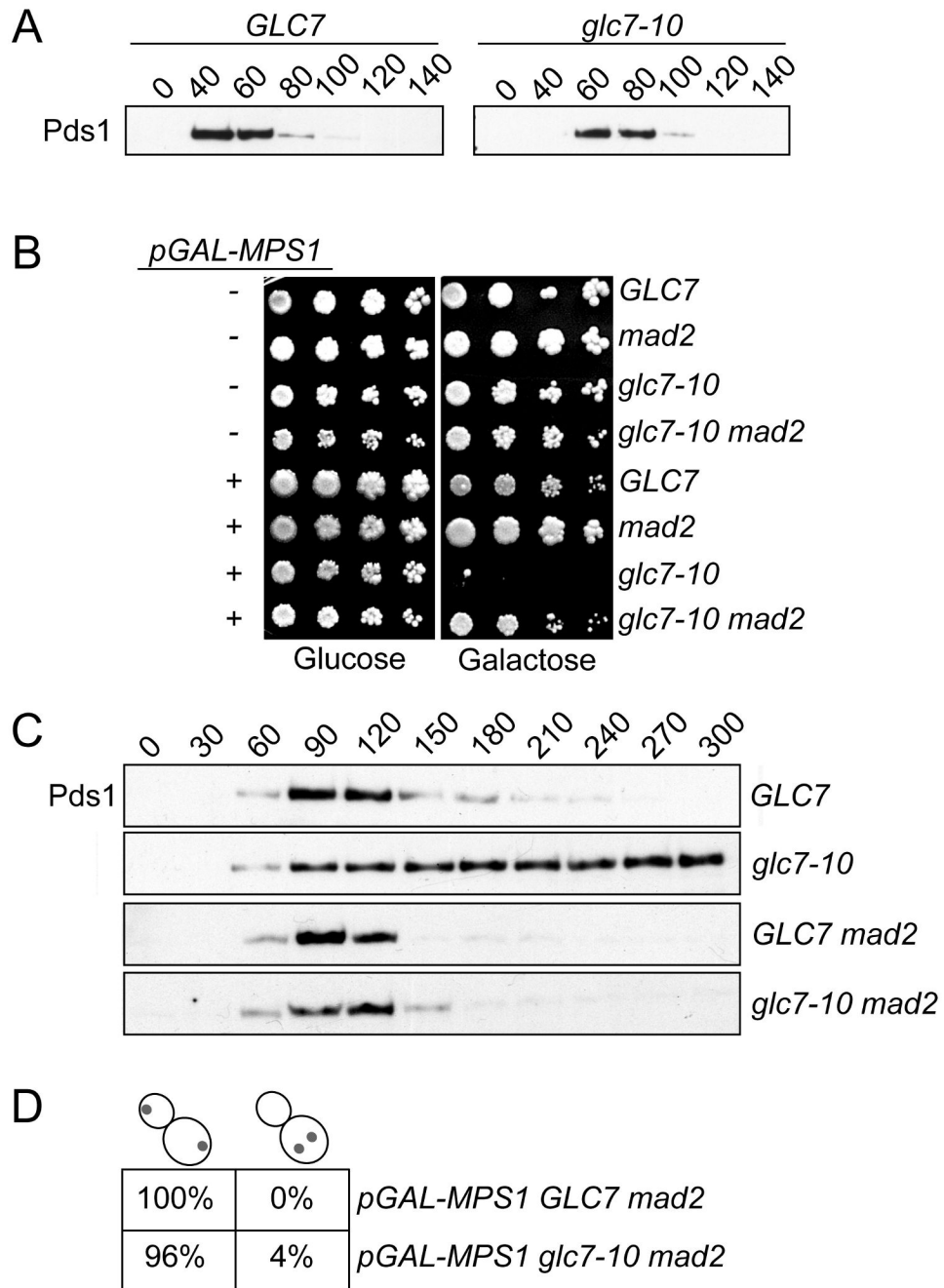
Glc7 overexpression leads to chromosome missegregation and bypasses the spindle checkpoint. A and B. *WT* and *pGAL-GLC7* strains (SBY2076 and SBY2077) were arrested in G1 and released into galactose media. The percentage of budded cells was scored every 20 min (A) and Pds1 levels (B) were analyzed by immunoblotting. C. Cells containing Clb2-myc with *GLC7* (SBY3116) or *pGAL-GLC7* (SBY3113) were treated as in (A). D. *WT* (SBY214) and *pGAL-GLC7* (SBY2973) cells with GFP-marked ChrIV were treated as in (A) and scored by microscopy for sister chromatid segregation at anaphase. Cells were categorized based on sister segregation to opposite poles (left), the mother cell (middle), or the daughter cell (right). E. *WT* and *mcd1-1* strains containing a control (*WT*, SBY1650 and

mcd1-1, SBY1849) or *pGAL-GLC7* vector (*WT*, SBY1293 and *mcd1-1*, SBY1848) were released from G1 into galactose media at 37 degrees and Pds1 levels were monitored at the indicated time points (min). F. *WT* cells containing a vector control (SBY8259), *pGAL-GLC7* (SBY8260) or catalytically inactive *pGAL-GLC7-H65K* (SBY8261) were released from G1 into nocodazole at 30 degrees and lysates were immunoblotted for Pds1 at the indicated time points.

**Figure 2.**

Glc7-10 mutants are delayed in exit from a Gal-Mps1-induced spindle checkpoint arrest. A. *GLC7* (SBY8289) and *glc7-10* mutant (SBY8290) cells containing *pGAL-MPS1* were arrested in galactose media at 23 degrees for 3 hours to activate the spindle checkpoint. The cells were then shifted to 35 degrees to inactivate Glc7-10 for 30 min and subsequently released into glucose media at 35 degrees. The levels of Pds1 (top arrow) and Mps1 (middle arrow) were monitored at the indicated time points. Tubulin is shown as a loading control. B. Segregation of a fluorescent chromosome to opposite poles (left) or the same pole (right) was scored in the cells from (A) that had entered anaphase 60 min after release into glucose

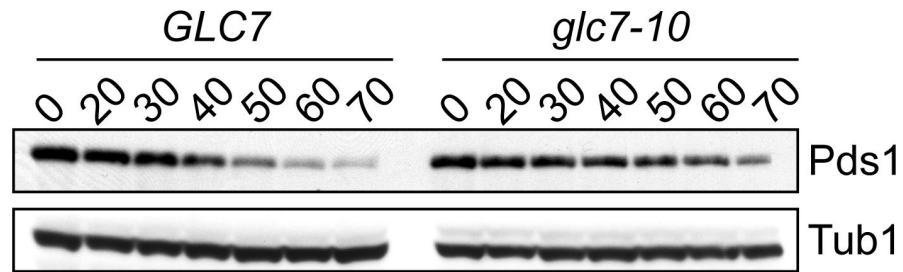
media. C. The experiment described in (A) was performed on *GLC7* (SBY3854) and *glc7-10* mutant (SBY3856) cells that were plated for viability at 23 degrees on glucose media.

**Figure 3.**

Glc7-10 cells are sensitive to increased Mps1 activity due to spindle checkpoint activation. A. *GLC7* (SBY8132) and *glc7-10* (SBY2083) cells containing Pds1-myc were arrested in G1 at 23 degrees and then released to 30 degrees. Pds1 levels were monitored by immunoblot. B. Five-fold serial dilutions of the following strains were plated on glucose or galactose media at 30 degrees: *WT* (SBY1308), *mad2* (SBY2377), *glc7-10* (SBY1306), *glc7-10 mad2* (SBY2400), *pGAL-MPS1* (SBY3670), *pGAL-MPS1 mad2* (SBY3844), *pGAL-MPS1 glc7-10* (SBY3680) and *pGAL-MPS1 glc7-10 mad2* (SBY3846). C. *pGAL-MPS1 GLC7* (SBY3854) or *pGAL-MPS1 glc7-10* (SBY3856) cells with either *MAD2* (SBY3854 and SBY3856) or *mad2Δ* (SBY7883 and SBY7884) were released from a G1 arrest at 23

degrees into galactose media at 30 degrees. After one hour, glucose was added to repress Mps1. Pds1 levels were monitored every 30 min. D. Chromosome IV segregation to opposite poles (left) or the same pole (right) was monitored in large-budded cells at 180 min in the experiment described in (C) for *pGAL-Mps1 GLC7 mad2* (SBY8137) and *pGAL-MPS1 glc7-10 mad2* (SBY8136) cells.

A Nocodazole release



B *pMET-CDC20* release

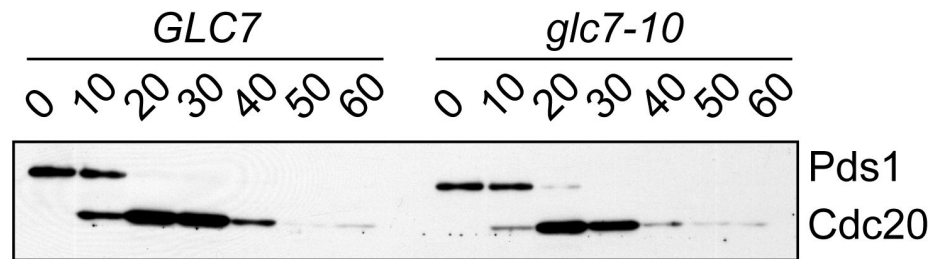


Figure 4.

Glc7-10 cells are defective in mitotic exit specifically after spindle checkpoint activation. A. *GLC7* (SBY8132) and *glc7-10* (SBY2083) cells were released from a nocodazole arrest at 30 degrees and Pds1 levels were monitored at the indicated time points (min). B. *pMET-HA-CDC20* cells with *GLC7* (SBY4635) or *glc7-10* (SBY4636) were arrested in metaphase with methionine and then released into media without methionine at 30 degrees. Pds1 and Cdc20 levels were monitored by immunoblotting at the indicated time points (min).