Analysis of Protein Phosphatase-1 and Aurora Protein Kinase Suppressors Reveals New Aspects of Regulatory Protein Function in *Saccharomyces cerevisiae*

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Abstract

Protein phosphatase-1 (PP1) controls many processes in eukaryotic cells. Modulation of mitosis by reversing phosphorylation of proteins phosphorylated by aurora protein kinase is a critical function for PP1. Overexpression of the sole PP1, Glc7, in budding yeast, *Saccharomyces cerevisiae*, is lethal. This work shows that lethality requires the function of Glc7 regulatory proteins Sds22, Reg2, and phosphorylated Glc8. This finding shows that Glc7 overexpression induced cell death requires a specific subset of the many Glc7-interacting proteins and therefore is likely caused by promiscuous dephosphorylation of a variety of substrates. Additionally, suppression can occur by reducing Glc7 protein levels by high-copy Fpr3 without use of its proline isomerase domain. This divulges a novel function of Fpr3. Most suppressors of Glc7 overexpression also suppress aurora protein kinase, ipl1, temperature-sensitive mutations. However, high-copy mutant SDS22 genes show reciprocal suppression of GLC7 overexpression induced cell death or ipl1 temperature sensitivity. Sds22 binds to many proteins besides Glc7. The N-terminal 25 residues of Sds22 are sufficient to bind, directly or indirectly, to seven proteins studied here including the spindle assembly checkpoint protein, Bub3. These data demonstrate that Sds22 organizes several proteins in addition to Glc7 to perform functions that counteract Ipl1 activity or lead to hyper Glc7 induced cell death. These data also emphasize that Sds22 targets Glc7 to nuclear locations distinct from Ipl1 substrates.

Citation: Ghosh A, Cannon JF (2013) Analysis of Protein Phosphatase-1 and Aurora Protein Kinase Suppressors Reveals New Aspects of Regulatory Protein Function in *Saccharomyces cerevisiae*. PLoS ONE 8(7): e69133. doi:10.1371/journal.pone.0069133

Editor: Michael Polymenis, Texas A&M University, United States of America

Received March 18, 2013; Accepted June 1, 2013; Published July 22, 2013

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Funding: This work was supported by the National Science Foundation and the Department of Molecular Microbiology and Immunology. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

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# Introduction

Protein phosphatase-1 (PP1) regulates many processes in eukaryotic organisms [1]. The single PP1 of budding yeast, Glc7, regulates glycerol metabolism, transcription, translation initiation, membrane fusion, sporulation, mitosis, and other processes [2,3]. The Glc7 catalytic subunit associates at least 25 different noncatalytic regulatory subunits to produce distinct PP1 holoenzymes. Noncatalytic subunits confer substrate specificity and subcellular localization to the PP1 holoenzymes. Although Glc7 finds many subcellular locations, the majority concentrates in the nucleolus [4]. Proline isomerases, Fpr3 and Fpr4, bind Glc7 in the nucleolus [5,6], Fpr3 regulates meiosis via inhibition of Glc7 [6,7]. Fpr4 modulates histone H3 and H4 lysine methylation by means of its histone proline isomerase activity [8]. Glc7 dephosphorylates histone H3 [9].

Glc7 activity is essential for cell viability in part because of dephosphorylation of nuclear proteins. Sds22 and Ypi1 facilitate nuclear Glc7 translocation by forming a trimeric complex [10,11]. Shp1 also facilitates Glc7 nuclear import by an undefined mechanism [12]. Sds22 appears to use a nuclear localization signal in its N-terminus independently from Ypi1 because a Sds22(1–25)-lacZ fusion is nuclear localized [13]. Within the nucleus, proteins Fin1 and Spc105 target Glc7 to kinetochores [14–16]. Glc7 dephosphorylation of kinetochore proteins promotes mitotic spindle attachment [17–21]. The protein kinases Ipl1 and Mps1 phosphorylate kinetochore proteins that Glc7 dephosphorylates [16,22] and reducing Glc7 activity suppresses *ipl1* lethality of temperature-sensitive mutations [23,24]. The opposing Ipl1 and Glc7 activities ensure that chromosomes achieve a bipolar attachment to the spindle. The spindle assembly checkpoint (SAC) guarantees that cells with at least one chromosome unattached to the mitotic spindle halt in metaphase [25,26]. A complex program of Bub1, Bub3, Mad1, Mad2, Mad3 movement, protein phosphorylation, and conformational transitions orchestrate SAC function [26,27]. Glc7 function silences SAC function once all chromosomes achieve bipolar spindle attachment to allow transition from metaphase to anaphase.

Glc7 dephosphorylates other nuclear substrates besides those at the kinetochore. Some of those substrates modulate transcription termination or promote mRNA export [28–30]. Numerous proteins that bind to Sds22 [31] might be also Glc7 substrates. They include DNA helicases, Rvb1 and Rvb2, Tor1 complex subunit Kog1, ribosome biogenesis factor Nop6, Snf1 protein kinase subunit, Snf4, and 60S ribosomal protein Ygr130C [32–36].

The mammalian PP1 inhibitor-2 ortholog, Glc8, activates the majority of Glc7 protein phosphatase activity in vivo [37]. Glc8 must be phosphorylated to activate Glc7 [38,39]. The yeast Glc8...
kinase is the cyclin-dependent protein kinase, Pho85, associated with cyclins Pcl6 and Pcl7 [39]. Glc8 is not normally required for yeast viability; however, certain GLC7 alleles render Glc8 essential for viability [39].

The glyogen-deficient trait used to initially identify glc7 mutations comes from the failure of the Gac1-Glc7 complex activity in cytoplasmic glycogen particles to dephosphorylate glycogen synthase [40,41]. Glc7 further regulates carbon metabolism via association with Reg1 and Reg2 [42,43].

GLC7 is one of several genes that kill yeast cells when they are overexpressed [44]. High-copy GLC7 increases the chromosome gain frequency; a phenotype also shared by ipl1 mutations [23]. Only mutations in SFB1 have previously reported to suppress GLC7 overexpression lethality [45]. A goal of this work was to analyze suppressors of GLC7 overexpression to learn more about the mechanism of lethality, about regulation of Glc7 activity, and function of Glc7 interacting proteins. We discovered that many suppressors of GLC7 overexpression also suppress ipl1. However, we isolated SDS22 mutant genes that could dominantly suppress GLC7, but not ipl1 and vice a versa.

**Results**

**Recessive Suppressors of Glc7 Overexpression**

The cause of cell death upon Glc7 overexpression is unknown. Characterization of suppressors of this trait reveals novel aspects of Glc7 function. PP1 enzymes like Glc7 function as holoenzymes containing alternative noncatalytic subunits [3,46,47]. *S. cerevisiae* possesses several Glc7 noncatalytic subunits and if one or more of them produced a Glc7 holoenzyme responsible for cell death, then deletion of the noncatalytic subunit gene should suppress Glc7 overexpression. Therefore, we tested whether deletions of well characterized noncatalytic genes, GLC1, REG1, GLC8, or REG2 suppressed Glc7 overexpression. Galactose induction of GAL1p-GLC7 overexpressed Glc7 in these assays. Wild-type, gac1, and reg1 cells reduced growth on galactose medium revealing the cell death overexpression was clear from the relative growth on YEP-galactose. In Figure 1A cells reduced growth on galactose medium revealing the cell death overexpressed Glc7 in these assays. Wild-type, suppressed Glc7 overexpression. Galactose induction of GAC1 overexpression. Therefore, we tested whether deletions of well characterized noncatalytic subunits [39]. Glc8 is not normally required for viability [39]. Our finding the heterozygous SDS22/S56am genotype as a suppressor of Glc7 overexpression implicates the Glc7-Sds22 holoenzyme in the Glc7-induced cell death.

We were curious whether the heterozygous SDS22/S56am allele was special in its suppression of GLC7 or if any SDS22 null allele could suppress. The SDS22/S56am mutation truncates the encoded Sds22 protein such that only residues preceding the leucine-rich repeats, which bind Glc7, would be expressed. We considered the possibility that this truncated Sds22 protein functioned as a dominant-negative. However, results consistent with that contention were not reproducible. Instead we favor the explanation that SDS22/S56am merely functions as a suppressor of Glc7 overexpression because it reduces the concentration of Sds22 in a diploid.

The observation that complete heterozygous deletion, sd22A1/, suppressed Glc7 as well as smaller deletions corroborates this conclusion (Figure 1C). Therefore, the Sds22-Glc7 holoenzyme must also be participate in lethal dephosphorylations that occur upon Glc7 overexpression.

**Fpr3 Dominantly Suppresses Glc7 by Reducing Glc7 Protein**

We screened libraries of wild-type genes in high-copy, 2 μ vectors for genes that suppress Glc7 overexpression to identify additional dominant suppressors of Glc7 (Materials and Methods). Extensive screening identified the FPR3 encoded proline isomerase as the sole dominant, high-copy Glc7 suppressor (Figure 2A). The *S. cerevisiae* genome encodes twelve proline isomerases and that encoded by FPR4 is most similar to FPR3 [48]. FPR4 also suppresses Glc7 slightly (Figure 2A).

We wanted to know how Fpr3 suppressed GLC7 overexpression. Comparison of Fpr3 and Fpr4 amino acid sequences showed two shared domains in addition to the C-terminal proline isomerase homologous domain (Figure 2B). GLC7 suppression by Fpr3 needs the central Fpr4 homologous region because deletion compromised suppression (Figure 2C). Surprisingly, deletion of the proline isomerase domain via the ochre nonsense mutation, K302oc, and many other mutations including deletion of residues 294 to 411 did not compromise GLC7 suppression. Therefore, Fpr3 suppresses Glc7 by using a function other than its proline isomerase activity.

Glc7 suppressors could reduce the levels of bulk Glc7 protein levels in the cell. Indeed, Glc7 protein levels declined with increased expression of Fpr3 (Figure 3). In contrast, glc8 and pcl6 pcl7 mutants that lack phospho-Glc8 or ipl1 a Glc7 suppressor that modulates Glc7 nuclear import [12], display no change in bulk Glc7 protein levels. Hence, Fpr3 exploits a unique mechanism of GLC7 suppression; it reduces Glc7 protein levels.

**Sds22 and Fpr3 Suppress ipl1**

Because Glc7 dephosphorylates kinetochore proteins phosphorylated by Ipl1, several mutations that reduce Glc7 function suppress temperature-sensitive ipl1 mutations [24,38]. Therefore, we tested ipl1 suppression by Fpr3 and Sds22. Wild-type and mutant FPR3 genes suppressed ipl1 identically to their GLC7 suppression; on the contrary, high-copy FPR4 did not suppress (Figure 4A). This is consistent with the weaker GLC7 suppression by FPR4 compared to FPR3. Notably, ipl1 suppression also did not require the proline isomerase domain of Fpr3. These results are consistent with ipl1 suppression due to reduced Glc7 function.

So far, all suppressors of ipl1 also suppress Glc7 overexpression and vice a versa. High-copy SDSL2 suppression of ipl1 was
previously reported [24]; however, potential suppression by reducing SDS22 gene dosage was not. To test this possibility, isogenic homozygous ipl1 diploids were constructed. We found homozygous ipl1 diploids to be more temperature-sensitive than ipl1 haploids; however, high-copy SDS22 suppression was evident at 37°C and heterozygous sds22 D/SDS22 failed to suppress ipl1 (Figure 4B). Therefore, increases, but not decreases of SDS22 gene dosage suppress ipl1. Suppression of Glc7 overexpression by changing SDS22 gene dosage was completely reciprocal to that of ipl1 suppression. These initial results show that SDS22 uniquely distinguishes suppression of Glc7 overexpression from ipl1 suppression.

Additional mutations illustrated facile ipl1 suppression by high-copy SDS22. Our earlier results showed that SDS22-S56am does not function as a dominantly-negative Glc7 suppressor. Remarkably, high-copy SDS22-S56am suppressed ipl1 (Figure 4C). Consistent with titrations of Sds22-S56am binding proteins, low-copy SDS22-S56am does not suppress ipl1 (data not shown). The SDS22-S56am allele encodes an Sds22 protein that lacks all leucine-rich repeats (LRRs), which mediate Glc7 binding (Figure 5A). If specific loss of Glc7 binding by Sds22 created a dominant ipl1 suppressor, we should be able create such suppressing alleles via mutations predicted to reduce Glc7 affinity. S. cerevisiae Sds22 leucine-rich repeat residues D119, D273 and W275 are homologous to human Sds22 residues, which mediate PP1 affinity [49]. Mutating these residues to alanine did not compromise ipl1 suppression (Figure 5B). Immunoblotting failed to detect D119A and W275A missense mutant proteins from crude extracts. Robust ipl1 suppression by SDS22-D119A and –W275A despite their undetectable protein expression indicates these two Sds22 proteins must have potent suppression activity (Figure 5E). These two Sds22-myc3 proteins are obscured by background proteins on more sensitive immunoblots (data not shown). We attempted to impair ipl1 suppression by deleting two LLRs (D119–127), four LLRs (A81–171), Ypl1 binding residues (A251–323), or N-terminal residues (A2–56); however these high-copy mutant SDS22 genes also suppressed ipl1 to various degrees (Figure 5C). Results from these and many other mutants (data not shown)

Figure 1. Recessive suppressors of Glc7 overexpression. A) JC746-9D (wt), JFY183 (gac1), JC1287-1C (reg1), JC938-5C (glc8), and JC1583 (reg2) transformed with GAL1p-GLC7 plasmids, p2562 or pKC978 (even rows) or control plasmids pRS315 or pRS316 (odd rows) were grown in selective raffinose medium and then serial five-fold dilutions were spotted on –Ura or –Leu glucose, YEP-galactose, or –Ura or –Leu galactose plates. B) Phospho-Glc8 is required for Glc7 overexpression lethality. JC938-5C (glc8) transformed with pYT251 (GAL1p-GLC7) and either p1945 (GLC8), pYT115 (GLC8-T118A), or p1614 (GAL1p-GLC8) were grown on –Trp –Ura galactose (Gal) or glucose (Glc). C) Diploids JC746, JC746/V76B8, JC746/RG200 and JC1378 transformed with pYT251 (GAL1p-GLC7) or pRS314 (control) were grown on –Trp galactose. The SDS22 genotypes of the host strains are shown. In all panels, the galactose medium induced GLC7 expression from the GAL1 promoter.

doi:10.1371/journal.pone.0069133.g001
vector did not suppress (Figure 6B) required it to be high-copy; expression from a single-copy degree to complement sds22 [50]. Elevation of wild-type SDS22 most frequently act via competition with the wild-type protein suppression show that it is a dominant negative allele. Such alleles suppression by SDS22-RM45 Sds22 besides Glc7 [31,51]. We confirmed a subset of these Figure 6C activity of Sds22-RM45 (Figure 5A and D), yet encoded proteins expressed at levels comparable to wild-type (Figure 5E). SDS22-RM45 contains mutations E79G, L159Q, and L295I and SDS22-RM107 mutations F63L, Y141H, and E210T (Figure 5A). High-copy SDS22-RM45 could not suppress ipl1 even if wild-type Sds22 levels were reduced (Figure 4B). Thus, SDS22 suppression of ipl1 can be compromised by multiple missense mutations. These findings make it unlikely that high-copy SDS22 suppresses ipl1 strictly by promoter titration because no other mutations were present in these mutant SDS22 genes that failed to suppress.

Sds22 that Fails to Bind Glc7 Suppresses Glc7 Overexpression

Testing GLC7 suppression by high-copy wild-type and mutant SDS22 genes revealed intriguing aspects of Sds22. Wild-type and most mutant SDS22 genes did not suppress Glc7 overexpression (Figure 6A). The exceptional alleles, SDS22-RM45 and to a lesser degree SDS-A81–171, did suppress Glc7. Suppression by SDS22-RM45 required it to be high-copy; expression from a single-copy vector did not suppress (Figure 6B). The failure of SDS22-RM45 to complement sds22A (Figure 5A) and its dominant GLC7 suppression show that it is a dominant negative allele. Such alleles most frequently act via competition with the wild-type protein [50]. Elevation of wild-type SDS22 consistently diminished GLC7 suppression by SDS22-RM45 consistent with a dominant negative activity of Sds22-RM45 (Figure 6C).

High-throughput studies reported many other proteins bind to Sds22 besides Glc7 [31,51]. We confirmed a subset of these interactions by purifying potential Sds22 binding proteins from yeast as GST fusion proteins and testing if HA3-Sds22 copurified. This methodology verified that Kog1, Nop6, Rvb1, Rvb2, Snf4, and Ygr130C bound to Sds22 (Figure 7A). The variable yields of HA3-Sds22 copurified were attributable to differences in GST fusion expression and degradation. We intended to use Bub3 as a negative control, but fortuitously discovered that it also bound to Sds22 in this assay. We successfully used histone acetyltransferase subunit, Abc1, as negative control instead. Binding of Sds22-RM45 to this collection of proteins was indistinguishable from wild-type Sds22 (Figure 7A).

Two-hybrid assays evaluated Glc7 interaction with mutant Sds22 proteins. As expected from of the lack of leucine-rich repeats, Sds22-S56am did not interact with Glc7 (Figure 7B). Of the other mutant Sds22 proteins tested, only the RM45 mutant showed no apparent Glc7 affinity. Surprisingly, even Sds22 mutant proteins lacking two or more leucine-rich repeats (ΔA81–124, ΔA81–171, and ΔA243–325) retained detectable Glc7 affinity by this assay. Failure of Sds22-RM45 to bind Glc7 can explain its dominant negative function if this mutant protein diminished the other Sds22-binding proteins from binding to the wild-type Sds22-Glc7 complex.

Dominant ipl1 suppression by high-copy SDB22-S56am (Figure 4C) suggested that truncated Sds22(1–55) protein bound to proteins other than Glc7 to mediate suppression. High-copy HA3-SDS22-S56am also suppressed ipl1 although it was weaker than SDB22-S56am (data not shown). This result is consistent with the Sds22 N-terminus playing an important role in ipl1 suppression. We sought to analyze Sds22 binding proteins for affinity to Sds22(1–55); however, we were unable to detect HA3-Sds22-
S56am protein from yeast extracts. Instead we used a Sds22(1–25)-
LacZ fusion, which showed Sds22 nuclear localization previously
[13]. None of the GST fusion proteins tested bound to β-
galactosidase (Figure 8D). In contrast, five of seven GST fusions
bound to Sds22(1–25)-LacZ greater than the negative control
(Figure 8C). These results support the binding of Bub3, Kog1, Rvb1, Rvb2, and Snf4 to residues 1–25 of Sds22. Therefore,
Sds22 residues 1–25, not only supplies a nuclear localization signal
for Sds22, but it promotes binding to at least five other proteins.
Note, that these results do not imply simultaneous or direct Sds22
interaction to all or any of these proteins.

Discussion

The molecular details of cell death caused by overexpression of
GLC7 remain elusive. However, our discovery that Sds22, Shp1,
Reg2, and phospho-Glc8 must all be functional for this cell death
strongly implicates the importance of nuclear-localized substrates
because Sds22 and Shp1 promote Glc7 nuclear localization
[11,12,14]. The Reg2 role in glucose transcription repression and
maltose permease proteolysis [42,43] suggests functions of the
Reg2-Glc7 holoenzyme in and outside the nucleus. The most parsimonious conclusion at this point is that substrates must be promiscuously dephosphorylated by both Reg2-Glc7 and Sds22-
Glc7 holoenzymes to kill yeast cells. Some and perhaps all of these
substrates reside in the nucleus. Our results illustrate that
Reg1 and Reg2 have distinct functions because reg1 fails to suppress Glc7 overexpression, whereas reg2 suppresses
(Figure 1A).

GLC7 suppression could result from displacement of Glc7 from
critical substrates, reducing phosphatase activity, or diminishing
Glc7 protein levels. We found examples for each of these
mechanisms. Phospho-Glc8 increases global Glc7 protein activity
via a proposed chaperone function [37,52]; therefore, glc8 mutants
have less activity in many if not all Glc7 holoenzymes. Absence of
phospho-Glc8 suppresses Glc7 (Figure 1B). Viable glc7
missense mutations were previously isolated based upon their glycogen-
deficient or sporulation-deficient traits [52]. They compromise
binding to subsets of Glc7 regulatory subunits and some have
enzymatic activity reductions [37,52]. All ten mutant GLC7 genes
we tested failed to kill when overexpressed (data not shown). A
GLC7 allele with the intron deleted retained the ability to kill when
overexpressed. This finding illustrates that death from Glc7
overexpression is not a result of taxing the cellular mRNA splicing
machinery. Together, these findings show that it is the fully active
Glc7 enzyme that kills when it is overexpressed.

High-copy FPR3 or FPR4 suppression of GLC7 overexpression
could be via their proline isomerase activity or by their nucleolar
partitioning. Proline isomerization is particularly attractive
because there are several conserved prolines found in all PP1
enzymes, proline isomerization is a rate limiting step in protein
folding, and PP1 enzymes are notoriously difficult to fold in
heterologous systems [47,53,54]. Furthermore, Fpr3 proline
isomerase activity inhibits Glc7 to modulate meiotic progression
[6]. However, it is clear the proline isomerase domain (PPI) of
Fpr3 does not suppress mitotic lethality of Glc7 overexpression
because its deletion actually enhanced suppression of GLC7 by
Fpr3 (Figure 2C). In addition to the FPR3-K302am nonsense
mutation, FPR3-V303am, and FPR3(A294–411), which truncate
the PPI domain also failed to suppress (data not shown). Fpr3
overproduction did not result in a detectable change in Glc7
subcellular localization (Kelly Tatchell, personal communication).
Fpr3 exploits a novel mechanism compared to other GLC7
suppressors; it reduced the total Glc7 protein levels (Figure 3). We
propose the chaperone activity of Fpr3 is exploited like that of
some other proline isomerases to catalyze Glc7 degradation [55–
57].

High-copy FPR3 or FPR4 also suppress ubiquitin ligase tom1
mutations [58]. Tom1 promotes destruction of Dia2, Cdc6 and
other proteins [59,60] and high-copy FPR3 might promote
destruction of Tom1 targets similar to Glc7. However, we found
that glc7 missense mutations or glc8Δ suppress tom1 (data not
shown), which suggests that high-copy FPR3 and FPR4 suppress

Figure 4. Dominant suppressors of ipl1. A) Suppression of ipl1 by several high-copy genes. JC1126-15B (ipl1-1) transformed with plasmids
(pRS426, p2665, p2509, p2510, p2615, and p2613 respectively) with the indicated genotypes were incubated on –Ura plates at the indicated
temperatures. Each spot had approximately 105 cells. B) SDS22-RM45 is not a dominant suppressor of ipl1. Homozygous ipl1 diploid strains, JC1630
(SDS22/SDS22) and JC1631 (SDS22/SDS22.4) transformed with pRS316 (control), pAG108 (SDS22), or pAG-RM45 (SDS22-RM45) were incubated on
–Ura plates at the indicated temperatures. C) Suppression of ipl1 by high-copy SDS22-S56am. JC1126-15B transformed with pAG108 (SDS22) or p2665
(SDS22-S56am) were incubated on –Ura plates at the indicated temperatures. Fivefold serial dilutions were spotted and grown for three days in
panels B and C.

doi:10.1371/journal.pone.0069133.g004
tom1 via their inhibition of Glc7. These findings further connect Glc7 and Tom1 function to mRNA nuclear export [61].

Through analysis of GLC7 and ipl1 suppressors, we learned additional information about Glc7 interacting proteins. Several Glc7 interacting protein genes in high-copy suppress temperature-sensitive ipl1 mutations [24]. Ipl1 phosphorylates several proteins that Glc7 dephosphorylates. Because of this antagonism, reduction of Glc7 activity on these shared substrates suppresses ipl1 temperature-sensitive mutations. High-copy GLC7 or FPR3 suppress ipl1 by reducing activity of all Glc7 holoenzymes. The ipl1 suppression by many of the genes encoding Glc7 interacting proteins was explained by displacement of Glc7 away from Ipl1 [24]. Such displacement allows Ipl1 substrates to increase phosphorylation. These two mechanisms for ipl1 suppression cannot explain how high-copy SDS22 suppresses. Sds22 is nuclear and increasing Sds22 levels would unlikely displace Glc7 from the nucleus [11]. Furthermore, Sds22 promotes Glc7 function [3].

Suppression of GLC7 and ipl1 by SDS22 reveal nuances of Sds22 function. The observation that halving SDS22 gene dosage in SDS22/sds22 diploids suppresses GLC7, but not ipl1 can be rationalized by the former suppression demanding smaller reductions in Glc7 activity than the later. Second, high-copy SDS22 suppression of ipl1, but not GLC7 suggests that Sds22 targets Glc7 to nuclear locations distinct from Ipl1 substrates. Other studies revealed discrete pools of nuclear PP1 [62]. Third, our attempts to reduce Glc7 binding by missense mutations or LRR deletions demonstrate redundant Glc7 binding by the LRR’s. Indeed, five LRRs are sufficient to bind PP1 in a histone variant [63].

Explaining suppression of GLC7 and ipl1 by SDS22 alleles is more complicated. Complexity arises because Sds22 is a scaffold, which binds many other proteins. We confirmed binding to Rvb1, Rvb2, Kog1, Nop6, Snf4, and Ygr130C in this work using different techniques than reported previously [31]. We fortuitously...
discovered that Sds22 also binds Bub3 (Figure 7). Therefore, overexpression of Sds22 could displace these proteins from their normal location and thus impair their function. For example, attenuation of Tor complex 1 function suppresses \( \text{ip}1/1 \) [64]. Kog1 is a Tor complex 1 component [36] and Kog1 binds to Sds22 attenuating of Tor complex 1 function suppresses overexpression of Sds22 could displace these proteins from their normal location and thus impair their function. For example, attenuation of Tor complex 1 function suppresses \( \text{ip}1/1 \) [64]. Kog1 is a Tor complex 1 component [36] and Kog1 binds to Sds22.

Materials and Methods

Yeasts and Strains

The genotypes of yeast strains used in this work are listed in Table 1. Note that strains JC482D, JC746, and JC1630 are diploid. The Gal1p-GLC7::URA3 was made by integration of pKC1048 at the GLC7 locus. The \( \text{sd}22(1–25):\text{Tn}1 \) was from integration of the plasmid V76B7 after linearization with NotI. It forms lac\( \beta \)-Gal from the \( \text{Tn}1 \) transposon in frame to the \( \text{SDS}22 \) codon 25 [13]. The \( \text{sd}22(1–116):\text{URA}3 \) allele was introduced by transformation with pRG200 after digestion with EcoRI and XhoI. Complete deletion \( \text{sd}22A::\text{HIS3} \) was made by transformation with a PCR fragment made using template pRS305 [65]. Plasmids used to make \( \text{g}l\text{c}7::\text{URA}3 \), \( \text{reg}2::\text{URA}3 \), and Gal1p-lac\( \beta \) have been described previously [40,42,66]. The \( \text{shp}1::\text{URA}3 \) allele was made by transformation with EcoRI and NdeI digested p2608. JC1126-15B was derived from four serial backcrosses of an \( \text{ip}1/1 \) strain [23] to JC746-9D. JC1353-17B is derived from crossing EJ758 [67] and JC746-9D. JC1630 was derived from HO-induced diploidization of JC1126-15B [52].

Mating, transformation, sporulation, and tetrad analysis were performed by procedures previously described [68]. Rich (yeast extract-peptone [YPE]) or synthetic omission media contained glucose, galactose, or raffinose at 2% (w/v) [68]. For growth comparison assays, the cell concentration in exponentially growing cultures was determined by absorbance at 600 nm. By appropriate dilution in water, equal cell numbers were spotted on plates in serial five-fold dilutions.

Plasmid Construction

Most plasmids used in this work are described in Table 3. High-copy plasmids used the 2\( \mu \) origin of replication and low-copy plasmids contained a centromere (CEN). Several plasmids were constructed by recombination in yeast [69]. \( \text{SDS}22 \) amplified from yeast DNA by PCR and recombined into YCP50 produced plasmid p2431 and contains an \( \text{SDS}22 \) gene with a \( \text{NoI} \) site just before the termination codon. Plasmid pAG101 was made by transferring a \( \text{SDS}22 \) \( \text{PulI}::\text{NoI} \) fragment to pRS416. A \( \text{NoI} \) fragment encoding the triplet mcyc epitope from pMPY-3sMYC [70] was transferred to pAG101 to yield pAG108. Plasmid p2453 was made by recombination of p705-3 with a yeast genomic PCR fragment. An \( \text{ApI}::\text{NoI} \) fragment from p2453 was transferred to pRS314 to make p2518. Plasmids p2603 contains the \( \text{SDS}22(1–25):\text{lac}\beta \) fusion driven by the \( \text{SDS}22 \) coding sequence resulting in removal of codons after 116. The 4851-bp p2533 plasmid was made by deleting a 581 frag.

Plasmid pK978, pKC1048 and pYT251 contain the \( \text{Gal1L} \) promoter from pBM272 [71] driving \( \text{GLC7} \) transcription in pRS316, YIp5 or pRS314 respectively. Plasmid p2608 has a 3604-bp EcoRI-XhoI \( \text{SHPI} \) DNA fragment in pBluescript II KS[+] with \( \text{URA}3 \) inserted into the \( \text{SDS}22 \) coding region. Plasmid p2757 was made by swapping restriction fragment with p2518 and p2752. The \( \text{2m} \) \( \text{LEU2} \) \( \text{GAL}1::\text{SDS}22 \) fusion plasmids used in Figure 7 were made by restriction fragment swapping with p2446, which was derived from pACT2-SDS22 [52]. All \( \text{2m} \) \( \text{URA}3 \) \( \text{CUP1p-GST} \) fusion plasmids [67] used here were DNA sequenced and compared to the S. cerevisiae S288c sequence.

Quick-Change (Stratagene) or “Round the horn PCR” (http://openwetware.org/wiki/’Round-the-horn_site-directed_mutagenesis) mutagenesis introduced \( \text{SDS}22 \) or \( \text{FPR3} \) mutations and were confirmed by DNA sequencing. The primers and details are available upon request.

![Figure 6. Dominant GLC7 suppression by mutant SDS22. A)](image-url) JC746-9D transforms with pRS314 (odd rows) or pYT251 (Gal1p-GLC7, even rows) and plasmids with the indicated genotypes were grown on selective galactose or glucose plates. B) JC746-9D/pYT251 additionally transformed with pRS314, pAG108, p2757, or pAG-RM45 were grown on selective galactose or glucose plates. C) JC746-9D/ pYT251 transforms with plasmid combinations pRS426+ pRS315 (control), pAG108+ pRS315 (SDS22), pRS426+ p2752 (SDS22-RM45), or pAG108+ p2752 (SDS22 SDS22-RM45) were grown on selective galactose or glucose plates. For all panels, fivefold serial dilutions were spotted on plated and grown for three days.

doi:10.1371/journal.pone.0069133.g006

Table 1

| Yeast Strain | Genotype |
|--------------|----------|
| JC482D       | \( \text{ip}1/1 \) |
**Figure 7. Sds22 binding proteins.**

A) GST fusion proteins were purified from JC746-9D transformed with p2518 (HA3-Sds22) or p2757 (HA3-Sds22-RM45) and indicated GST fusions as described (Materials and Methods). The immunoblot of crude extracts probed with anti-GST antibody in top image. The arrows point to full-length proteins or specific degradation product. Immunoblots of the affinity-purified GST fusion mixture probed with anti-HA antibody in bottom images. B) Two-hybrid assay of Sds22 interaction with Glc7. The β-galactosidase activity of three independent PJ69-4A transformants with pAS1-GLC7 and indicated Gal4AD-Sds22 fusions were assayed. The control is transformed with pRS315. The average and standard deviation is reported. Immunoblots of crude extracts probed with anti-Gal4AD antibody showed equivalent Gal4AD-Sds22 expression for each mutant fusion protein. The Sds22-S56am and RM45 fusions produced β-galactosidase activity that was comparable to the negative control. In contrast, every other fusion had activity significantly higher (two-tailed t-test, p<0.01).

doi:10.1371/journal.pone.0069133.g007
Four spontaneous, independent, galactose-resistant revertants of JC482D/pKC1048 were isolated and analyzed. After sporulation and tetrad dissection, two of the revertants produced no galactose-sensitive haploid progeny. These revertants likely suffered a slower mutation and 60-fold lower expression (data not shown). A third revertant harbored a suppressor mutation unlinked to GLC7, as determined by anti-GST antibody. When overexpressed like panel C, all lanes have equal signal. In panels B-D, the “-” lane is JC353-17B/pRS314 detected with anti-GST antibody. This panel shows that SDS22(1–25)-LacZ affinity (Figure 8) or JC1353-17B/p2603 detected with anti-GST antibody. This result indicated that the recessive lethal trait was linked to SDS22.

Genetic and DNA sequence analysis showed that SDS22 loci from diploid JC908-2 retrieved by gapped plasmid repair [74] were either wild-type or contained an amber mutation at SDS22 codon 56 (SDS22-S56am). To confirm the SDS22-S56am mutation in JC907, we isolated JC907 derivatives that contained amber mutation. Several spontaneous His+ revertants of JC907 were selected and then analyzed by tetrad analysis. Those with extragenic his4-539 suppressors (i.e. those with putative amber suppressors) suppressed SDS22-S56am and allowed viability and became sensitive to galactose. These results confirmed that SDS22-S56am suppressed Glc7 overexpression lethality in a diploid.

### Immunoblotting Experiments

Crude extracts were prepared from exponentially grown cells by glass bead vortexing in extract buffer (50 mM Tris HCl pH 7.5, 150 mM NaCl, 0.1% (v/v) Triton X-100, 1 mM DTT, 10% (v/v) glycerol, 1X Complete Protease Inhibitor Cocktail (Roche Molecular Biochemicals) and 2 mM PMSF [11]. Protein concentrations were determined by Bradford assays using bovine serum albumin standards (Pierce). SDS-PAGE, blotting and detection by chemiluminescence was as described [37] with anti-HA and anti-Pgk1 antibodies (Santa Cruz Biotechnology, Inc and Molecular Probes respectively). Densitometry of films used ImageJ software.

### Table 1. Summary of suppression by SDS22 genes.

| SDS22 allele | Protein Binding | Suppression |
|--------------|-----------------|-------------|
|              | Other proteins* | GLC7 overexpression | ip1 |
| sds22.1/+    | +               | +           | -   |
| high-copy SDS22 | +       | +           | -   |
| high-copy SDS22-S56am | - | -           | +   |
| high-copy SDS22-MM45 | + | -           | -   |

*The seven other SDS22 binding proteins are Bub3, Kog1, Nop6, Rvb1, Rvb2, Snf4, and Ygr130C. The five SDS22-S56am-binding proteins are Bub3, Kog1, Rvb1, Rvb2, and Snf4 (missing Nop6 and Ygr130C). This is based on sds22(1–25)-LacZ affinity (Figure 8). doi:10.1371/journal.pone.0069133.t001

### Isolation of Genomic Dominant GLC7 Overexpression Suppressors

Four spontaneous, independent, galactose-resistant revertants of JC482D/pKC1048 were isolated and analyzed. After sporulation and tetrad dissection, two of the revertants produced no galactose-sensitive haploid progeny. These revertants likely suffered a recessive lethal trait and it was genetically unlinked to GLC7. A third revertant harbored a suppressor mutation unlinked to GLC7, because approximately one-half the Ura+ spores were galactose-resistant. The extragenic suppressor in that revertant was not analyzed further. The fourth revertant, JC907, produced only two viable spores per tetrad. About one-half the viable spore progeny were Ura+ and all Ura+ haploids were galactose-sensitive. These observations indicated a dominant GLC7 suppressor in JC907 that had a recessive lethal trait and it was genetically unlinked to GLC7. The integrated pKC1048 (URA3) plasmid was evident from JC907 by 3-fluoro-orotic acid resistance selection [73] to produce strain JC908-2, which continued to display two viable spores per tetrad.

### Mapping the Recessive Lethal Trait in JC908-2

Sporulation and tetrad dissection of strain JC908-2 yields two viable haploid spores per tetrad. TnUR43 transposons were integrated into JC908-2 by transformation with plasmids from the Triples collection [13] after Ncol digestion. Transposons were chosen that integrated URA3 at 73 distinct locations spaced approximately 150 Kb throughout the genome and thus at least one was guaranteed to show linkage to any locus. Tetrad analysis showed that sds22::TrxUR43 from plasmid V768 failed to recombine with the JC908-2 recessive lethal trait; either all spores were inviable (sds22::TrxUR43/sds22-S56am) or only two viable Ura+ spores were found (sds22::TrxUR43/+). This result indicated the recessive lethal trait was linked to SDS22.

### High-copy Suppressors of GLC7 Overexpression

JC482D/pKC1048 was transformed with wild-type yeast libraries constructed in the 2µ, YEp13, vector [75]. Suppressors were selected on –Leu –Ura galactose medium. Plasmids retrieved from transformants that grew on this selective medium were analyzed by restriction mapping, DNA sequence analysis, subcloning, and GLC7 suppression analysis. Only plasmids containing FPR3 were isolated by this scheme.

### Analysis of GLC7 and ipl1 Suppressors

Sporulation and tetrad dissection of strain JC908-2 yields two viable haploid spores per tetrad. TnUR43 transposons were integrated into JC908-2 by transformation with plasmids from the Triples collection [13] after Ncol digestion. Transposons were chosen that integrated URA3 at 73 distinct locations spaced approximately 150 Kb throughout the genome and thus at least one was guaranteed to show linkage to any locus. Tetrad analysis showed that sds22::TrxUR43 from plasmid V768 failed to recombine with the JC908-2 recessive lethal trait; either all spores were inviable (sds22::TrxUR43/sds22-S56am) or only two viable Ura+ spores were found (sds22::TrxUR43/+). This result indicated the recessive lethal trait was linked to SDS22.

### Genetic and DNA sequence analysis showed that SDS22 loci from diploid JC908-2 retrieved by gapped plasmid repair [74] were either wild-type or contained an amber mutation at SDS22 codon 56 (SDS22-S56am). To confirm the SDS22-S56am mutation in JC907, we isolated JC907 derivatives that contained amber nonsense suppressors. The his4-539 mutation in JC907 is an amber mutation. Several spontaneous His+ revertants of JC907 were selected and then analyzed by tetrad analysis. Those with extragenic his4-539 suppressors (i.e. those with putative amber suppressors) suppressed SDS22-S56am and allowed viability and became sensitive to galactose. These results confirmed that SDS22-S56am suppressed Glc7 overexpression lethality in a diploid.
GST Pull-down Experiments

Cells that express galactose and copper inducible proteins were grown in minimal medium with 2% (w/v) raffinose to A600 = 0.7–1.0 and induced with 2% (w/v) galactose and 0.5 mM CuSO4 for 2–4 hours. GST fusion proteins were purified as described [67] except that binding and washes used buffer containing 250 mM NaCl. In pull-downs with Sds22(1–25)-LacZ, an additional wash with RIPA buffer (50 mM Tris, pH 8, 150 mM NaCl, 1% (v/v) NP40, 0.5% (w/v) sodium deoxycholate, 0.1% (w/v) SDS) was required to reduce the background binding of the negative control GST-Ahc1. In each experiment, the affinity purification was from equivalent crude extract protein masses (1–2 mg). Immunoblots of crude extracts and GST affinity-purified proteins were probed with anti-GST, anti-HA, or anti-β-galactosidase antibodies (Santa Cruz Biotechnology, Inc and Molecular Probes).

Two-hybrid Analysis

DNA binding domain plasmid, pAS1-GLC7, has been described previously [52]. SDS22 mutations were transferred to p2644 by restriction fragment swapping and β-galactosidase activity of P069-4A transformants were assayed in triplicate as described [76]. Statistics of activities were compared with a two-tailed t-test assuming unequal variances. Immunoblots probed with anti-Gal4AD (Sigma) antibody showed equivalent Gal4AD-Sds22 expression for each mutant fusion protein.

Random Mutagenesis of SDS22

SDS22 DNA that encodes residues 28–299 was amplified from p2533 in an error-prone PCR that contained 0.5 mM MnCl2 [77]. The PCR product was co-transformed with gel-purified BglII-BamHI digested pAG108 into JC1126-15B (ipl1-1) yeast cells. Yeast transformants selected on –Ura plates circularized the plasmid by in vivo recombination [69]. After growth to 2–3 mm diameter, colonies were replica printed to fresh -Ura plates and incubated at 30°C and 39°C to screen for temperature-sensitive (ts) transformants. Plasmid DNA retrieved from ts transformants was retransformed into JC1126-15B to confirm the ts trait and the SDS22 DNA sequence determined starting at base 2500 relative to the start codon. One of three plasmids isolated, pAG-RM12, had a large SDS22 deletion. Plasmid pAG-RM45 had E79G, L159Q, and L295I and pAG-RM102 had F65L, Y141H, and I210T SDS22 mutations.

Table 2. Yeast strains used in this work.

| Strains       | Genotype                          | Source       |
|---------------|-----------------------------------|--------------|
| JC482         | MATa leu2 ura3-52 his4-539         | [79]         |
| JC482D        | MATa leu2 ura3-52 his4-539         | [80]         |
| JC482D/pKC1048| JC482D, GAL1p-GLC7::URA3/+        | This work    |
| JC746-9D      | MATa leu2 ura3-52 his3 can1 trp1-1 | [80]         |
| JC746         | MATa leu2 ura3-52 his3 can1 trp1-1 | [80]         |
| JC746/RG200   | MATa leu2 ura3-52 his3 can1 trp1-1 | This work    |
| JC746/V768B   | MATa leu2 ura3-52 his3 can1 trp1-1 | This work    |
| JC907         | JC482D/RK1048, sds22-535am/+      | This work    |
| JC908-2       | JC482D, sds22-535am/+             | This work    |
| JC938-SC      | JC746-9D, glu3::HIS3              | This work    |
| JC1126-15B    | MATa leu2 ura3-52 his3 trp1-1     | This work    |
| JC1287-1C     | JC746-9D, reg1::LEU2             | [37]         |
| JC1338-20A    | MATa pcl6::kanMX4 pcl7::kanMX4 ura3 his3 leu2 | [39] |
| JC1353-17B    | MATa his3::IS000 leu2-3,112 ura3-52 pep4::HIS3 trp1 | This work |
| JC1378        | JC746, sds22::HIS3/+             | This work    |
| JC1535        | SBY625, shp1::URA3               | This work    |
| JC1552-17A    | SBY625, glu3::HIS3               | This work    |
| JC1583        | JC746-9D, reg2::URA3             | This work    |
| JC1624        | JC1353-17B, leu2-3,112::GAL1p-lacZ::LEU2 | This work |
| JC1630        | MATa leu2 ura3-52 his3 trp1-1     | This work    |
| JC1631        | SBY625, glu3::HIS3               | This work    |
| JFY183        | JC482, gal1::LEU2                | [81]         |
| P169-4A       | MATa trp1-901 leu2-3,112 ura3-52 his3-200 gal4 gal80 lys2::GAL1-HIS3 GAL2-ADE2 met2::GAL7-lacZ | [82] |
| SBY625        | W303-1A, GLC7-HA3::HIS3           | Sue Biggins  |
| W303-1A       | MATa ade2-1 can1-100 trp1-1 his3-11,15 leu2-3,112 ura3-1 | [83] |

doi:10.1371/journal.pone.0069133.t002

Analysis of GLC7 and ipl1 Suppressors

PLOS ONE | www.plosone.org 10 July 2013 | Volume 8 | Issue 7 | e69133
Table 3. Plasmids used in this work.

| Plasmid Code | Description |
|--------------|-------------|
| p1614        | CEN URA3 GAL1p-GLC8 [84] |
| p1945        | CEN URA3 GLC8 [40] |
| p2431        | CEN URA3 SDS22 This work, KF113850 |
| p2453        | CEN URA3 GAL1p-HA3-SDS22 This work |
| p2509        | 2μ URA3 LEU2d CUP1p-GLC8 [67] |
| p2508        | 2μ URA3 LEU2d CUP1p-GST-YGR130C [67] |
| p2510        | 2μ URA3 LEU2d CUP1p-GST-FPR4 [67] |
| p2511        | 2μ URA3 LEU2d CUP1p-GST-BUB3 [67] |
| p2518        | CEN TRP1 GAL1p-HA3-SDS22 This work |
| p2521        | 2μ URA3 LEU2d CUP1p-GST-SNF4 [67] |
| p2522        | 2μ URA3 LEU2d CUP1p-GST-KOG1 [67] |
| p2526        | 2μ URA3 LEU2d CUP1p-GST-AHC1 [67] |
| p2533        | SDS22 This work |
| p2539        | 2μ URA3 LEU2d CUP1p-GST-RVB1 [67] |
| p2540        | 2μ URA3 LEU2d CUP1p-GST-NOP6 [67] |
| p2541        | 2μ URA3 LEU2d CUP1p-GST-RVB2 [67] |
| p2562        | CEN LEU2 GAL1p-GLC7 This work |
| p2603        | CEN TRP1 SDS22(1-27)-lacZ This work |
| p2608        | shp1::URA3 This work |
| p2613        | 2μ URA3 LEU2d CUP1p-GST-FPR3(1121-167) This work |
| p2615        | 2μ URA3 LEU2d CUP1p-GST-FPR3-K302oc This work |
| p2644        | 2μ LEU2 GAL4AD-SDS22 This work |
| p2665        | 2μ URA3 SDS22-SS6am This work |
| p2752        | 2μ LEU2 SDS22-RM45-myc3 This work |
| p2757        | CEN TRP1 GAL1p-HA3-SDS22-RM45-myc3 This work |
| p705-3       | CEN URA3 GAL1p-HA3 [85] |
| pAG101       | 2μ URA3 3DS22 This work |
| pAG108       | 2μ URA3 3DS22-myc3 This work, KF113851 |
| pAG109       | 2μ URA3 3DS22-D119A-myc3 This work, KF113852 |
| pAG110       | 2μ URA3 3DS22-D273A-myc3 This work, KF113853 |
| pAG111       | 2μ URA3 3DS22-W275A-myc3 This work, KF113854 |
| pAG117       | 2μ URA3 3DS22-A81-127-myc3 This work, KF113855 |
| pAG118       | 2μ URA3 3DS22-A81-171-myc3 This work, KF113856 |
| pAG119       | 2μ URA3 3DS22-A251-323-myc3 This work, KF113846 |
| pAG120       | 2μ URA3 3DS22-A251-323-myc3 This work, KF113846 |
| pAG-RM45     | 2μ URA3 3DS22-RM45-myc3 This work, KF113848 |
| pAG-RM107    | 2μ URA3 3DS22-RM107-myc3 This work, KF113849 |
| pAS1-GLC7    | 2μ TRP1 GAL4(1-147)-GLC7 [52] |
| pBM272       | CEN URA3 GAL1p [71] |
| pKC978       | CEN URA3 GAL1p-GLC7 This work |
| pKC1048      | Integrative URA3 GAL1p-GLC7 This work |
| pRG200       | Integrative sdh221(1-116):URA3 This work |
| pRS303       | Integrative HIS3 [72] |
| pRS314       | CEN TRP1 [72] |
| pRS315       | CEN LEU2 [85] |
| pRS316       | CEN URA3 [72] |
| pRS426       | 2μ URA3 [86] |
| pYT115       | CEN URA3 GLC8-T118A [39] |
| pYT251       | CEN TRP1 GAL1p-GLC7 This work |
| V7688        | Integrative SDS22(1-25)::mTnURA3 [13] |
| YCp50        | CEN URA3 [87] |
| YCp50-HA-GLC7| CEN URA3 HA-GLC7 [88] |
| Ypl5         | Integrative URA3 [89] |
Genbank Submissions
DNA sequences of mutant SDS22 restriction fragments in plasmids were submitted to Genbank. Accession numbers of p2431, pAG108, pAG109, pAG110, pAG111, pAG117, pAG118, pAG119, pAG120, pAG-RM45, and pAG-RM107 are indicated in Table 3.

Acknowledgments
We are grateful to Sue Biggins, Bruce Futcher, Bart Lesage, David Mitchell, and Mike Syder for strains and plasmids. We thank Jianhong Zheng, Raad Gitan, Jill Adams, Ge Gao, and Karen Clemens for plasmid and strain constructions. We appreciate comments from Judy Wall and Jason Furrer about the manuscript.

Author Contributions
Conceived and designed the experiments: JFC AG. Performed the experiments: JFC AG. Analyzed the data: JFC AG. Contributed reagents/materials/analysis tools: JFC AG. Wrote the paper: JFC AG.

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