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# Inactivation of Pmc1 vacuolar $Ca^{2+}$ ATPase causes $G_2$ cell cycle delay in *Hansenula polymorpha*

Anastasia V. Fokina,[1] Svyatoslav S. Sokolov,[2] Hyun A. Kang,[3] Tatyana S. Kalebina,[2] Michael D. Ter-Avanesyan[1] and Michael O. Agaphonov[1,*]

[1]A.N. Bach Institute of Biochemistry of the Russian Academy of Sciences; [2]Molecular Biology Department; Faculty of Biology; Lomonosov Moscow State University; Moscow, Russia; [3]Department of Life Science; Chung-Ang University; Seoul, Korea

**Key words:** yeast, cell cycle, $G_2$ delay, Hog1 kinase, Wee1 kinase, calcium homeostasis

The vacuolar $Ca^{2+}$ ATPase Pmc1 is involved in maintenance of a low $Ca^{2+}$ concentration in cytosol in yeast cells. Here we observed that increase of $Ca^{2+}$ cytosolic concentration in yeast *Hansenula polymorpha* due to inactivation of Pmc1 resulted in sensitivity to sodium dodecyl sulfate (SDS). To elucidate the mechanisms of the observed effect, a screening for mutations suppressing SDS sensitivity of the *H. polymorpha pmc1* mutant was performed. As a result, three genes were identified. Two of them, designated as their *Saccharomyces cerevisiae* orthologs *CCH1* and *HOG1* encoded the plasma membrane voltage-gated high-affinity calcium channel and the MAP kinase involved in osmoregulation, respectively. The third gene, designated as *WEE1,* coded for the ortholog of Wee1/Swe1 kinase involved in cell cycle regulation by inhibiting of the $G_2$/M transition. Detailed analysis of this mutant demonstrated that suppression of *pmc1* SDS sensitivity by the *wee1* mutation depended on an accompanying chromosomal rearrangement, whereas inactivation of *WEE1* in the absence of this rearrangement caused SDS sensitivity. Expression of a chimeric protein containing an N-terminal portion of Wee1 in the *pmc1* mutant led to abnormal morphology characteristic of $G_2$ delay. Our data indicate that cytosolic $Ca^{2+}$ rise causes SDS sensitivity in *H. polymorpha* through the activation of the Wee1 kinase, which is mediated by the Hog1 kinase. Wee1 has a dual role in the manifestation of SDS sensitivity in the *H. polymorpha pmc1* mutant. Mechanisms of influence of the obtained mutations on the $G_2$/M transition are discussed.



## Introduction

The role of $Ca^{2+}$ as a second messenger in signal transduction in eukaryotes requires maintenance of its free cytosolic concentration ($[Ca^{2+}]$) at very low levels (reviewed in ref. 1). In yeast cells $Ca^{2+}$ sequestration occurs in secretory organelles by means of the Golgi apparatus membrane Pmr1 pump and in the vacuole by means of $Ca^{2+}$ ATPase Pmc1 and $H^+$/$Ca^{2+}$ antiporter Vcx1.[2-4] Loss of Pmc1 leads to an increase in cytosolic $[Ca^{2+}]$ and decrease in vacuolar $[Ca^{2+}]$. This rise of cytosolic $[Ca^{2+}]$ is alleviated due to the presence of the Golgi apparatus Pmr1 $Ca^{2+}$/$Mn^{2+}$ ATPase, inactivation of which is synthetically lethal with the loss of Pmc1. The synthetic lethality of *pmr1* and *pmc1* is due to an elevated concentration of cytosolic $[Ca^{2+}]$, since it can be rescued by inactivation of the calcineurin.[2] Calcineurin is a $Ca^{2+}$/calmodulin-dependent protein phosphatase consisting of the catalytic A and regulatory B subunits.[5] In *Saccharomyces cerevisiae,* the former subunit is encoded by two functionally redundant genes, *CNA1* and *CNA2*, while the latter one is encoded by the single *CNB1* gene. One of the calcineurin targets is the Crz1 transcription factor which regulates the expression of various genes implicated in ion homeostasis (e.g., *PMR1*, *PMR2*, *PMC1*) and cell wall integrity (e.g., *FKS2*).[6,7]

Requirement of calcineurin for cell growth was revealed in strains defective in pathways involved in cell wall integrity control. For example, the loss or inhibition of the calcineurin pathway exacerbates the cell wall integrity defects caused by an impairment of Mpk1 MAP kinase cascade.[8,9] In particular, inactivation of the *CNB1* gene in a *mpk1*Δ mutant led to the need of an osmostabilizer for growth. The HOG pathway (high osmolarity of glycerol), which is implicated in the response to an elevation of osmolarity,[10] antagonizes calcineurin in the control of cell wall integrity, since its inhibition restores ability of the *cnb1*Δ *mpk1*Δ double mutant to grow without an osmostabilizer.[11] These pathways regulate distinct processes of bud emergence: calcineurin negatively regulates actin polarization at the bud site at an early step of bud emergence, whereas the HOG pathway promotes bud emergence at a step after actin has become polarized.[11]

Activation of calcineurin increases the expression of Wee1 kinase, which is involved in the $G_2$ cell cycle regulation. In *S. cerevisiae*, this kinase is encoded by the *SWE1* gene (*Saccharomyces WEE1*).[12] Phosphorylation of Cdc28 by Swe1 leads to $G_2$ delay. This is opposed by the Mih1 protein phosphatase, which activates the Cdc28/Clb complex promoting $G_2$/M transition. Recruitment of Swe1 to the bud neck by Hsl1-Hsl7 causes its rapid degradation, leading to an activation of the Cdc28/Clb

*Correspondence to: Michael O. Agaphonov; Email: agaphonov@inbi.ras.ru
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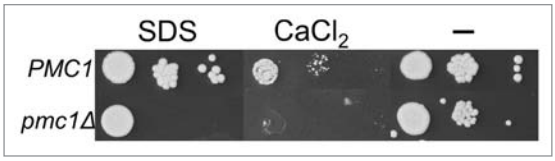


**Figure 1.** Sensitivity of *pmc1*Δ mutant to SDS and $CaCl_2$. Serial 1:10 dilutions of cell suspensions of DL1-L (*PMC1*) and DL1-Δpmc1 (*pmc1*Δ) were applied onto YPD (-), YPD supplemented with 0.15 M $CaCl_2$ ($CaCl_2$), or YPD containing 0.005% SDS (SDS) and incubated at 37°C for 2 d.

complex.[13] The HOG pathway is also involved in this phase of the cell cycle regulation, since activated Hog1 interacts and directly phosphorylates a residue within the Hsl7-docking site of the Hsl1 checkpoint kinase, which results in delocalization of Hsl7 from the septin ring and leads to Swe1 accumulation.[14]

Cdc28 is not the only Wee1 substrate. For example, in Drosophila, Wee1 may phosphorylate Kinesin-5 to regulate its activity.[15] In yeast and mammalian cells, Wee1 was shown to phosphorylate a conserved tyrosine residue in the N-domain of a subpopulation of nuclear-localized Hsp90 in a cell cycle-dependent manner.[16] This modulates Hsp90 chaperone function toward a subset of client proteins, including Wee1 itself. In yeast, the Hsp90 Y24F mutation prevents Hsp90 phosphorylation by Wee1 and leads to Wee1 destabilization.[17] In human cells, Hsp90-mediated stabilization of Wee1 depends on the ubiquitin-specific protease USP50.[18]

In this study we have observed that in *Hansenula polymorpha* manifestations of the cytosolic $[Ca^{2+}]$ rise can be alleviated by a defect of Hog1 or Wee1 and that, upon high cytosolic $[Ca^{2+}]$, expression of a chimera containing the N-terminal portion of Wee1, leads to alterations in cell morphology, which were previously shown to be characteristic of $G_2$ delay in *S. cerevisiae*.

## Results

**Inactivation of *PMC1* leads to SDS sensitivity.** Two *H. polymorpha* strains defective for the *PMC1* gene were isolated by searching SDS-sensitive clones among transformants bearing randomly integrated linear DNA fragments (see Materials and Methods). One of these mutants, DLQS1, was obtained in *H. polymorha* DL-1, while another one, S67, was detected in *H. polymorpha* CBS4732. The *PMC1* wild-type allele was cloned by complementation of the SDS sensitivity of the DLQS1 strain. This gene was able to complement SDS sensitivity of S67 as well (data not shown). Targeted disruption of *PMC1* in the DL-1 (**Fig. 1**) and CBS4732 (data not shown) strains confirmed that its inactivation led to SDS sensitivity. It had been previously shown that *S. cerevisiae pmc1* mutants are unable to grow at high $Ca^{2+}$ concentrations.[2] The same phenotype was observed in the *H. polymorpha pmc1* mutants (**Fig. 1**). In contrast to *H. polymorpha*, disruption of *PMC1* in *S. cerevisiae* did not cause increased sensitivity to SDS (data not shown). As a rule, osmo-remediable detergent sensitivity indicates a cell envelope defect. However, the SDS sensitivity of *H. polymorpha pmc1* mutants was not alleviated in presence of an osmotic stabilizer (0.5–1.2 M sorbitol), and no alteration in cell wall carbohydrate composition or in cell wall protein pattern was revealed in this mutant (data not shown). Moreover, the growth of the *pmc1* mutant was noticeably retarded at high sorbitol concentrations even in absence of a detergent (data not shown).

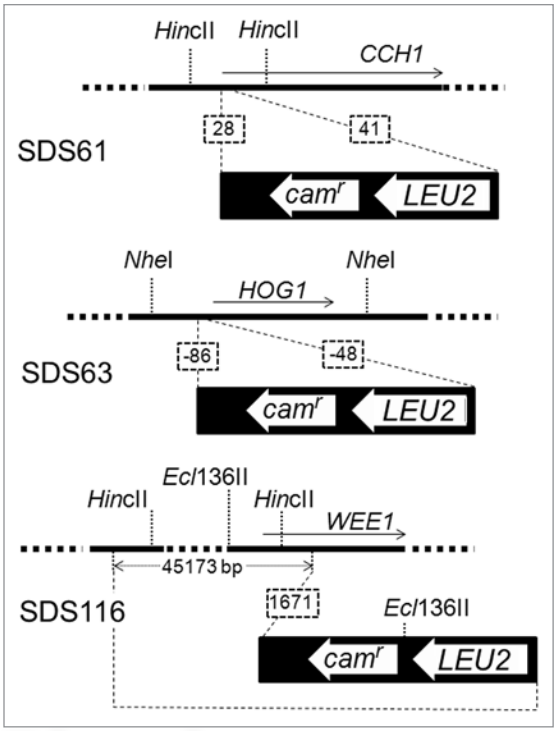


**Figure 2.** Schemes of pCLHXdHS integration in SDS61, SDS63 and SDS116 mutants. Lines, chromosome sequences; bar, pCLHXdHS sequence. Arrows indicate positions of the identified ORFs. Boxed numbers indicate positions of integration sites respectively the ORFs. "45173 bp" indicate distance between sequences adjoining the plasmid integration deduced from the *H. polymorpha* DL-1 genome database.

**Inactivation of *CCH1* or *HOG1* suppresses manifestations of *PMC1* deficiency.** To elucidate the mechanism responsible for the effects of Pmc1p on the cell wall integrity, we performed a screen for mutations suppressing SDS sensitivity of the *pmc1* mutant s67. Mutations were obtained by random integration of the linearized pCLHXdHS plasmid. Several clones able to grow on SDS-containing medium were selected, and the chromosomal loci adjoining the plasmid integration sites were identified in three of them, SDS61, SDS63 and SDS116 (see Materials and Methods and **Fig. 2**). The SDS61 clone possessed the plasmid integrated into the *CCH1* gene coding for a component of the plasma membrane high-affinity calcium channel. Plasmid integration in SDS63 occurred in the *HOG1* gene coding for the mitogen-activated protein kinase responsible for osmoregulation. Targeted disruption of the *CCH1* or *HOG1* genes also suppressed SDS sensitivity of the s67 strain (**Fig. 3**). These genes are orthologs of *S. cerevisiae CCH1* and *HOG1,* since their inactivation in a *H. polymorpha* strain with wild-type *PMC1* led to phenotypes that are characteristic of the corresponding *S. cerevisiae* mutants. In particular, the *hog1* mutant was hypersensitive to a high osmolarity, while the *cch1* mutant was unable to grow at low $Ca^{2+}$ concentrations (data not shown).

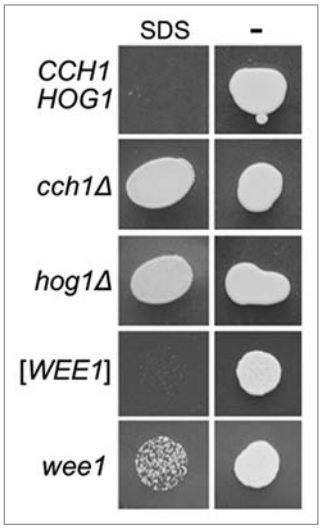


**Figure 3.** Suppression of *pmc1* SDS sensitivity by *cch1*, *hog1* and *wee1* mutations. Cell suspension were applied onto YPD (-), or YPD supplemented with 0.004% SDS (SDS) and incubated at 37°C for 2 d. *CCH1 HOG1*, the S67 strain; *cch1*Δ, S67Δcch1 strain; *hog1*Δ, S67Δhog1 strain; [*WEE1*], SDS116 strain transformed with the pKAF48 plasmid bearing wild type *WEE1*; *wee1*, untransformed SDS116 strain.

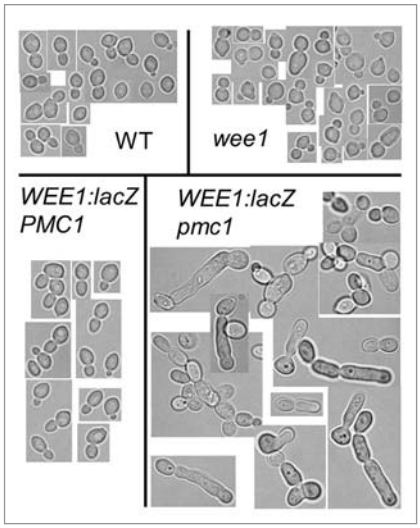


**Figure 4.** Cells of DL1-L transformants. WT, transformant bearing only an empty *LEU2*-containing vector; *wee1*, DL1-L disrupted for *WEE1* by *LEU2*; *WEE1:lacZ*, DL1-L bearing pKAM613 plasmid, which was transformed with an empty *LEU2*-containing vector (*PMC1*), or disrupted for *PMC1* by *LEU2* (*pmc1*).



**Suppression of *pmc1* by inactivation of *WEE1* depends on genetic background.** In contrast to SDS61 and SDS63, the sequences adjoining the transforming DNA fragment in the SDS116 clone were found to belong to different genomic loci (**Fig. 2**). The integrated plasmid was flanked by a portion of a gene coding for a homolog of the Wee1/Swe1 kinase and by a sequence lacking presumable ORFs and originating from another genomic locus. This could be due to a chromosomal rearrangement accompanied the plasmid integration. The identified gene was designated as *WEE1,* since, similarly to the *S. cerevisiae swe1*Δ mutation,[19] its targeted disruption led to sensitivity to drugs, inducing ER stress (data not shown). Besides, this mutation affected cell morphology, since in contrast to wild-type cells, which were olive-shaped, most of the *wee1*Δ cells were spherical or were sometimes elongated at the side opposite the budding site (**Fig. 4**).

Surprisingly, while genomic integration of the plasmid bearing wild-type *WEE1* abolished SDS resistance of the SDS116 strain (**Fig. 3**), targeted disruption of *WEE1* did not suppress, but rather aggravated, SDS sensitivity of the *pmc1*Δ mutant (**Fig. 5**). Inactivation of *WEE1* in a strain with wild-type *PMC1* also led to SDS sensitivity, which, however, was less pronounced then in *pmc1* mutants (data not shown).

To elucidate the role of the *wee1* mutation in suppression of *pmc1* SDS sensitivity in SDS116, we studied inheritance of this phenotype in meiotic progeny of a diploid obtained in the cross of SDS116 with the 1B27 strain bearing wild-type *WEE1* and *PMC1*. This diploid was homozygous for the *leu2* and *ura3::ADE2* alleles and heterozygous for the *wee1* and *pmc1* alleles obtained by integration of DNA fragments carrying *LEU2* and *URA3* markers, respectively. This enabled monitoring of *wee1* and *pmc1* segregation by leucine and uracil prototrophy. All 21 tested *pmc1 wee1* ($Leu^+$ $Ura^+$) segregants were less sensitive to SDS than *pmc1* ($Leu^-$ $Ura^+$) segregants, indicating that *pmc1* suppression depended on the *wee1* allele. However, targeted disruption of *WEE1* in three *pmc1* ($Leu^-$ $Ura^+$) segregants even slightly enhanced their SDS sensitivity. Clones obtained by targeted disruption of *PMC1* in three *wee1* ($Leu^+$ $Ura^-$) segregants were less sensitive to SDS than *PMC1* disruptants bearing the *WEE1* wild-type allele. Obtained data allowed a suggestion that suppression of *pmc1*Δ was due to expression of a portion of *WEE1* remaining in the SDS116 strain. To verify this hypothesis, the *WEE1* wild-type allele in a *pmc1*Δ strain was replaced with the truncated *WEE1* allele isolated from SDS116. However, this did not alleviate the *pmc1* SDS sensitivity (data not shown). Taken together, our data indicate that suppression of the *pmc1* SDS sensitivity by *WEE1* disruption depends on the chromosomal rearrangement that occurred in the SDS116 strain.

**Expression of a chimera containing the N-terminal portion of *WEE1* in *pmc1*-Δ strain causes abnormal cell morphology.** Since in *S. cerevisiae,* expression of the *SWE1*/*WEE1* gene is positively regulated by the $Ca^{2+}$/calmodulin-dependent protein phosphatase calcineurin,[12] one could suggest that in *H. polymorpha*, an increase of cytosolic [$Ca^{2+}$] also induces *WEE1* expression. To test this, a portion of *WEE1*, which included its promoter and ORF fragment lacking the protein kinase domain, was fused to the *E. coli lacZ* ORF. Then, *H. polymorpha* transformants possessing this *WEE1:lacZ* cassette were obtained. Disruption of *PMC1* led to only a slight increase in the β-galactosidase activity (from 13.3 ± 0.7 to 15.9 ± 1.0 U per mg of total cellular protein). Interestingly, *pmc1* disruptants expressing *WEE1:lacZ* grew noticeably slower than *pmc1* strains lacking this construct (data not shown). Besides the decreased growth rate, inactivation of *PMC1* in the *WEE1:lacZ*-expressing transformants led to alterations in cell morphology such as an abnormal cell shape

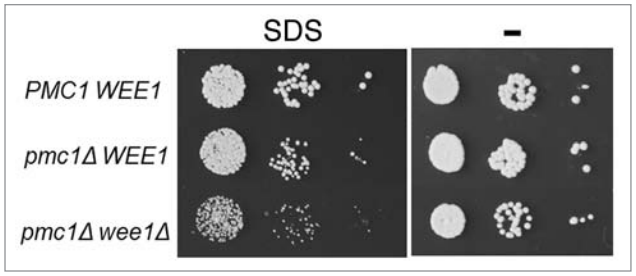


**Figure 5.** Growth of the 1B (*PMC1 WEE1*), 1BΔpmc1 (*pmc1Δ WEE1*) and 1BΔpmc1Δwee1 (*pmc1Δ wee1Δ*) strains on YPD (-) and YPD supplemented with 0.004% SDS (SDS).

and elongated buds (**Fig. 4**). At the same time, cell morphology was not noticeably affected, neither in wild-type cells expressing *WEE1:lacZ*, nor in *pmc1* cells lacking this construct (data not shown), indicating that the observed effect was due to the combination of these factors.

## Discussion

In yeast, the Pmc1 vacuolar $Ca^{2+}$ ion pump is involved in the sequestration of cytosolic $Ca^{2+}$ into the vacuole. Disruption of this process in *S. cerevisiae* by *pmc1* mutations leads to sensitivity to elevated [$Ca^{2+}$] in culture medium.[2] Here, the same phenotype was observed in *H. polymorpha pmc1* mutants, indicating the same role of this $Ca^{2+}$ pump in ion homeostasis. However, unlike in *S. cerevisiae*, inactivation of the *H. polymorpha PMC1* gene additionally led to detergent sensitivity.

We have identified three genes, *CCH1*, *WEE1* and *HOG1*, whose inactivation suppresses the *pmc1* detergent sensitivity. Importantly, *CCH1* encodes a subunit of the plasma membrane high-affinity $Ca^{2+}$ channel involved in the $Ca^{2+}$ influx.[20] This indicates that the detergent sensitivity is due to an increased cytosolic [$Ca^{2+}$]. Since in *S. cerevisiae,* expression of the *WEE1* ortholog (*SWE1*) is positively regulated by the $Ca^{2+}$/calmodulin-dependent protein phosphatase, calcineurin,[12] one could expect that *H. polymorpha WEE1* is also a target of calcineurin regulation. Therefore, *pmc1* detergent sensitivity can be mediated by an increase of *WEE1* expression in response to elevation of cytosolic [$Ca^{2+}$]. However, we observed only a slight *WEE1* promoter activation in *pmc1*Δ cells. Such slight increase of *WEE1* expression by itself is unlikely to lead to the severe SDS sensitivity we observed in *pmc1* mutants.

The third gene encoded the Hog1 MAP kinase, which is a key component of the HOG signaling pathway required for survival at high environmental osmolarity (reviewed in ref. 10). Inhibition of this pathway rescues cell wall integrity in *S. cerevisiae cnb1*Δ *mpk1*Δ double mutant, indicating an antagonistic relationship between the HOG and $Ca^{2+}$/calcineurin signaling pathways.[11] Suppression of the *pmc1* SDS sensitivity by inactivation of *HOG1* could indicate similar negative effect of the HOG pathway on cell wall integrity in *H. polymorpha*. However, our data suggest that the *pmc1* detergent sensitivity is mediated by a mechanism, in which $Ca^{2+}$-mediated signaling and the HOG pathway are not antagonized. It is noteworthy that Hog1 is implicated in cell cycle regulation (reviewed in ref. 21). Its involvement in $G_2$ cell cycle regulation is mediated by Swe1/Wee1, which inhibits Cdc28. The Hsl1-Hsl7 complex promotes Swe1/Wee1 degradation, which results in activation of the Cdc28/Clb complex promoting $G_2$/M transition. Phosphorylation of Hsl1 by Hog1 rescues Swe1/Wee1 from degradation, thus leading to its accumulation.[13] This warrants the suggestion that in *H. polymorpha, pmc1* detergent sensitivity is related to a $G_2$ delay due to Wee1 accumulation. Indeed, inactivation of *PMC1* in cells expressing the *WEE1:lacZ* construct led to cell morphology alterations that were very similar to those observed in *S. cerevisiae* upon $G_2$ arrest. In particular, similar effects were observed when Swe1 was overexpressed in *S. cerevisiae* cells under the control of *GAL1* promoter.[22] One can suggest that in *H. polymorpha,* the fusion protein containing only the N-terminal portion of Wee1 rescues wild-type Wee1 from degradation by competing with it for interaction with the Hsl1-Hsl7 complex. According to this model, the increase of cytosolic [$Ca^{2+}$] due to *PMC1* inactivation induces phosphorylation of Hsl1 by Hog1, which, in turn, leads to Wee1 release that is boosted in presence of the Wee1:lacZ fusion protein. Although this protein was expressed under control of the native *WEE1* promoter, which provides a relatively low expression level, its concentration in the cytosol could be much higher than that of wild-type Wee1, since the latter is transported to the nucleus and undergoes degradation, while the chimeric protein possibly escapes these pathways.

Interestingly, the targeted inactivation of *WEE1* caused increased SDS sensitivity on its own and slightly increased the SDS sensitivity of the *pmc1*-Δ mutant. Suppression of *pmc1* SDS sensitivity was observed only in strains bearing the *wee1* allele obtained by random integration of a transforming DNA fragment. This integration was accompanied by a chromosomal rearrangement, which possibly affected some gene(s) mediating the negative effect of Wee1 loss on SDS resistance and, thus, allowed suppression of *pmc1* SDS sensitivity. We suggest that the primary process leading to the SDS sensitivity at high cytosolic [$Ca^{2+}$] is boosted by Wee1 and by an unknown component (X), which is negatively regulated by Wee1 (**Fig. 6**). Inactivation of either Wee1 or this hypothetical component X would not alleviate SDS sensitivity. On the contrary, deletion of *WEE1* aggravated SDS sensitivity of *pmc1* and led to SDS sensitivity on its own in a wild-type strain. According to the suggested model (**Fig. 6**) this can be due to activation of the hypothetical component X. Inactivation of Hog1 may relieve the direct effect of Wee1 while maintaining its inhibitory effect on the component X, thus mimicking loss of both Wee1 and the component X. A search for mutations suppressing SDS sensitivity in a *H. polymorpha pmc1 wee1* strain might identify this hypothetical component.

## Materials and Methods

**Yeast strains, culture conditions and genetic methods.** *H. polymorpha* cells were cultivated at 37°C in YPD (1% Yeast extract, 2% Peptone and 2% glucose) or in synthetic complete media SC [0.67% yeast nitrogen base (Difco), supplemented with 2%

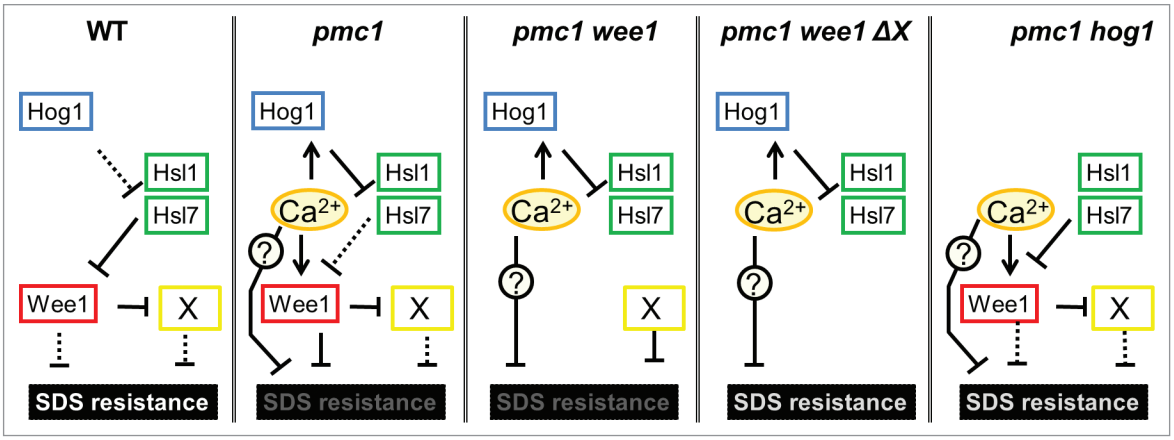


**Figure 6.** Hypothetical scheme of events leading to SDS sensitivity. Arrows and lines with bars indicate positive and negative influence, respectively. Dotted lines indicate that influence is depressed. In wild-type cells (WT), Wee1 is downregulated by interaction with the Hsl1-Hsl7 complex, which however does not prevent inhibition of the hypothetical component X involved in control of SDS resistance (see text). Inactivation of *PMC1* (*pmc1*) leads to activation of Hog1, which releases Wee1 from interaction with Hsl1-Hsl7. Wee1 in turn affects SDS resistance. Inactivation of *WEE1* (*pmc1 wee1*) abolishes its influence on SDS sensitivity, but activates the hypothetical component, which causes SDS sensitivity on its own. Suppression of *pmc1* by *wee1* may be observed upon inactivation of this component (*pmc1 wee1 ΔX*). Inactivation of *HOG1* (*pmc1 hog1*) restores the negative effect of Hsl1-Hsl7 on Wee1, but residual Wee1 activity is sufficient to inhibit the hypothetical component. Incomplete suppression of SDS sensitivity by *hog1* mutation may be due to presence of an alternative pathway marked by "?," which mediates the effect of high cytosolic [$Ca^{2+}$] on this phenotype.



glucose]. *H. polymorpha* strains were crossed, and hybrids were sporulated on maltose-containing medium. *H. polymorpha* was transformed according to the modified lithium acetate method.[23] Ability of *H. polymorpha* strains to grow in a $Ca^{2+}$ shortage was studied using EGTA-containing medium as described earlier in reference 24.

Strains used in this work are listed in **Table 1**. The DLQS strain resulted from an accidental inactivation of the *PMC1* gene in DLQ due to random integration of *LEU2*-containing fragment of *Eco*RV-digested pSS2.[25] The SDS-sensitive S67 mutant was selected among transformants of the u23M25 strain obtained by random integration of the *Pst*I-linearized pKNR50 plasmid. This mutant was defective for *PMC1,* since its SDS-sensitivity could be complemented by a single copy integration of a plasmid possessing the wild-type *H. polymorpha* DL-1 *PMC1* gene (data not shown). The SDS61, SDS63, SDS116 strains with increased resistance to SDS were obtained via random integration of the *Eco*RV-linearized pCHLXdHS plasmid into the genome of the S67 strain. The DLΔpmc1 strain was obtained by transformation of the DL1-L strain, with the *PMC1* disruption cassette bearing the *LEU2* selectable marker (*Eco*RI- and *Dra*I-digested pKAF2 plasmid). The 1BΔpmc1 strain was obtained by transformation of the 1B strain with the *PMC1* disruption cassette bearing the *Zeo*$^r$ selectable marker (*Mlu*I- and *Bgl*II-digested pKAI41 plasmid). The 1BΔpmc1Δwee1 was obtained by transformation of the 1BΔpmc1 strain with *WEE1* disruption cassette (*Pst*I-digested pCAF45 plasmid). The S67Δcch1 and S67Δhog1 strains were obtained by transformation of the S67 strain with *CCH1* disruption cassette (*Hin*cII-digested p61H plasmid) and *HOG1* disruption cassette (*Nhe*I-digested pCAF41 plasmid), respectively. The 1B27 strain was obtained by disruption of the *URA3* gene with *ADE2* selectable marker in the 1B strain.

**Construction of plasmids and identification of mutant loci in the SDS61, SDS63 and SDS116 clones.** The pSS5 plasmid containing the *PMC1* wild-type gene was isolated from *H. polymorpha* DL-1 genomic library[25] by complementation of SDS sensitivity of the DLQS strain. To obtain *PMC1* disruption cassettes, the *Bam*HI-*Mlu*I fragment of the pSS5 plasmid containing *PMC1* was inserted into the pUK21 vector.[26] Then the *PMC1 Nhe*I-*Hin*dIII and *Dra*I-*Asp*718 internal fragments were replaced with the *Xba*I-*Hin*dIII fragment of AMIpL1 [27] to obtain the pKAF2 plasmid, and with the *Pvu*II-*Bsr*GI fragment of pGAPZα-a (Invitrogen) to obtain the pKAI41 plasmid. The pCLHXdHS plasmid was obtained by deleting the *Hin*dIII-SalI fragment in pCLHX.[28] As a result, this plasmid does not possess a *Hin*cII cleavage site. *Hin*cII digestion and self-ligation of chromosomal DNA of the SDS61, SDS63 and SDS116 clones, obtained by random integration of pCLHXdHS, produced p61H, p63H and p116H plasmids, respectively. These plasmids contained chromosomal sequences adjoining the integration sites. Sequencing of these plasmids revealed that in the SDS61 and SDS63 clones, the pCLHXdHS sequence replaced short portions of *CCH1* and *HOG1* loci, respectively (**Fig. 2**). The recombination pattern in the SDS116 clone was more complex, since p116H possessed sequences located between recombination sites at a distance of 45,173 bp (**Fig. 2**). Possibly, this 45,173 bp fragment was excised from the chromosome by recombination with the pCHLXdHS sequence forming a circular molecule, which could re-integrate into the genome via recombination at a site within the captured 45,173 bp fragment. The p63N plasmid possessing the *H. polymorpha HOG1* ORF was obtained by *Nhe*I digestion (**Fig. 2**) and self-ligation of the SDS63 chromosomal DNA. The pCAF41 plasmid was obtained by replacing the *Xba*I-*Bsr*GI fragment of p63N with the *Xba*I-*Bsr*GI fragment of pJJ282.[29] The p116E plasmid containing a portion

**Table 1.** Yeast strains used in this study

| Strain | Genotype (Description) | Reference |
|---|---|---|
| DLQ* | *leu2 mox::uPA*-$Q^{302}$ | 33 |
| DLQS1* | *leu2 mox::uPA*-$Q^{302}$ *pmc1::LEU2* (resulted from random integration of *LEU2*-contaning DNA fragment into the *PMC1* locus of the DLQ strain) | This study |
| DL1-L | *leu2* | 28 |
| DLΔpmc1* | *leu2 pmc1::LEU2* | This study |
| 1B27** | *leu2 ade2 ura3::ADE2* | This study |
| u23M25** | *leu2 ade2 ura3::ADE2*** | 34 |
| S67** | *leu2 ade2 ura3::ADE2 pmc1::URA3*** (resulted from random integration of *URA3*-contaning DNA fragment into the *PMC1* locus of the u23M25 strain) | This study |
| S67Δcch1** | *leu2 ade2 ura3::ADE2 pmc1::URA3 cch1::LEU2**** | This study |
| S67Δhog1** | *leu2 ade2 ura3::ADE2 pmc1::URA3 hog1::LEU2**** | This study |
| 1B** | *leu2 ade2* | 23 |
| 1BΔpmc1** | *leu2 ade2 pmc1::Zeo*$^r$ | This study |
| 1BΔpmc1Δwee1** | *leu2 ade2 pmc1::Zeo*$^r$ *wee1::LEU2* | This study |
| SDS61 | (resulted from random integration of *LEU2*-contaning plasmid into the *CCH1* locus of the S67 strain) | This study |
| SDS63 | (resulted from random integration of *LEU2*-contaning plasmid into the *HOG1* locus of the S67 strain) | This study |
| SDS116 | (resulted from random integration of *LEU2*-contaning plasmid into the *WEE1* locus of the S67 strain accompanied with a chromosomal rearrangement) | This study |

*A derivative of H. polymorpha DL-1 (*Ogataea parapolymorpha* ATCC26012); **A derivative of *H. polymorpha* CBS4732 (*Ogataea polymorpha* ATCC34438); ***Strain possesses expression cassettes of three recombinant proteins,[34] which are not indicated in the genotype because they were not used in this study.



of the *WEE1* ORF was recovered from SDS116 chromosomal DNA by *Ecl*136II digestion (**Fig. 2**). The *Stu*I-*Mlu*I fragment of the *WEE1* sequence in p116E was replaced with the *LEU2* selectable marker to obtain pCAF45. The pKAF48 plasmid was obtained by insertion of *H. polymorpha* DL-1 *WEE1* into the pKAM556 vector.[30] The *WEE1* gene was amplified by PCR with the 5'-GGA ACA CGA AAG TAG ATG GC-3' and 5'-AAG GGT ATG TAT CGA AAT GG-3' primers. The pKNR50 plasmid was obtained by inserting of the *Pst*I-*Sma*I fragment of pJJ244 [29] between the *Pst*I and *Sma*I sites of pUK21.[26] To obtain pKAM613, the *Bam*HI-*Nde*I fragment of pKAF48 was replaced with the *Bam*HI-*Vsp*I fragment of Yep368 bearing the *E. coli lacZ* gene.[31]

**β-galactosidase activity assay.** To determine β-galactosidase activity in *H. polymorpha* transformants, overnight YPD cultures were diluted to $OD_{600}$ = ~0.5 and cultured for 4 h. Then β-galactosidase activity was assayed as described previously in reference 32 and normalized to total cellular protein.

**GenBank accession numbers.** GenBank accession numbers of *H. polymorpha PMC1*, *HOG1*, *WEE1* and *CCH1* sequences are JN015194, JN015195, JN015196 and JN015197, respectively.

### Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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### References

1. Cui J, Kaandorp JA, Sloot PMA, Lloyd CM, Filatov MV. Calcium homeostasis and signaling in yeast cells and cardiac myocytes. FEMS Yeast Res 2009; 9:1137-47; PMID:19678847; http://dx.doi.org/10.1111/j.1567-364.2009.00552.x.
2. Cunningham KW, Fink GR. Calcineurin-dependent growth control in *Saccharomyces cerevisiae* mutants lacking *PMC1*, a homolog of plasma membrane $Ca^{2+}$ ATPases. J Cell Biol 1994; 124:351-63; PMID:7507493; http://dx.doi.org/10.1083/jcb.124.3.351.
3. Pozos TC, Sekler I, Cyert MS. The product of *HUM1*, a novel yeast gene, is required for vacuolar $Ca^{2+}$/$H^+$ exchange and is related to mammalian $Na^+$/$Ca^{2+}$ exchangers. Mol Cell Biol 1996; 16:3730-41; PMID:8668190.
4. Miseta A, Kellermayer R, Aiello DP, Fu L, Bedwell DM. The vacuolar $Ca^{2+}$/$H^+$ exchanger Vcx1p/Hum1p tightly controls cytosolic $Ca^{2+}$ levels in *S. cerevisiae*. FEBS Lett 1999; 451:132-6; PMID:10371152; http://dx.doi.org/10.1016/S0014-5793(99)00519-0.
5. Aramburu J, Rao A, Klee CB. Calcineurin: from structure to function. Curr Top Cell Regul 2000; 36:237-95; PMID:10842755; http://dx.doi.org/10.1016/S0070-2137(01)80011-X.
6. Miyakawa T, Mizunuma M. Physiological roles of calcineurin in *Saccharomyces cerevisiae* with special emphasis on its roles in $G_2$/M cell cycle regulation. Biosci Biotechnol Biochem 2007; 71:633-45; PMID:17341827; http://dx.doi.org/10.1271/bbb.60495.
7. Levin DE. Cell wall integrity signaling in *Saccharomyces cerevisiae*. Microbiol Mol Biol Rev 2005; 69:262-91; PMID:15944456; http://dx.doi.org/10.1128/MMBR.69.2.262-91.2005.
8. Garrett-Engele P, Moilanen B, Cyert MS. Calcineurin, the $Ca^{2+}$/calmodulin-dependent protein phosphatase, is essential in yeast mutants with cell integrity defects and in mutants that lack a functional vacuolar H(+)-ATPase. Mol Cell Biol 1995; 15:4103-14; PMID:7542741.
9. Nakamura T, Ohmoto T, Hirata D, Tsuchiya E, Miyakawa T. Genetic evidence for the functional redundancy of the calcineurin- and Mpk1-mediated pathways in the regulation of cellular events important for growth in *Saccharomyces cerevisiae*. Mol Gen Genet 1996; 251:211-9; PMID:8668132.
10. Hohmann S, Krantz M, Nordlander B. Yeast osmoregulation. Methods Enzymol 2007; 428:29-45; PMID:17875410; http://dx.doi.org/10.1016/S0076-6879(07)28002-4.

11. Shitamukai A, Hirata D, Sonobe S, Miyakawa T. Evidence for antagonistic regulation of cell growth by the calcineurin and high osmolarity glycerol pathways in *Saccharomyces cerevisiae*. J Biol Chem 2003; 279:3651-61; PMID:14583627; http://dx.doi.org/10.1074/jbc.M306098200.
12. Mizunuma M, Hirata D, Miyahara K, Tsuchiya E, Miyakawa T. Role of calcineurin and Mpk1 in regulating the onset of mitosis in budding yeast. Nature 1998; 392:303-6; PMID:9521328; http://dx.doi.org/10.1038/32695.
13. Clotet J, Escoté X, Adrover MA, Yaakov G, Garí E, Aldea M, et al. Phosphorylation of Hsl1 by Hog1 leads to a $G_2$ arrest essential for cell survival at high osmolarity. EMBO J 2006; 25:2338-46; PMID:16688223; http://dx.doi.org/10.1038/sj.emboj.7601095.
14. Asano S, Park JE, Sakchaisri K, Yu LR, Song S, Supavilai P, et al. Concerted mechanism of Swe1/Wee1 regulation by multiple kinases in budding yeast. EMBO J 2005; 24:2194-204; PMID:15920482; http://dx.doi.org/10.1038/sj.emboj.7600683.
15. Garcia K, Stumpff J, Duncan T, Su TT. Tyrosines in the kinesin-5 head domain are necessary for phosphorylation by Wee1 and for mitotic spindle integrity. Curr Biol 2009; 19:1670-6; PMID:19800237; http://dx.doi.org/10.1016/j.cub.2009.08.013.
16. Mollapour M, Tsutsumi S, Donnelly AC, Beebe K, Tokita MJ, Lee MJ, et al. Swe1$^{Wee1}$-dependent tyrosine phosphorylation of Hsp90 regulates distinct facets of chaperone function. Mol Cell 2010; 37:333-43; PMID:20159553; http://dx.doi.org/10.1016/j.molcel.2010.01.005.
17. Mollapour M, Tsutsumi S, Neckers L. Hsp90 phosphorylation, Wee1 and the cell cycle. Cell Cycle 2010; 9:2310-6; PMID:20519952; http://dx.doi.org/10.4161/cc.9.12.12054.
18. Aressy B, Jullien D, Cazales M, Marcellin M, Bugler B, Burlet-Schiltz O, et al. A screen for deubiquitinating enzymes involved in the $G_2$M checkpoint identifies USP50 as a regulator of HSP90-dependent Wee1 stability. Cell Cycle 2010; 9:3815-22; PMID:20930503; http://dx.doi.org/10.4161/cc.9.18.13133.
19. Chen Y, Feldman DE, Deng C, Brown JA, De Giacomo AF, Gaw AF, et al. Identification of mitogen-activated protein kinase signaling pathways that confer resistance to endoplasmic reticulum stress in *Saccharomyces cerevisiae.* Mol Cancer Res 2005; 3:669-77; PMID:16380504; http://dx.doi.org/10.1158/1541-7786.MCR-05-0181.
20. Locke EG, Bonilla M, Liang L, Takita Y, Cunningham KW. A homolog of voltage-gated Ca(2+) channels stimulated by depletion of secretory Ca(2+) in yeast. Mol Cell Biol 2000; 20:6686-94; PMID:10958666; http://dx.doi.org/10.1128/MCB.20.18.6686-94.2000.
21. Clotet J, Posas F. Control of cell cycle in response to osmostress: lessons from yeast. Methods Enzymol 2007; 428:63-76; PMID:17875412; http://dx.doi.org/10.1016/S0076-6879(07)28004-8.
22. Booher RN, Deshaies RJ, Kirschner MW. Properties of *Saccharomyces cerevisiae* wee1 and its differential regulation of p34$^{CDC28}$ in response to $G_1$ and $G_2$ cyclins. EMBO J 1993; 12:3417-26; PMID:8253069.
23. Bogdanova AI, Agaphonov MO, Ter-Avanesyan MD. Plasmid reorganization during integrative transformation in *Hansenula polymorpha.* Yeast 1995; 11:343-53; PMID:7785335; http://dx.doi.org/10.1002/yea.320110407.
24. Chechenova MB, Romanova NV, Deev AV, Packeiser AN, Smirnov VN, Agaphonov MO, et al. C-terminal truncation of alpha-COP affects functioning of secretory organelles and calcium homeostasis in *Hansenula polymorpha.* Eukaryot Cell 2004; 3:52-60; PMID:14871936; http://dx.doi.org/10.1128/EC.3.1.52-60.2004.
25. Agaphonov MO, Sokolov SS, Romanova NV, Sohn JH, Kim SY, Kalebina TS, et al. Mutation of the protein-O-mannosyltransferase enhances secretion of the human urokinase-type plasminogen activator in *Hansenula polymorpha.* Yeast 2005; 22:1037-47; PMID:16200504; http://dx.doi.org/10.1002/yea.1297.
26. Vieira J, Messing J. New pUC-derived cloning vectors with different selectable markers and DNA replication origins. Gene 1991; 100:189-94; PMID:1905257; http://dx.doi.org/10.1016/0378-1119(91)90365-I.
27. Agaphonov MO, Trushkina PM, Sohn JH, Choi ES, Rhee SK, Ter-Avanesyan MD. Vectors for rapid selection of integrants with different plasmid copy numbers in the yeast *Hansenula polymorpha* DL1. Yeast 1999; 15:541-51; PMID:10341417; http://dx.doi.org/10.1002/(SICI)1097-0061(199905)15:7<541::AID-YEA392>3.0.CO;2-G.
28. Sohn JH, Choi ES, Kim CH, Agaphonov MO, Ter-Avanesyan MD, Rhee JS, et al. A novel autonomously replicating sequence (ARS) for multiple integration in the yeast *Hansenula polymorpha* DL-1. J Bacteriol 1996; 178:4420-8; PMID:8755868.
29. Jones JS, Prakash L. Yeast *Saccharomyces cerevisiae* selectable markers in pUC18 polylinkers. Yeast 1990; 6:363-6; PMID:2220072; http://dx.doi.org/10.1002/yea.320060502.
30. Agaphonov M, Romanova N, Choi ES, Ter-Avanesyan M. A novel kanamycin/G418 resistance marker for direct selection of transformants in *Escherichia coli* and different yeast species. Yeast 2010; 27:189-95; PMID:20014045.
31. Myers AM, Tzagoloff A, Kinney DM, Lusty CJ. Yeast shuttle and integrative vectors with multiple cloning sites suitable for construction of *lacZ* fusions. Gene 1986; 45:299-310; PMID:3026915; http://dx.doi.org/10.1016/0378-1119(86)90028-4.
32. Casadaban MJ, Martinez-Arias A, Shapira SK, Chou J. Beta-galactosidase gene fusions for analyzing gene expression in *Escherichia coli* and yeast. Methods Enzymol 1983; 100:293-308; PMID:6312261; http://dx.doi.org/10.1016/0076-6879(83)00063-4.
33. Agaphonov MO, Romanova NV, Trushkina PM, Smirnov VN, Ter-Avanesyan MD. Aggregation and retention of human urokinase type plasminogen activator in the yeast endoplasmic reticulum. BMC Mol Biol 2002; 3:15; PMID:12366865; http://dx.doi.org/10.1186/1471-2199-3-15.
34. Agaphonov M, Romanova N, Sokolov S, Iline A, Kalebina T, Gellissen G, et al. Defect of vacuolar protein sorting stimulates proteolytic processing of human urokinase-type plasminogen activator in the yeast *Hansenula polymorpha.* FEMS Yeast Res 2005; 5:1029-35; PMID:16181812; http://dx.doi.org/10.1016/j.femsyr.2005.07.003.