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Huihui Xu, Malcolm Whiteway, Linghuo Jiang

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# The tricarboxylic acid cycle, cell wall integrity pathway, cytokinesis and intracellular pH homeostasis are involved in the sensitivity of *Candida albicans* cells to high levels of extracellular calcium

Huihui Xu[1,2], Malcolm Whiteway[3] and Linghuo Jiang[2*]

[1]School of Biotechnology, Jiangnan University, Wuxi 214122, China

[2]Laboratory for Yeast Molecular and Cell Biology, the Research Center of Fermentation Technology, School of Agricultural Engineering and Food Science, Shandong University of Technology, Zibo 255000, China;

[3]Department of Biology, Concordia University, Montreal, Quebec, Canada H4B 1R6

[*]To whom correspondence should be addressed. Tel/fax No. 011-86-531-8580-4371, Email: linghuojiang@sdut.edu.cn.

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**Abstract**

Through a genetic screen we have identified 21 genes whose inactivation renders *Candida albicans* cells sensitive to high levels of extracellular calcium. These genes are involved in the tricarboxylic acid cycle, cell wall integrity pathway, cytokinesis, intracellular pH homeostasis, magnesium transport, as well as DNA damage response and repair processes. The calcium sensitivity due to inactivation of nine of these genes can be partially or completely suppressed by cyclosporine A, an inhibitor of calcineurin. Therefore, the calcium sensitivity of nearly a half of these 21 mutations is at least partially due to the activation of calcium/calcineurin signaling. Our work provides a basis for further understanding the regulation of calcium homeostasis in this important human fungal pathogen.

Keywords: GRACE, calcium; screen, calcineurin; yeast

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**Introduction**

Calcium ions are essential for cells and regulate numerous cellular processes in both prokaryotes and eukaryotes, from bacteria to humans [Tang and Luan 2017; Bond et al. 2017; Espeso 2016; Plattner and Verkhratsky 2015; Medler 2010]. In eukaryotes cytosolic calcium homeostasis is maintained by $Ca^{2+}$ transporters and sequestrators in the plasma and organellar membranes. Regulation of calcium homeostasis and the calcium/calcineurin signaling pathway are highly conserved in eukaryotic cells. For example, functional counterparts of yeast calcium channels, pumps and exchangers exist and have similar roles in mammalian cells [Serra-Cardona et al. 2015; Cui et al. 2009a]. Under normal growth conditions, environmental $Ca^{2+}$ enters the *S. cerevisiae* cytosol through unknown transporters "X" and "M" [Cui et al. 2009b]. Transient increases in cytosolic $Ca^{2+}$ activate the calcium/calcineurin signaling pathway. Sustained $Ca^{2+}$ accumulation in the cytosol is harmful to cells, and this is prevented by a $Ca^{2}$ sequestration system composed of the calcium pump Pmc1 and the $Ca^{2+}/H^{+}$ exchanger Vcx1 of the vacuole as well as the calcium pump Pmr1 and $Ca^{2+}/H^{+}$ exchanger Gdt1 of the ER/Golgi secretory pathway [Colinet et al. 2017; Cyert and Philpott 2013; Cyert 2003]. The calcium/calcineurin signaling functions in ion homeostasis, cell wall biogenesis and morphogenesis, and is highly conserved in human fungal pathogens including *Candida albicans* [Thewes 2014; Gow et al. 2011]. Our previous studies have demonstrated that the plasma membrane protein Rch1 is a novel negative regulator of calcium uptake in both *S. cerevisiae* and *C. albicans* [Zhao et al. 2016; Alber et al. 2013; Jiang et al. 2012].

In recent years, with the emergence of drug-resistant fungal species and increases in immunocompromised patients due to organ transplantation, AIDS and cancer therapy, human fungal infections have significantly increased [Liu et al. 2015; Yu et al. 2015]. *C. albicans* remains as one of leading causes of systemic fungal infection in immunocompromised patients [Dadar et al. 2018; Gow et al. 2011; Chauhan et al. 2006]. Many functional counterparts of calcium homeostasis and calcium/calcineurin signaling components have been identified and characterized in *C. albicans* [Wang et

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al. 2018; Liu et al. 2015; Wang et al. 2015; Thewes 2014]. Calcineurin is an attractive antifungal drug target, and its inhibitors (FK506 or cyclosporin A) can be combined with azoles or echinocandins for use against multidrug-resistant *Candida* species [Yu et al. 2015]. Therefore, inhibition of calcineurin is considered as a new therapeutic strategy for combating the calcineurin-mediated drug resistance of *C. albicans* [Li et al. 2015; Steinbach et al. 2007]. However, calcineurin regulates virulence via distinct mechanisms between *C. albicans* and another human pathogenic fungus *Cryptococcus neoformans* [Kraus and Heitman 2003]. Therefore, elucidation of various components in the calcium/calcineurin signaling pathway in *C. albicans* is important to fully exploit potential new targets for development of new antifungal drugs against candidasis.

The GRACE (gene replacement and conditional expression) library of conditional mutants with one allele of a target gene deleted and the other placed under the control of a tetracycline-repressive promoter, provides a functional genomics tool that was successfully used to assess gene essentiality in *C. albicans* [Roemer et al. 2003]. This library has been used for mechanism-of-action studies of various compounds against *C. albicans* as well as for studies on the role of essential genes with respect to pathogen virulence in a murine model of candidiasis [Wilson et al. 2014; Becker et al. 2010; Xu et al. 2007]. In addition, a modified GRACE library (version 1) has been used to screen for genes involved in white-opaque switching that identified *OFR1* as a key regulator for maintaining the cryptic mating phenotype in *C. albicans* [Sun et al. 2016], and has also been used to study the interactions between genes and antifungal drugs [Chen et al. 2018]. To further understand the effects of calcium on *C. albicans* cells, we have screened the GRACE library of 2358 conditional mutants and identified a total of 21 genes whose conditional repression leads to the sensitivity of *C. albicans* cells to high levels of extracellular calcium.

**Materials and Methods**

*Strains and Media*

The wild type *C. albicans* strain (CaSS1) and the GRACE library of conditional

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mutants used in this study were described previously [Roemer *et al.* 2003]. Yeast cells were grown and maintained at 30℃in YPD medium (1% yeast extract, 2% peptone, 2% glucose) or SD medium (0.67% yeast nitrogen base without amino acids, 2% glucose, and auxotrophic amino acids as needed). Chemicals were purchased from Sangon Biotech (Shanghai, China) except that 5-fluoro-orotic acid (5-FOA) and cyclosporine A (CsA) were purchased from Sigma (Beijing, China).

***Genetic screen for calcium-sensitive mutations***

The GRACE library of 2358 conditional mutants was used for primary screens for calcium-sensitive strains. We replicated this mutant collection onto YPD plates supplemented with 100 μg/ml tetracycline alone or together with 0.4 M $CaCl_2$. The plates were incubated at 30◦C for 2–3 days. A mutant with a relative colony size reduced by more than 30% as compared to the average size of its surrounding mutants on YPD plates supplemented with 100 μg/ml tetracycline and 0.4 M $CaCl_2$ but not on YPD plates containing 100 μg/ml tetracycline alone, was identified as a calcium-sensitive mutant (Supplementary Figure 1). This primary screen was repeated two times. Sensitive mutants were subjected to a secondary screen by a serial dilution assay method as described (Zhang et al., 2013; Zhao et al., 2010; Ruan *et al*. 2007). In brief, they were individually streaked from the original collection onto YPD plates. They were then inoculated into liquid YPD medium for growth overnight to stationary phase, serially diluted by 10 fold increments and spotted onto YPD plates with or without supplemented tetracycline as well as YPD plates supplemented with both tetracycline and $CaCl_2$ to retest their sensitivity to calcium in comparison to their wild-type strain CaSS1. The secondary screen was repeated three times. Functional categories of relevant genes were identified using the Munich Information Center for Protein Sequences (MIPS) analysis (https://www.helmholtz-muenchen.de/ibis/).

***Phenotype suppression assay for calcium-sensitive mutants***

The suppressive effects of cyclosporine A (CsA) on the phenotypes of calcium-sensitive mutants were assayed by adding 50 μg/ml CsA into YPD plates

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with or without $CaCl_2$. The growth of calcium-sensitive mutants was examined on these plates by the serial dilution assay method as described above.

***Construction of unconditional mutants***

To obtain unconditional mutants for the calcium-sensitive GRACE conditional mutants identified above, we grew them at 30 °C in liquid YPD overnight. Overnight cultures were diluted by 100 times and spread on synthetic complete (SC) plates supplemented with 0.1% 5-FOA to counter select *URA3*. After incubation at 30°C 2-5 days, individual *URA3*$^-$ colonies were purified on SC plates with 5-FOA, and the *URA3*$^-$ phenotypes were confirmed by streaking them on SD-URA and YPD medium, respectively. Colonies growing on YPD plate but not on SD-URA plate are *URA3*$^-$ mutants that have also lost their transactivator cassette [Roemer *et al.* 2003], which leads to the promoter inactivation of the allele with the tetracycline-repressive promoter. Like mutants in the modified GRACE library (version 1) used in previous studies [Chen et al. 2018; Sun et al. 2016], these unconditional *URA3*$^-$ mutants harbor one allele of a target gene deleted and the other with an inactivated promoter independent of tetracycline. A control *URA3*$^-$ wild type CaSS1 strain was constructed similarly.

**Results**

***Screen for calcium sensitivity of conditional GRACE mutants in the presence of tetracycline***

To further explore the effects of calcium on *C. albicans* cells, we screened the GRACE library of 2358 conditional mutants for calcium sensitivity in the presence of tetracycline. As compared to their growth on YPD plates, GRACE mutants for 10 genes, *BIG1*, *ORF19.4341*, *ORF19.5053*, *CKS1*, *ALR1*, *SWC4, ORF19.3242*, *ORF19.4312*, *RAD53* and *SMC6*, showed growth defect on YPD plates with tetracycline (Figure 1). Nevertheless, these 10 GRACE mutants showed further reduced growth on YPD plates containing tetracycline and 0.2M or 0.4M $CaCl_2$ as

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compared to their growth on YPD with tetracycline alone (Figure 1), indicating they are calcium-sensitive in the presence of tetracycline. In contrast, GRACE mutants for 13 genes, *ACO1*, *IDH1*, *MIT1*, *CRZ1*, *CDC55*, *HOF1*, *HSL1*, *MOB2*, *RIM21*, *CKS1*, *RCH1*, *ALR1* and *ORF19.7511*, showed no growth defect on YPD plates with tetracycline, but they were sensitive to 0.2M or 0.4M $CaCl_2$ in the presence of tetracycline as compared to their growth on YPD with tetracycline alone (Figure 1). Furthermore, these GRACE mutants were not sensitive to 1M sorbitol, a hyperosmolar solution, in the presence of tetracycline (data not shown), suggesting they were specifically sensitive to calcium ions but not to high osmolarity derived from the high concentration of calcium ions. Three of our currently identified genes, *CRZ1*, *MIT1* and *RCH1,* have been reported to confer calcium sensitivity in previous studies [Karababac et al. 2006; Mille et al. 2004; Jiang et al. 2012].

Functional classification showed that these 21 genes can be categorized into the following groups: metabolism (6), cell cycle and DNA processing (5), transcription (3), protein fate (folding, modification, destination) (1), regulation of metabolism and protein function (2), cellular transport, transport facilities and transport (3), and unknown functions (1) [Table 1].

***Suppression of calcium sensitivity of GRACE mutants by the calcineurin inhibitor cyclosporine A***

Calcium sensitivity of these 21 mutants could potentially be caused by activation of the calcium/calcineurin signaling pathway in *C. albicans* cells [Jiang et al. 2012]. We therefore asked whether their calcium sensitivity could be suppressed by cyclosporine A (CsA), a specific inhibitor of calcineurin. The calcium sensitivity of nine GRACE mutant strains of *IDH1*, *RIM21*, *CKS1*, *RCH1*, *CRZ1*, *CDC55*, *HOF1*, *HSL1* and *MOB2* was partially or completely suppressed by CsA, but that for other GRACE mutants was not [Figure 1]. Consistent with previous studies [Jiang et al. 2012], the calcium sensitivity of the deletion mutant of *RCH1* was also suppressed by CsA. In contrast, the GRACE mutants for *BIG1*, *MIT1*, *ORF19.4341*, *RAD53*,

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*ORF19.3242* and *SMC6* became more sensitive to calcium in the presence of CsA [Figure 1], indicating an additive effect between calcium and CsA in inhibiting their growth.

To confirm the phenotypes of GRACE mutants described above, we successfully constructed ten unconditional mutants, *CRZ1*, *IDH1*, *HSL1*, *RCH1*, *RIM21*, *MIT1*, *CKS1*, *MOB2*, *CDC55* and *ACO1*, that lacked the transactivator for the Tet promoter that controls the expression of the remaining ORF of these target genes, and therefore both of their alleles were not functional even in the absence of tetracycline [Chen et al. 2018; Sun et al. 2016; Roemer *et al.* 2003]. These unconditional mutants showed similar calcium-sensitive phenotypes to their corresponding GRACE mutants [Figure 2A]. The calcium sensitivity of eight unconditional mutants for *CRZ1*, *IDH1*, *HSL1*, *RCH1*, *RIM21*, *CKS1*, *MOB2* and *CDC55* was partially or completely suppressed by CsA, but that of unconditional mutants for *MIT1* and *ACO1* was not [Figure 2B]. This is consistent with what we have observed for the equivalent GRACE mutants in the presence of tetracycline [Figure 1].

**Discussion**

To discover more players in the regulation of calcium homeostasis in *C. albicans* through a genetic approach, we have screened a conditional inactivation allele library to identify 21 genes whose inactivation leads to *C. albicans* sensitivity to high levels of extracellular calcium. Except for *CRZ1*, *MIT1* and *RCH1*, the other 19 genes identified in this screen have not been previously reported to be related to calcium sensitivity of *C. albicans*. Eight of the genes identified in this study are involved in metabolism and its regulation (Table 1). *ACO1* and *IDH1* encode an aconitase and a subunit of the NAD (+)-dependent isocitrate dehydrogenase complex, respectively, both of which are components of the tricarboxylic acid (TCA) cycle [Chen et al. 2005; Cupp and McAlister-Henn 1992]. Mutations in the human functional homolog *ACO2* of yeast *ACO1* are responsible for non-syndromic autosomal recessive optic neuropathies [Metodiev et al. 2014]. Interestingly, both mutations of the human *IDH1*

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gene and overexpression of the *ATP2A2* gene encoding the sarcoplasmic reticulum $Ca^{2+}$-ATPase isoform 2 (SERCA2), a human homolog of yeast Pmr1, have been detected in secondary glioblastoma [Li et al. 2017], which suggests a potential link between the TCA cycle and calcium homeostasis in this human genetic disease. This is further supported by the observation that expression of *ACO1* is affected by the treatment of various antifungal drugs in *C. albicans* [Liu et al. 2005], since it is well-known that the calcium/calcineurin signaling is related to the tolerance of *C. albicans* cells to antifungal drugs [Jiang et al. 2012; Karababa et al. 2006; Sanglard et al. 2003; Cruz et al. 2002].

*BIG1* encodes an integral membrane protein of the endoplasmic reticulum and is required for beta-1,6-glucan synthesis, filamentation, adhesion and virulence in *S. cerevisiae* and *C. albicans* [Umeyama et al. 2006; Azuma et al. 2002]. As the homolog of the *S. cerevisiae SUR1* gene, *C. albicans MIT1* encodes a synthase for one of membrane sphingolipids, mannose-inositol-phosphoceramide, and its inactivation leads to calcium sensitivity and reduced virulence in a systemic mouse model [Mille et al. 2004]. These results support previous observations that cell wall integrity is related to the regulation of calcium homeostasis in *C. albicans* [Jiang et al. 2018a and 2018b; Bates et al. 2005].

*CDC55* encodes the regulatory subunit B of protein phosphatase 2A, which positively regulates mitotic entry at the G2/M phase transition and negatively regulates mitotic exit [Baro et al. 2013]. Hof1 regulates actin cytoskeleton organization and is required for cytokinesis, actin cable organization, and secretory vesicle trafficking [Graziano et al. 2014]. Hsl1 is probably a protein kinase that colocalizes with a septin complex to the bud neck during yeast growth or to a potential septation site during hyphal growth, functions to determine morphology during the cell cycle of both yeast-form and hyphal cells and is required for full virulence and kidney colonization in mouse systemic infection in *C. albicans* [Umeyama et al. 2005; Wightman et al. 2004]. As well, *CKS1* encodes a regulatory subunit and adaptor for the cyclin-dependent protein kinase Cdc28 (Cdk1) and is required for G1/S and G2/M phase transitions and budding in *S. cerevisiae*

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[Koivomagi et al. 2011]. Furthermore, the DNA damage response protein kinase Rad53 is required for cell-cycle arrest in *S. cerevisiae* and involved in regulation of DNA-damage-induced filamentous growth in *C. albicans* [Hoch et al. 2013; Shi et al. 2007]. Taken together, these data indicate that calcium ions might play a role in the regulation of cytokinesis, which is supported by our recent observation on the dynamic movement of Rch1, the novel negative regulator of calcium uptake, in the plasma membrane during cytokinesis in *S. cerevisiae* [Zhao et al. 2016; Jiang et al. 2012].

*RIM21* encodes a plasma membrane pH-sensor protein, a component of the RIM101 pathway; and functions in cell wall construction and alkaline pH response in *S. cerevisiae* [Obara et al. 2012]. In our previous study we have demonstrated that a downstream target of Rim101 negatively regulates the expression of *PMR1* [Zhao et al. 2013b], which links the Rim101 pathway to the regulation of calcium homeostasis in *S. cerevisiae*. Our observation in this study that inactivation of *RIM21* leads to calcium sensitivity of *C. albicans* cells suggests that a similar link between the Rim101 pathway and calcium homeostasis might exist in *C. albicans*. Mucosal layers of the oral cavity with a pH of 7.0 and the vaginal cavity with a pH of 4.0 are two human sites colonized and infected by *C. albicans*. A previous study has shown that ambient pH strongly affects the wall proteome of *C. albicans* [Sosinska et al. 2011]. Future work on the elucidation of Rim21 functions in *C. albicans* could reveal the detailed link between pH regulation and calcium homeostasis in this human fungal pathogen.

*SWC4* encodes a subunit of either the NuA4 histone acetyltransferase complex or the Swr1p complex that incorporates Htz1p into chromatin [Zhou et al. 2010]. *SMC6* encodes a component of the SMC5-SMC6 complex; which plays a key role in the removal of X-shaped DNA structures that arise between sister chromatids during DNA replication and repair in *S. cerevisiae* [Bermudez-Lopez et al. 2010]. *ALR1* encodes the plasma membrane $Mg^{2+}$ transporter in *S. cerevisiae* [Graschopf et al. 2001]. Magnesium ions could suppress the calcium sensitivity of a large amount of yeast deletion mutants [Zhao et al. 2013a]. Therefore, it is not surprising to observe

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that *C. albicans* cells lacking *ALR1* confers calcium-sensitivity.

In conclusion, through a genetic approach we have identified that, in addition to the well-studied calcium/calcineurin signaling pathway, the tricarboxylic acid cycle, the cell wall integrity pathway, cytokinesis and intracellular pH homeostasis are involved in the sensitivity of *C. albicans* cells to high levels of extracellular calcium. Our work provides a basis for further understanding the regulation of calcium homeostasis in *C. albicans*, which would help discover potential targets for development of new antifungal drugs against this important human fungal pathogen.

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**Figure Legends**

**Figure 1. Phenotypes of conditional GRACE mutants of genes in each functional category.** Cells of the wild-type CaSS1 and 27 GRACE mutants identified from the GRACE library screen were grown at 30$^{o}$C in liquid YPD overnight, serially diluted by 10 times and spotted on YPD plates with or without supplemented reagents as indicated, respectively. Mutants were arranged into groups according to their gene categories, and top and bottom panels of each category represent plates without and with 50 μg/ml CsA supplemented, respectively. Mutants showing growth defect on YPD plate in the presence of tetracycline were indicated with their names underlined. Plates were incubated for 2-5 days at 30$^{o}$C. CsA, cyclosporine A.

**Figure 2. Phenotypes of unconditional GRACE mutants.** Cells of the wild-type CaSS1 and 10 GRACE mutants without a *URA3* marker were grown at 30$^{o}$C in liquid YPD overnight, serially diluted by 10 times and spotted on YPD plates with or without $CaCl_2$ in the absence (**A**) or presence (**B**) of CsA. Plates were incubated for 2-5 days at 30$^{o}$C. CsA, cyclosporine A.

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**Table 1 List of 21 genes whose GRACE mutants are sensitive to $CaCl_2$ in the presence of tetracycline**

| Systemic name | Standard name | Systemic name | Standard name | Systemic name | Standard name |
|---|---|---|---|---|---|
| **Metabolism (6)** | | | | | |
| CR_08210C_A | *ACO1* | C1_10830W_A | *BIG1* | C2_09150W_A | *MIT1* |
| C1_09630W_A | *IDH1* | C1_07830C_A | *orf19.5053* | C5_03120W_A | *orf19.4341* |
| **Cell cycle and DNA processing (5)** | | | | | |
| C2_01600C_A | *CDC55* | CR_00400C_A | *SWC4* | C4_00370W_A | *HOF1* |
| C5_02840C_A | *HSL1* | C1_00620W_A | *MOB2* | | |
| **Transcription (3)** | | | | | |
| C3_05780C_A | *CRZ1* | C5_02900W_A | *orf19.4312* | CR_01120C_A | *orf19.3242* |
| **Protein fate (folding, modification, destination) (1)** | | | | | |
| C5_01950C_A | *RIM21* | | | | |
| **Regulation of metabolism and protein function (2)** | | | | | |
| C5_04070C_A | *CKS1* | C3_03810W_A | *RAD53* | | |
| **Cellular transport, transport facilities and transport (3)** | | | | | |
| C3_02370C_A | *ALR1* | CR_00230W_A | *orf19.7511** | C4_00360C_A | *RCH1* |
| **Unknown (1)** | | | | | |
| C7_01580W_A | *SMC6* | | | | |

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Highlights

- A genetic screen identified 21 calcium-sensitive gene mutations
- Calcium sensitivity of 9 gene mutations can be suppressed by cyclosporine A
- Tricarboxylic acid cycle is involved in the calcium sensitivity of *Candida albicans*
- Cytokinesis and pH homeostasis are involved in the calcium sensitivity

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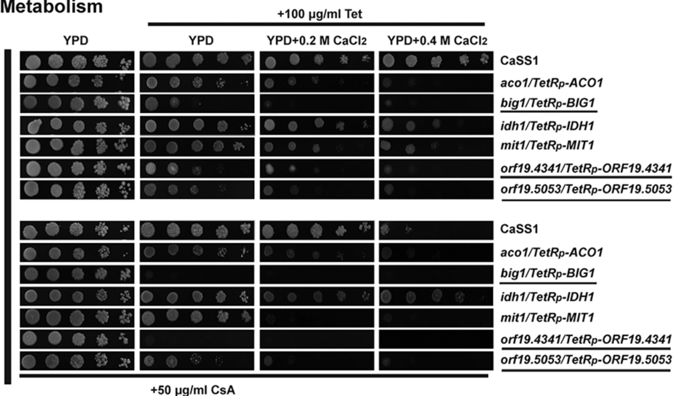


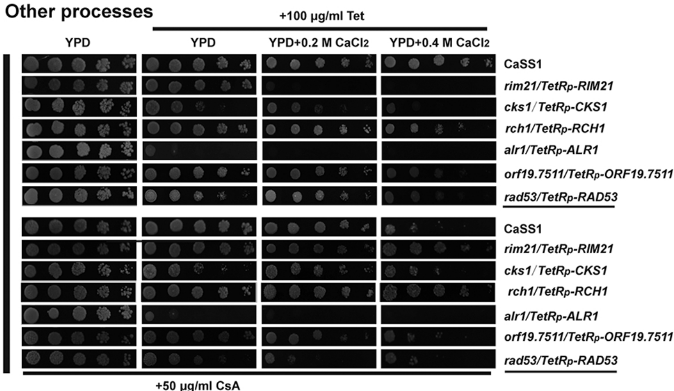


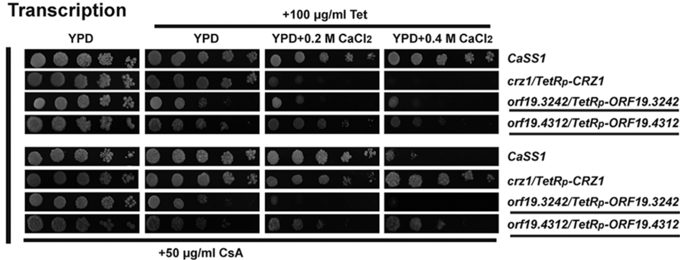


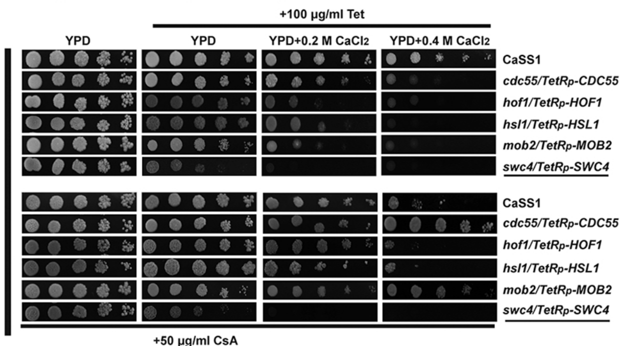


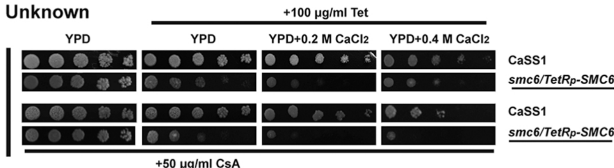


Figure 1

**A**

YPD | YPD+0.2 M $CaCl_2$ | YPD+0.4 M $CaCl_2$

CaSS1
*crz1/TetRp-CRZ1*
*idh1/TetRp-IDH1*
*hsl1/TetRp-HSL1*
*rch1/TetRp-RCH1*
*rim21/TetRp-RIM21*
*mit1/TetRp-MIT1*
*cks1/TetRp-CKS1*
*mob2/TetRp-MOB21*
*cdc55/TetRp-CDC55*
*aco1/TetRp-ACO1*

**B**

YPD | YPD+0.2 M $CaCl_2$ | YPD+0.4 M $CaCl_2$

CaSS1
*crz1/TetRp-CRZ1*
*idh1/TetRp-IDH1*
*hsl1/TetRp-HSL1*
*rch1/TetRp-RCH1*
*rim21/TetRp-RIM21*
*mit1/TetRp-MIT1*
*cks1/TetRp-CKS1*
*mob2/TetRp-MOB21*
*cdc55/TetRp-CDC55*
*aco1/TetRp-ACO1*

+50 μg/mL CsA

Figure 2