Conserved and Diverged Functions of the Calcineurin-Activated Prz1 Transcription Factor in Fission Yeast

Kate Chatfield-Reed, Lianne Vachon, Eun-Joo Gina Kwon, and Gordon Chua[1]
Department of Biological Sciences, University of Calgary, Calgary, Alberta T2N 1N4, Canada

ABSTRACT Gene regulation in response to intracellular calcium is mediated by the calcineurin-activated transcription factor Prz1 in the fission yeast *Schizosaccharomyces pombe*. Genome-wide studies of the Crz1 and CrzA fungal orthologs have uncovered numerous target genes involved in conserved and species-specific cellular processes. In contrast, very few target genes of Prz1 have been published. This article identifies an extensive list of genes using transcriptome and ChIP-chip analyses under inducing conditions of Prz1, including $CaCl_2$ and tunicamycin treatment, as well as a *Δpmr1* genetic background. We identified 165 upregulated putative target genes of Prz1 in which the majority contained a calcium-dependent response element in their promoters, similar to that of the *Saccharomyces cerevisiae* ortholog Crz1. These genes were functionally enriched for Crz1-conserved processes such as cell-wall biosynthesis. Overexpression of *prz1*$^+$ increased resistance to the cell-wall degradation enzyme zymolyase, likely from upregulation of the *O*-mannosyltransferase encoding gene *omh1*$^+$. Loss of *omh1*$^+$ abrogates this phenotype. We uncovered a novel inhibitory role in flocculation for Prz1. Loss of *prz1*$^+$ resulted in constitutive flocculation and upregulation of genes encoding the flocculins Gsf2 and Pfl3, as well as the transcription factor Cbf12. The constitutive flocculation of the *Δprz1* strain was abrogated by the loss of *gsf2*$^+$ or *cbf12*$^+$. This study reveals that Prz1 functions as a positive and negative transcriptional regulator of genes involved in cell-wall biosynthesis and flocculation, respectively. Moreover, comparison of target genes between Crz1/CrzA and Prz1 indicate some conservation in DNA-binding specificity, but also substantial rewiring of the calcineurin-mediated transcriptional regulatory network.

KEYWORDS fission yeast; calcium; transcription factor; cell wall; flocculation

CALCINEURIN is a highly conserved phosphatase central to Ca^{2+} signaling. In metazoans, calcineurin regulates a wide array of Ca^{2+}-dependent processes including T-cell activation (Clipstone and Crabtree 1992), cardiac hypertrophy (Molkentin *et al.* 1998), neutrophil motility (Hendey *et al.* 1992), apoptosis (Wang *et al.* 1999), angiogenesis (Graef *et al.* 2001), and memory development (Mansuy *et al.* 1998). One of the primary effectors of calcineurin is the NFAT family of transcription factors, which translocates into the nucleus to regulate target genes when dephosphorylated (reviewed in Macian 2005). In fungi, the activity of the Crz1 C_2H_2 zinc-finger transcription factor is modulated by calcineurin in a similar way (reviewed in Thewes 2014). Crz1 orthologs have been identified in various fungal species and their function appears conserved in cell-wall-related processes and resistance to external stressors (Thewes 2014).

The *Saccharomyces cerevisiae* Crz1 is localized in the cytosol under optimal growth conditions, but is activated and rapidly translocated into the nucleus through dephosphorylation by calcineurin in response to exogenous Ca^{2+} (Stathopoulos-Gerontides *et al.* 1999). In addition to exogenous Ca^{2+}, Crz1 is activated by numerous external stresses including high salt, prolonged exposure to α-factor, alkaline pH, antifungal compounds, blue light, nutrient deprivation, heavy metals, and ethanol (Matheos *et al.* 1997; Stathopoulos and Cyert 1997; Edlind *et al.* 2002; Serrano *et al.* 2002; Zakrzewska *et al.* 2005; Zhang and Rao 2007; Ruiz *et al.* 2008; Araki *et al.* 2009; Ferreira *et al.* 2012; Bodvard *et al.* 2013). Transcriptome profiling of *CRZ1* was initially performed with Ca^{2+} or Na^+ treatment that identified

doi: 10.1534/genetics.115.184218
Manuscript received October 29, 2015; accepted for publication February 10, 2016; published Early Online February 19, 2016.
Available freely online through the author-supported open access option.
Supplemental material is available online at www.genetics.org/lookup/suppl/doi:10.1534/genetics.115.184218/-/DC1.
[1]Corresponding author: University of Calgary, 2500 University Dr. N.W., Calgary, AB T2N 1N4, Canada. E-mail: gchua@ucalgary.ca

163 target genes (Yoshimoto *et al.* 2002), and this list has been expanded subsequently with similar profiling under alkaline stress, nutrient deprivation, and transcription factor overexpression (Viladevall *et al.* 2004; Chua *et al.* 2006; Ruiz *et al.* 2008). The Crz1 target genes are known to function in ion homeostasis, small-molecule transport, cell-wall maintenance, lipid and sterol metabolism, and vesicle transport. Many of these target genes contain the calcineurin-dependent response element (CDRE) motif [5′-GNGGC(G/T)CA-3′] in their promoter (Yoshimoto *et al.* 2002). Crz1 binds this motif, which was originally discovered in the promoter of *FKS2*, and is sufficient to drive the transcriptional activation of a reporter gene (Stathopoulos and Cyert 1997; Yoshimoto *et al.* 2002).

In *Schizosaccharomyces pombe*, the Ppb1 calcineurin catalytic subunit dephosphorylates the transcription factor Prz1 in response to elevated Ca^{2+} levels (Hirayama *et al.* 2003). Similar to other Crz1 orthologs, dephosphorylation of Prz1 causes nuclear translocation and transcriptional regulation of its target genes through binding of a CDRE-like motif (5′-AGCCTC-3′) (Deng *et al.* 2006) or a Ca^{2+}-dependent response element (5′-CAACT-3′) (Hamasaki-Katagiri and Ames 2010). Loss of *prz1*$^+$ produces a normal phenotype under optimal growth conditions, but results in hypersensitivity to Ca^{2+} and reduced mating efficiency (Hirayama *et al.* 2003; Sun *et al.* 2013). In contrast, the calcineurin Δ*ppb1* strain exhibits a more severe phenotype with additional defects in cytokinesis, cell polarity, and chloride hypersensitivity (Yoshida *et al.* 1994; Hirayama *et al.* 2003). These defects are not suppressed by *prz1*$^+$overexpression, indicating that Prz1 is not the sole target of calcineurin (Hirayama *et al.* 2003). In addition to Ca^{2+}, activation of Prz1 occurs upon exposure to NaCl, DTT and tunicamycin (ER stressors), micafungin (a β-glucanase inhibitor), and heat shock, when assayed by a CDRE-regulated reporter, nuclear translocation, or *prz1*$^+$ messenger RNA levels (Hirayama *et al.* 2003; Deng *et al.* 2006). In addition, *prz1*$^+$ overexpression activates the CDRE-regulated reporter, thus indicating positive autoregulation (Koike *et al.* 2012). The response to diverse external stimuli indicates that Prz1 must regulate genes involved in multiple cellular processes as observed in other fungal orthologs. However, the current target gene list of Prz1 is far from complete. Only five target genes have been identified: *pmc1*$^+$ (Hirayama *et al.* 2003), *pmr1*$^+$ (Maeda *et al.* 2004), *ncs1*$^+$ (Hamasaki-Katagiri and Ames 2010), *cmk1*$^+$ (Cisneros-Barroso *et al.* 2014), and *prz1*$^+$ (Deng *et al.* 2006). This is in contrast to other fungal Crz1 orthologs that have more extensive target gene lists, ranging from dozens up to several hundred genes, functioning in cellular processes such as cell-wall biosynthesis, ion transport, lipid metabolism, and vesicle transport (Yoshimoto *et al.* 2002; Karababa *et al.* 2006; Hagiwara *et al.* 2008; Chen *et al.* 2012; Soriani *et al.* 2008).

Here, we substantially expand the number of putative target genes for Prz1 by transcriptome and ChIP-chip profiling and uncover novel biological roles in reproduction, cell-wall structure, and flocculation. We discovered that the DNA-binding specificity of the calcineurin-responsive transcription factors is conserved between budding and fission yeasts, but considerable differences exist among orthologous target genes. The role in cell-wall biosynthesis is conserved between Prz1 and its orthologs, and several putative target genes for this role were identified. Finally, we show that Prz1 directly represses target genes implicated in flocculation.

Materials and Methods

Yeast strains, media, and general methods

Supplemental Material Table S1, contains a list of yeast strains used in this study. Strains were grown in yeast extract with supplements (YES) or EMM and supplemented with adenine (225 mg/liter), leucine (225 mg/liter), uracil (225 mg/liter), thiamine (15 μM), geneticin (150 mg/liter), and nourseothricin (100 mg/liter) when required. Calcium chloride and tunicamycin (T7765: Sigma Aldrich, St. Louis) were added to YES medium at 0.15 M and 2.5 μg/ml, respectively. Cell-wall sensitivity was assayed with 0.5 μg/ml micafungin (A13270-1: AdooQ Bioscience, Irvine, CA) and 25 U/ml Zymolyase 100T (E1005: Zymo Research, Irvine, CA). Deletion and epitope-tagged strains were constructed by a PCR-based stitching method as described in Kwon *et al.* (2012). All constructed strains were verified by colony PCR and sequencing of the amplicons. For deletion strains, the entire open reading frame (ORF) was replaced with the KanMX6 or NatMX4 cassettes, while for endogenously tagged *prz1* strains, GFP and HA epitopes were PCR-amplified from pYM27 and pYM14 plasmids, respectively (Janke *et al.* 2004) and inserted in-frame at the C-terminal end of the *prz1*$^+$ ORF. Functionality of the *prz1-HA* and *prz1-GFP* strains was determined by comparing their growth to wild type on 0.15 M $CaCl_2$-containing medium. Overexpression of *prz1*$^+$ with the *nmt1* or *nmt41* promoter was accomplished by cloning the ORF into the *pREP1/pREP2* and *pREP41* vectors, respectively. Standard genetic and molecular methods were performed as described in Moreno *et al.* (1991).

Microarray expression profiling

Wild-type and Δ*prz1* cultures were concurrently grown in 100 ml liquid YES at 30° for 16–20 hr to a matching cell density of ~8 × 10^6 cells/ml before harvesting. Calcium chloride and tunicamycin treatment were 0.15 M for 0.5 hr and 2.5 μg/ml for 1.5 hr, respectively, prior to harvesting. The Δ*pmr1* and Δ*prz1* cultures were also grown concurrently in 100 ml liquid YES at 30° for 16–20 hr to a matching cell density of ~8 × 10^6 cells/ml. For *prz1*$^+$ overexpression, cultures of the *prz1OE* strain and empty vector control were concurrently grown in 100 ml of EMM (supplemented with adenine and uracil) without thiamine at 30° for 18–22 hr to ~8 × 10^6 cells/ml and then harvested. The same procedure was used in the heterologous overexpression of *S. cerevisiae CRZ1* with the *nmt1* or *nmt41* promoter in the Δ*prz1* strain. Sample preparation for hybridization to 8 × 15,000 Agilent *S. pombe* expression microarrays and scanning were carried

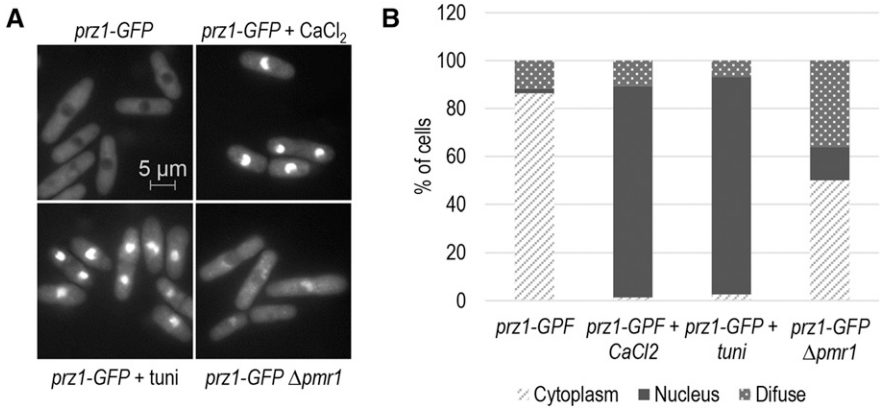

Figure 1 Intracellular localization of endogenously controlled Prz1-GFP. (A) Wild-type cells expressing endogenously controlled Prz1-GFP were exponentially grown in YES medium (top left) and treated with 0.15 M $CaCl_2$ (top right) or 2.5 μg/ml tunicamycin (bottom left) for 0.5 and 1.5 hr, respectively. The intracellular localization of endogenously controlled Prz1-GFP in *Δpmr1* cells grown in rich medium (bottom right). (B) Bar graph showing the percentage of cells in each category of Prz1-GFP localization from A. The data are from three replicates of ~100 cells each.

out as described in detail in Kwon *et al.* (2012). All microarray experiments were performed with a single dye-swap and normalized using the R Limma package with Lowess scaling (Smyth and Speed 2003), and the eBayes method was used to combine the replicates by fitting to a linear model (Smyth 2004). Hierarchical clustering was performed using the uncentered Pearson correlation with Cluster 3.0 (Eisen *et al.* 1999), and the tree image was generated using Java Treeview (Saldanha 2004).

ChIP-chip profiling

The endogenous C-terminal-tagged *prz1-HA* strain was grown in 200 ml liquid YES medium at 30° for 16–20 hr and treated with calcium chloride or tunicamycin as described above. A detailed description in sample preparation for hybridization to 4 × 44,000 Agilent *S. pombe* Genome ChIP-on-chip microarrays is found in Kwon *et al.* (2012). All ChIP-chip experiments were performed with dye swaps for two biological replicates. The data were normalized using the median correction method, and replicates were combined with eBayes from the R limma package (Smyth and Speed 2003; Smyth 2004). Significant peaks were identified using chIPOTle (Buck *et al.* 2005). To detect potential promoter occupancy, the peak was considered only when within 1000 bp (the average length of the sonicated DNA fragments) of the promoter region, defined as 0–1500 bp upstream of the start codon.

Motif and functional enrichment analyses

Motif searching for the DNA-binding specificity of Prz1 was carried out in MEME using promoter sequences consisting of 1000 bp upstream of the start codon (Bailey and Elkan 1994). The maximum size of the motif was set at 10 bp and at zero or one motif per promoter sequence. Functional enrichment was performed with the Princeton GO term finder (Boyle *et al.* 2004).

Crz1 target genes

A list of Crz1 target genes in *S. cerevisiae* was assembled from a literature review of genome-wide, as well as smaller scale, studies (Matheos *et al.* 1997; Yoshimoto *et al.* 2002; Chua *et al.* 2006; Fardeau *et al.* 2007; Hu *et al.* 2007; Cai *et al.* 2008; Ruiz *et al.* 2008). In most cases, the Crz1 target genes identified as significant were used. For the Chua *et al.* (2006) study, no specific cutoff was defined, and a $\log_{10}$-fold change of 3 was selected. The Crz1/CrzA target genes used for *Candida albicans*, *Candida glabrata*, *Aspergillus nidulans*, and *Aspergillus fumigatus* were from individual studies of each organism that performed transcriptome analysis using microarray experiments (Karababa *et al.* 2006; Hagiwara *et al.* 2008; Soriani *et al.* 2008; Chen *et al.* 2012).

The list of *S. pombe* genes with orthologs in *S. cerevisiae* was obtained from the V2.18 ortholog list (Wood *et al.* 2012). The orthologs in the other species were determined using Inparanoid data sets (Sonnhammer and Östlund 2015) and from the Fungal Orthogroups Repository (Wapinski *et al.* 2007), except for *A. fumigatus*, which relied solely on Inparanoid data.

Cell-wall degradation assays

Strains were grown with their respective controls as described for the expression microarray experiments. Wild-type and Δ*prz1* strains were grown in liquid YES medium, while genetic backgrounds containing *nmt1*-driven *prz1*$^+$ or empty vector were grown in liquid EMM minus thiamine for 18–24 hr. The cells were washed twice with 0.9% saline solution and resuspended in TE buffer at ~1.2×10^7 cells/ml. Three milliliters of cell suspension was transferred to test tubes in the presence and absence of 25 U/ml Zymolyase 100T (Zymo Research) and shaken at 37°. OD_{600} readings were taken every 15 min to assess the degree of cell-wall degradation. The significance of the different treatments was assessed at the 2-hr time point with an ANOVA followed by a two-tailed *t*-test.

Flocculation assays

Constitutive flocculation was assessed by inoculating cells in liquid YES at an initial cell density of ~10^7 cells/ml and growing at 30° in a shaking incubator. After 24 hr, 10 ml of culture was transferred to a 90-mm plastic petri dish and rotated slowly on an orbital low-speed shaker (Labnet International, Woodridge, NJ) for 10 min at room temperature. Images of flocs were acquired with a SPImager (S&P Robotics Inc., Toronto).

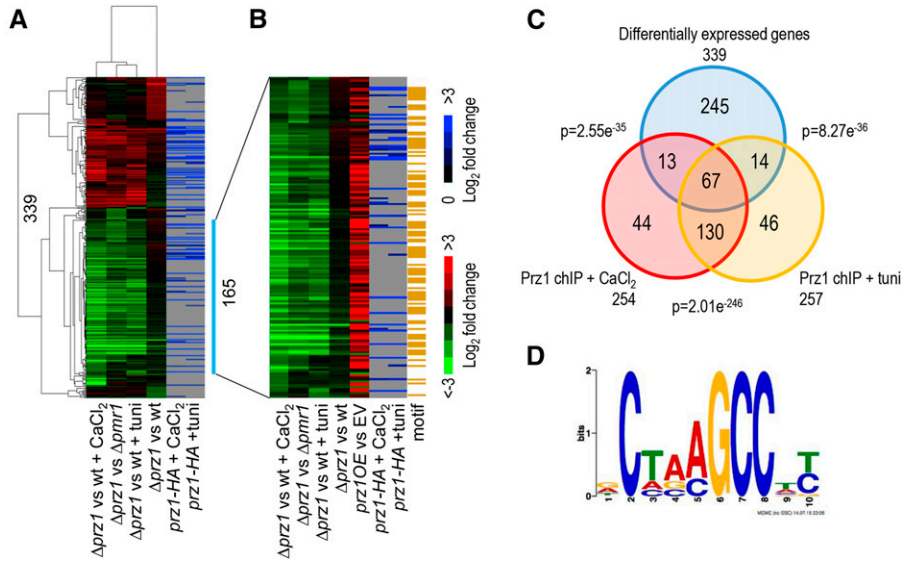

Figure 2 Identification of Prz1 target genes by transcriptome and ChIP-chip profiling. (A) The heat map shows two-dimensional hierarchical clustering of 339 genes that were differentially expressed by at least twofold in at least one of the microarray experiments. The first four columns of the heat map compare transcriptomes of the following conditions: the *Δprz1* strain and wild type, the *Δprz1* strain and wild type supplemented with 0.15 M $CaCl_2$ for 0.5 hr, the *Δprz1* strain and wild type supplemented with 2.5 μg/ml tunicamycin for 1.5 hr, and the *Δprz1* strain compared to the *Δpmr1* strain. All of the above experiments were performed in rich medium. In the heat map, genes upregulated and downregulated in the *Δprz1* strain relative to the control are indicated in red and green, respectively. The two rightmost columns in the heat map show ChIP-chip analysis of a *prz1-HA* strain treated with 0.15 M $CaCl_2$ or 2.5 μg/ml tunicamycin for 0.5 and 1.5 hr, respectively. (B) The heat map shows the expression profiles of 165 putative target genes that are positively regulated by Prz1. The first four columns of the heat map match the expression data from A while the fifth column shows the expression profiles of the same target genes upregulated in a *prz1*$^+$ overexpression strain compared to the empty vector (EV) control. The next two columns in the heat map show ChIP-chip analysis of a *prz1-HA* strain treated with 0.15 M $CaCl_2$ or 2.5 μg/ml tunicamycin for 0.5 and 1.5 hr, respectively. The rightmost column of the heat map shows the 91 genes containing the CDRE motif within their promoter in orange. The color bars indicate relative expression and ChIP enrichment ratios between experimental and control strains. All microarray expression and ChIP-chip experiments were performed in replicate with dye reversal. (C) The Venn diagram shows the overlap between the 339 differentially expressed genes in the transcriptome experiments and the genes identified from the ChIP-chip analysis with Prz1 promoter occupancy in the presence of $CaCl_2$ or tunicamycin. The significance of the overlap is indicated as *P*-values that were determined using a hypergeometric distribution. (D) A DNA motif generated by MEME from promoter analysis of the 165 putative target genes of Prz1. This motif is similar to the CDRE motif (5′-AGCCTC-3′) previously discovered in Deng *et al.* (2006).

Fluorescence microscopy

The intracellular localization of natively regulated Prz1-GFP was determined in the *prz1-GFP* strain treated with $CaCl_2$ or tunicamycin and in the *Δpmr1 prz1-GFP* strain. Cells were grown and treated as described for the expression microarray experiments. Images of live *prz1-GFP* cells were captured with a Zeiss Imager Z1 microscope and AxioCam MRM digital camera (Zeiss, Thornwood, NY). The proportion of cells containing Prz1-GFP predominantly in the cytoplasm, nucleus, or both compartments was determined manually in ~300 cells from three biological replicates.

Data availability

The microarray expression data have been submitted to the National Center for Biotechnology Information (NCBI) Gene Expression Omnibus Database (GSE77761). The ChIP-chip data also have been submitted to the NCBI Gene Expression Omnibus Database (GSE77761).

Results

Chemical and genetic activation of Prz1

Identification of target genes using genome-wide approaches requires that the transcription factor be in an active state. Dephosphorylation of Prz1 by calcineurin results in nuclear translocation and regulation of its target genes. Therefore, we first determined whether several types of chemical treatment and a change in genetic background resulted in activation of Prz1 by promoting its translocation into the nucleus. Several studies have examined the intracellular localization of GFP-tagged Prz1, but expression of the fusion protein was controlled by the *nmt1* promoter. The elevated expression in this strain could potentially increase the nuclear localization of Prz1 through mass action. As a result, we constructed a strain expressing a Prz1-GFP fusion protein under control of the native promoter to examine its nuclear localization in response to $CaCl_2$ and tunicamycin treatment, as well as deletion of *pmr1*$^+$. The *prz1-GFP* strain exhibited sensitivity to $CaCl_2$ comparable to wild type, indicating that the fusion protein was functional. When cells were grown in rich medium, Prz1-GFP was primarily localized in the cytoplasm and not in the nucleus in the majority (>85%) of cells (Figure 1, A and B). Only ~2% of these cells exhibited predominantly nuclear localization. In contrast, Prz1-GFP exhibited mostly nuclear localization (~90%) when exposed to 0.15 M $CaCl_2$ and 2.5 μg/ml tunicamycin for 0.5 and 1.5 hr, respectively (Figure 1, A and B). These results indicate that Prz1 is activated when treated with $CaCl_2$ and tunicamycin, which is in agreement with previous studies (Hirayama *et al.* 2003; Deng *et al.* 2006). We also investigated the intracellular localization of Prz1-GFP when *pmr1*$^+$, which encodes a Golgi Ca^{2+}/Mn^{2+} ATPase, is deleted. Loss of *pmr1*$^+$ is synthetic lethal with the Δ*prz1* strain (Ryan *et al.* 2012), suggesting that Prz1 function is required in the Δ*pmr1* background. Moreover, calcium homeostasis may be disrupted in the

Table 1 Gene ontology enrichment among the 165 target genes positively regulated by Prz1 using Princeton GO Term Finder

Gene ontology term	*P*-value	Gene list
Reproduction (GO:0000003)	$5.1e^{-4}$	*cdc1*$^+$, *dic1*$^+$, ***gpa1***$^+$, *gwt1*$^+$, *isp3*$^+$, *krp1*$^+$, *mam2*$^+$, *map1*$^+$, ***matPc***$^+$, *mcp2*$^+$, ***mcp5***$^+$, *mde6*$^+$, *mei2*$^+$, ***meu13***$^+$, *meu17*$^+$, ***meu22***$^+$, ***meu27***$^+$, *mfm2*$^+$, *mst2*$^+$, ***mug108***$^+$, ***mug133***$^+$, *mug136*$^+$, *mug63*$^+$, *mug8*$^+$, ***ncs1***$^+$, *pmp31*$^+$, ***ppk35***$^+$, *rec10*$^+$, ***scd2***$^+$, *set3*$^+$, **SPAPB1A10.08**, *ste11*$^+$, ***ste4***$^+$, *bgs1*$^+$, *cfr1*$^+$, ***pvg5***$^+$
Cell-wall organization or biogenesis (GO:0071554)	$4.9e^{-3}$	*bgs1*$^+$, *cfr1*$^+$, ***pvg5***$^+$, ***cfh2***$^+$, *gas2*$^+$, ***gmh2***$^+$, ***omh1***$^+$, *pun1*$^+$, ***pvg1***$^+$, ***rga5***$^+$, **SPAC13C5.05c**, **SPAC9G1.10c**, SPBC1198.07c, **SPBC19C7.05**, SPBC21B10.07
Membrane (GO:0016020)	$2.18e^{-6}$	***gda1***$^+$, *sen54*$^+$, SPAC977.02, ***mug133***$^+$, *ima1*$^+$, *pet2*$^+$, SPBC21B10.07, ***git3***$^+$, SPBC1271.03c, ***ppk35***$^+$, ***ncs1***$^+$, *pun1*$^+$, **SPAC23C11.06c**, **SPCPB1C11.02**, *gas2*$^+$, *krp1*$^+$, **SPAC14C4.07**, ***dnf1***$^+$, SPBC15C4.06c, *pmp31*$^+$, *imt2*$^+$, *mfm2*$^+$, *cki2*$^+$, ***frp1***$^+$, **SPAC18B11.03c**, SPCC1529.01, ***ost2***$^+$, *mam2*$^+$, ***omh1***$^+$, *fur4*$^+$, **SPAC212.01c**, *bgs1*$^+$, ***isp5***$^+$, *ggc1*$^+$, ***cfh2***$^+$, *rsn1*$^+$, *psd2*$^+$, **SPAC630.04c**, ***erg1***$^+$, **SPBC1348.03**, ***meu22***$^+$, *tco1*$^+$, ***bst1***$^+$, SPAC869.03c, *meu17*$^+$, **SPAC5D6.04**, **SPAC750.04c**, ***ppr3***$^+$, **SPAC1687.08**, **SPBC19C7.05**, ***mac1***$^+$, *gwt1*$^+$, ***yip5***$^+$, SPBC1198.07c, ***itr2***$^+$, ***gga21***$^+$, *pet1*$^+$, SPCC553.12c, *lcb4*$^+$, ***pvg1***$^+$, **SPAC869.05c**, SPCC794.03, **SPAC4C5.03**, *str1*$^+$, ***tvp15***$^+$, ***gmh2***$^+$, ***pmr1***$^+$, **SPCC4B3.02c**, *mfs1*$^+$, SPAC23D3.12, ***gpa1***$^+$, ***imt1***$^+$, ***pvg5***$^+$
Golgi apparatus (GO:005794)	$1.08e^{-5}$	*fmd2*$^+$, ***gda1***$^+$, *tco1*$^+$, ***bst1***$^+$, *cfr1*$^+$, ***mug133***$^+$, **SPAC869.05c**, *pet2*$^+$, SPAC869.03c, *meu17*$^+$, **SPAC3A11.10c**, ***omh1***$^+$, ***tvp15***$^+$, *fur4*$^+$, **SPBC19C7.05**, ***gmh2***$^+$, *pun1*$^+$, ***yip5***$^+$, **SPCC4B3.02c**, ***gga21***$^+$, SPAC23D3.12, *krp1*$^+$, *pet1*$^+$, **SPAC14C4.07**, ***dnf1***$^+$, *lcb4*$^+$, ***imt1***$^+$, *mug136*$^+$, ***pvg5***$^+$, ***pvg1***$^+$, *imt2*$^+$

The target genes that have the CDRE motif in their promoter (Figure 2D) are indicated in boldface type and those with an ortholog that is regulated by Crz1 in *S. cerevisiae* are underlined. Only the genes with enriched gene ontology terms are shown.

Δ*pmr1* strain (Maeda *et al.* 2004), which could result in activation of Prz1. The frequency of nuclear localization of Prz1-GFP in Δ*pmr1* cells (~14%) was greater than unperturbed wild-type cells (Figure 1, A and B). Interestingly, a substantial proportion of Δ*pmr1* cells (~38%) displayed levels of Prz1-GFP present in both the nucleus and cytoplasm. These results suggest a robust activation of Prz1 in response to $CaCl_2$ or tunicamycin treatments and intermediate activation in Δ*pmr1* cells compared to untreated wild type.

Identification of Prz1 target genes by genome-wide analyses

There are currently only a handful of known direct target genes of Prz1. To expand the list of Prz1 target genes, we performed transcriptome profiling and ChIP-chip analysis under the inducing conditions determined by our Prz1-GFP intracellular localization studies. Transcriptomes were compared between the *Δprz1* mutant with wild type exposed to 0.15 M $CaCl_2$ or 2.5 μg/ml tunicamycin, as well as between the untreated *Δprz1* and the *Δpmr1* strains (Table S2). As a control, transcriptome profiling was performed on the *Δprz1* and wild-type strains grown in rich medium (Table S2). We identified 339 genes that were differentially regulated by more than twofold with a $P < 0.001$ in at least one of the four expression microarray experiments (Figure 2A). Lower expression levels in the Δ*prz1* strain, relative to the wild-type or *Δpmr1* strain, represented positively regulated target genes of Prz1, while higher expression indicated negatively regulated targets. $CaCl_2$ treatment resulted in the most differentially expressed genes (150 lower and 67 higher in Δ*prz1*), followed by the Δ*pmr1* strain (109 lower and 67 higher in Δ*prz1*) and the tunicamycin treatment (94 lower and 51 higher in Δ*prz1*) (Figure 2A). In contrast, only 17 and 51 genes had lower and higher expression, respectively, in the *Δprz1* strain relative to wild type. This is consistent with previous observations that transcriptome profiling of most transcription-factor deletion strains does not uncover many direct target genes in *S. pombe* under optimal growth conditions (Chua 2013; Vachon *et al.* 2013). Hierarchical clustering revealed that the tunicamycin treatment and the *Δpmr1* strain expression profiles were the most similar (Figure 2A), which is in agreement with the observations that both tunicamycin and *pmr1*$^+$ are involved in ER stress (Dürr *et al.* 1998; Deng *et al.* 2006) and therefore may activate Prz1 in a similar way.

ChIP-chip profiling of a natively regulated Prz1-HA strain was performed to further confirm that the differentially expressed genes retrieved from the transcriptome studies are putative target genes of Prz1. These ChIP-chip experiments were carried out under inducing conditions of Prz1 in the presence of $CaCl_2$ or tunicamycin with the same dosages as the transcriptome studies. We found that Prz1 bound to the promoters of 254 and 257 genes during $CaCl_2$ and tunicamycin treatments, respectively, and the overlap of genes (197 genes or ~77%) between both treatments was substantial (Figure 2, B and C). Moreover, we detected Prz1 occupancy in its own promoter in response to both $CaCl_2$ and tunicamycin treatments (Table S3 and Table S4), which is in agreement with other studies (Deng *et al.* 2006). For the $CaCl_2$ treatment, 80 of the 254 promoter-bound genes (31.4%) were differentially expressed in the *Δprz1* strain ($P = 2.55e^{-35}$ using a hypergeometric distribution) (Figure 2, B and C). In response to tunicamycin treatment, 81 of the 257 promoter-bound genes (31.5%) were differentially expressed in the *Δprz1* strain ($P = 8.27e^{-36}$) (Figure 2, B and C). Overall, 94 of the 339 genes (27.7%) differentially expressed in the transcriptome experiments were bound in

Table 2 Gene ontology enrichment among the 92 target genes negatively regulated by Prz1 using Princeton GO Term Finder

Gene ontology term	*P*-value	Gene list
Small-molecule catabolic process (GO:0044282)	$3.0e^{-4}$	SPAC2F3.05c, *car1*$^+$, SPAC139.05, SPAC4H3.08, *gut2*$^+$, *gpd3*$^+$, *atd1*$^+$, *tms1*$^+$, SPCC1223.09, *eno102*$^+$
Ion transmembrane transport (GO:0034220)	$4.7e^{-4}$	*gti1*$^+$, *tgp1*$^+$, *pho1*$^+$, *mfs3*$^+$, *mug86*$^+$, SPCPB1C11.03, SPBPB2B2.01, SPBC36.02c, SPBC1683.01, *pho84*$^+$, *mae1*$^+$, SPBPB10D8.01, SPBC3H7.02, *pgt1*$^+$, SPCC794.04c

The genes that have an ortholog regulated by Crz1 in *S. cerevisiae* are underlined. Only the genes with enriched gene ontology terms are shown.

one of the ChIP-chip experiments (Figure 2, B and C). This amount of overlap between transcriptome and ChIP-chip analyses of Prz1 is comparable to a similar study on the fission yeast CSL transcription factors Cbf11 and Cbf12 (Převorovský *et al.* 2015).

Among the 339 differentially regulated genes, 165 genes were consistently lower in the *Δprz1* strain relative to wild type when treated with $CaCl_2$ or tunicamycin, indicating that Prz1 may positively regulate these target genes (Figure 2B). We next subjected these 165 putative target genes of Prz1 to gene ontology analysis using the Princeton GO Term Finder. The gene products were enriched for biological process categories such as reproduction ($P = 5.1e^{-4}$), cell-wall organization, or biogenesis ($P = 4.9e^{-3}$), as well as components of membranes ($P = 2.2e^{-6}$) and the Golgi apparatus ($P = 1.1e^{-5}$) (Table 1). Moreover, the vast majority of these target genes were also upregulated in the *prz1*$^+$ overexpression strain, and Prz1 was found to be associated with the promoters of 37 of these genes ($P = 2.3e^{-12}$) (Figure 2B). All the known target genes of Prz1 (*prz1*$^+$, *pmc1*$^+$, *pmr1*$^+$, *ncs1*$^+$, and *cmk1*$^+$) were also found within these 165 genes (Table 1). Strikingly, promoter analysis by MEME revealed a common motif in 91 of the 165 putative Prz1 target genes that closely resembled the CDRE sequence 5′-AGCCTC-3′ (Deng *et al.* 2006) (Figure 2D and Table S5). Altogether, these results indicate that the majority of these 165 genes are likely direct targets that are positively regulated by Prz1.

Interestingly, 92 genes were upregulated at least twofold in the *Δprz1* strain during $CaCl_2$ or tunicamycin treatment (Figure S1), indicating that Prz1 may also negatively regulate a different set of target genes. In addition, 46 of these genes were downregulated in the *prz1*$^+$ overexpression strain, and Prz1 was associated with the promoters of 40 of the 92 putative target genes ($P = 5.2e^{-25}$) (Figure S1). However, unlike the positively regulated genes, no common binding motif was detected by MEME. Gene ontology analysis of these 92 genes detected functional enrichment in ion transmembrane transport ($P = 4.7e^{-4}$) and small molecule catabolic processes ($P = 3.0e^{-4}$) (Table 2 and Table S6). Direct transcriptional repression of target genes has not been detected for Prz1 or confirmed in fungal Crz1 orthologs (Thewes 2014).

Orthologous target genes between S. pombe Prz1 and other fungal Crz1/CrzA

The known target genes of Prz1 are well conserved in *S. cerevisiae*. All of the known Prz1 target genes (*prz1*$^+$, *pmc1*$^+$, *pmr1*$^+$, *ncs1*$^+$, and *cmk1*$^+$) have budding yeast orthologs regulated by Crz1 with the exception of *ncs1*$^+$ (Hamasaki-Katagiri and Ames 2010). However, the proportion of the remaining Prz1 putative target genes that have Crz1 target gene orthologs was considerably lower. There were 24 Crz1 target gene orthologs among the 165 putative target genes upregulated by Prz1 (Table 1 and Table S5) (Matheos *et al.* 1997; Yoshimoto *et al.* 2002; Chua *et al.* 2006; Fardeau *et al.* 2007; Hu *et al.* 2007; Cai *et al.* 2008; Ruiz *et al.* 2008). The conservation of target genes is highest for the conserved biological process of cell-wall organization or biogenesis in which 40% of the Prz1 putative target genes annotated in this gene ontology category have Crz1 target gene orthologs (Table 1). Interestingly, there were eight putative target genes (*atd1*$^+$, *sua1*$^+$, *bfr1*$^+$, SPBC1683.01, *pho84*$^+$, *fhn1*$^+$, *plb1*$^+$, and SPAC513.07) seemingly repressed by Prz1 that had orthologs that were positively regulated by Crz1 (Yoshimoto *et al.* 2002; Chua *et al.* 2006; Ruiz *et al.* 2008). This phenomenon is further supported by the observation that the promoters of seven of these eight genes were bound by Prz1 in one or both of the ChIP-chip experiments (Table S6).

The number of orthologous target genes decreases with more distantly related fungi (Figure S2). Comparison with a study in *C. albicans* found that, of the 65 Crz1 targets, only 5 overlap with the 165 positively regulated targets and 2 overlap with the negatively regulated target genes in *S. pombe* (Karababa *et al.* 2006). A similar overlap was also observed between the 34 target genes of *C. glabrata* Crz1 (Chen *et al.* 2012). CrzA target genes in *A. nidulans* and *A. fumigatus* showed even less overlap with the *S. pombe* Prz1 target genes (Hagiwara *et al.* 2008; Soriani *et al.* 2008). Cell-wall organization is positively regulated by Prz1, as well as its orthologs in fungi, which would indicate conservation among the target genes implicated in that process. However, this conservation was not as extensive as expected. Only two Prz1 target genes with cell-wall functions (SPBC21B10.07 and *bgs1*$^+$) share orthologs in *S. cerevisiae* and *C. albicans*. Other cell-wall genes like *gas2*$^+$ and SPAC9G1.10c were conserved in the more distantly related species *C. glabrata* and *A. fumigatus*, respectively, while not sharing an ortholog in *S. cerevisiae*.

The difference in the number of orthologs suggests an increasingly distant relationship between Prz1 and its Crz1/CrzA orthologs over evolutionary time. However, it may reflect incomplete data because, unlike *S. cerevisiae*, the other targets are each determined from a single study

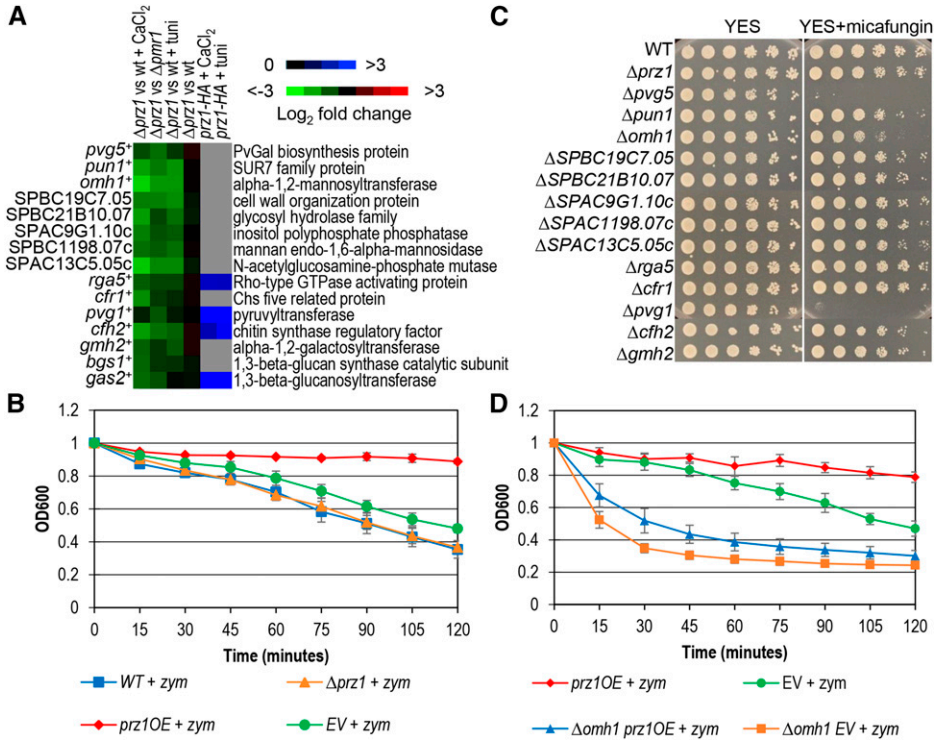

Figure 3 Characterization of putative target genes of Prz1 implicated in cell-wall-related processes. (A) The heat map shows relative expression and Prz1 promoter occupancy for 15 putative target genes annotated to function in cell-wall organization or biogenesis. The color bars indicate relative expression and ChIP enrichment ratios between experimental and control strains. All microarray expression and ChIP-chip experiments were performed in replicate with dye reversal. (B) Cell-wall degradation assays. Wild-type and Δ*prz1* strains were grown in liquid YES medium, while *nmt1*-driven *prz1*$^+$ or empty vector (EV) were grown in liquid EMM minus thiamine for 18–24 hr. The samples were adjusted to matching cell densities and transferred to test tubes in the presence of 25 U/ml Zymolyase 100T. The samples were shaken at 37°, and OD_{600} readings were taken every 15 min to assess the degree of cell-wall degradation. Overexpression of *prz1*$^+$ caused resistance to the cell-wall-degrading enzyme zymolyase ($P = 1.0e^{-4}$) while Δ*prz1* cells did not show significant sensitivity to zymolyase compared to wild type. (C) Spot dilution for micafungin sensitivity of deletion strains of the putative Prz1 target genes involved in cell-wall-related processes. Exponentially growing wild-type and deletion strains were pinned on solid YES medium containing 0.5 μg/ml micafungin and incubated at 30° for 3 days. (D) Cell-wall degradation assays. The Δ*omh1* strain was more sensitive to zymolyase treatment than wild type ($P = 1.6e^{-2}$). The zymolyase-resistant phenotype from overexpression of *prz1*$^+$ was abrogated by loss of *omh1*$^+$ ($P = 5.0e^{-4}$). The zymolyase experiments were repeated in triplicate, and error bars represent the standard error. The *P*-values were determined with ANOVA followed by a two-tailed *t*-test after 2 hr of zymolyase treatment.

(Karababa *et al.* 2006; Hagiwara *et al.* 2008; Soriani *et al.* 2008; Chen *et al.* 2012). The curation of the ortholog lists may also play a role in the overlap observed. The ortholog list between *S. cerevisiae* and *S. pombe* has been extensively refined (Wood *et al.* 2012), whereas with the other species orthology was determined using Inparanoid data sets (Sonnhammer and Östlund 2015) and the Fungal Orthogroups Repository (Wapinski *et al.* 2007). Although the overlap with these other species offers an interesting look at conserved elements, it is clearly an incomplete picture.

Prz1 activates target genes functioning in cell-wall synthesis and structure

The activation of Prz1 upregulates 15 putative target genes implicated in the biosynthesis and structure of the cell wall (Figure 3A; Table 1). Among these 15 genes, 9 contained a CDRE motif in the promoter while Prz1 occupancy was detected in 4 (Figure 3A; Table 1; Table S5). To further investigate this, we first determined whether Δ*prz1* and *prz1OE* strains possessed an altered cell-wall structure. The Δ*prz1* and *prz1OE* strains were tested for resistance to cell-wall degradation by zymolyase (a β-glucanase) relative to their controls. The short duration of the zymolyase assay was ideal because of the reduced fitness exhibited by the *prz1OE* strain (Koike *et al.* 2012; Vachon *et al.* 2013). We found that overexpression of *prz1*$^+$ with the *nmt1* promoter confers increased resistance to zymolyase ($P = 1.0e^{-4}$), suggesting that the upregulation of these target genes could enhance the strength of the cell wall (Figure 3B). In contrast, no change in resistance to zymolyase was observed in the Δ*prz1* strain compared to wild type (Figure 3B). We next attempted to identify the putative target genes that could be responsible for the increased resistance to zymolyase in the *prz1OE* strain. Loss of these target genes could potentially result in sensitivity to cell-wall-perturbing agents. Thirteen deletion strains of the Prz1 putative target genes implicated in cell-wall processes were assayed for growth sensitivity to the antifungal micafungin, which inhibits β-1,3-glucan production. The remaining two genes, *gas2*$^+$ and *bgs1*$^+$, the latter being an essential gene, were not available in the Bioneer deletion collection. Among the 13 deletion strains, only Δ*pvg1*, Δ*pvg5*, and Δ*omh1* were sensitive to micafungin (Figure 3C). Both *pvg1*$^+$ and *pvg5*$^+$ function in the synthesis of pyruvated galactose residues in N-linked glycans (Andreishcheva *et al.* 2004; Yoritsune *et al.* 2013), and *omh1*$^+$ encodes a putative α1,2-mannosyltransferase for synthesis of *O*-linked glycans (Ikeda *et al.* 2009). The Δ*prz1* strain, in addition to not being sensitive to zymolyase, was not sensitive to micafungin (Figure 3C). The lack of sensitivity is perhaps due to redundancy in the regulation of these cell-wall target genes by a transcription factor other than Prz1. We then overexpressed *prz1*$^+$ with the *nmt1* promoter in the Δ*pvg1*, Δ*pvg5*, and Δ*omh1* strains to determine if the zymolyase resistance exhibited in the *prz1OE* strain was affected by loss of these genes. Consistent

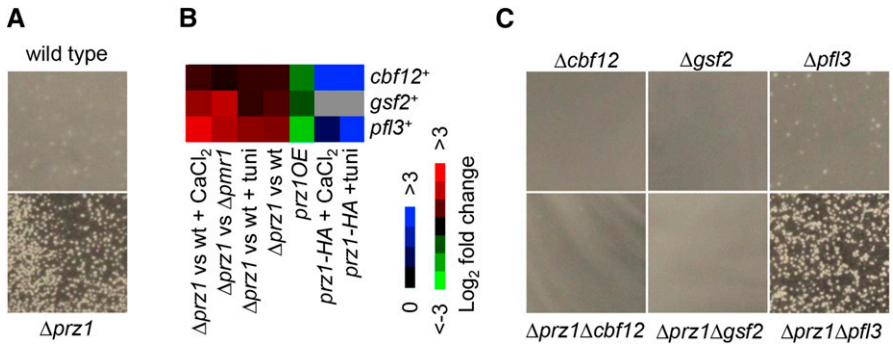

Figure 4 Constitutive flocculation of the *Δprz1* strain. (A) Wild type and the *Δprz1* strain were grown at an initial cell density of ~10^7 cells/ml in liquid YES medium for 24 hr at 30° and assayed for flocculation. (B) Negative regulation of flocculation genes by Prz1. The heat map shows relative expression and Prz1 promoter occupancy for the flocculation genes *cbf12*$^+$, *gsf2*$^+$, and *pfl3*$^+$. The color bars indicate relative expression and ChIP enrichment ratios between experimental and control strains. All microarray expression and ChIP-chip experiments were performed in replicate with dye reversal. (C) The *Δprz1* flocculation phenotype was abolished by loss of *cbf12*$^+$ or *gsf2*$^+$. Cells were assayed for flocculation as described for A.

with the micafungin assay, the *Δomh1* strain was more susceptible to degradation by zymolyase than the control after 2 hr of treatment ($P = 1.2e^{-2}$), while the *Δpvg1* and *Δpvg5* strains did not show a significant increase in sensitivity (Figure 3D and Figure S3). Indeed, loss of *omh1*$^+$ almost completely abrogated the zymolyase resistance caused by *prz1*$^+$ overexpression ($P = 5.0e^{-4}$). A similar genetic interaction was also observed in the *Δpvg1* background except the degree of abrogation was less ($P = 6.8e^{-3}$) (Figure S3). Together, these results indicate that the cell-wall function of Prz1 involves activation of its target genes *omh1*$^+$ and *pvg1*$^+$.

Prz1 repression of flocculation

The *Δprz1* strain exhibited a slightly crumbly texture on solid media that is often present in flocculent strains. To determine if *Δprz1* cells are flocculent, we cultured the strain in liquid YES and EMM with an initial density of ~10^7 cells/ml for 24 hr. The Δ*prz1* cells, but not wild type, formed large flocs in both EMM and YES media (Figure 4A). In contrast, overexpression of *prz1*$^+$ did not suppress the flocculation of wild-type cells in flocculation-inducing medium (data not shown). We next examined whether putative target genes in our genome-wide data could be responsible for the constitutive flocculent phenotype of *Δprz1* cells. Interestingly, several genes implicated in triggering flocculation were upregulated in the *Δprz1* strain. These upregulated genes (1.6- to 5.3-fold) encoded the flocculins Gsf2 and Pfl3 and the transcription factor Cbf12, which is known to activate *gsf2*$^+$ (Převorovský *et al.* 2015; Kwon *et al.* 2012) (Figure 4B). In addition, these three genes were downregulated in the *prz1OE* strain, and the promoters of *pfl3*$^+$ and *cbf12*$^+$ were bound by Prz1 in both $CaCl_2$ and tunicamycin treatments (Figure 4B). These results suggest that Prz1 inhibits flocculation by repression of *gsf2*$^+$, *pfl3*$^+$, or *cbf12*$^+$. To further confirm this hypothesis, we examined whether the constitutive flocculent phenotype of the *Δprz1* strain in YES medium could be abrogated in a Δ*gsf2*, Δ*pfl3*, or *Δcbf12* genetic background. Indeed, we discovered that loss of *gsf2*$^+$ or *cbf12*$^+$, but not *pfl3*$^+$, could abrogate the constitutive flocculation observed in *Δprz1* cells (Figure 4C).

Discussion

In this study, we substantially expanded the target gene list and implicated new functional roles for the calcineurin-responsive transcription factor Prz1 in *S. pombe*. Moreover, we presented genetic evidence that links putative target genes to these new functions of Prz1. Our results also demonstrate that identification of target genes by genome-wide approaches requires that the transcription factor be active. Transcriptome profiling of the Δ*prz1* strain grown in rich medium did not identify many direct target genes of Prz1. We used several inducing conditions that promote Prz1 activity, including chemicals (Ca^{2+} and tunicamycin), overexpression of *prz1*$^+$, and the Δ*pmr1* genetic background. Both Ca^{2+} and tunicamycin and have been shown to activate a CDRE-regulated reporter, and Ca^{2+} has also been shown to cause Prz1 to translocate to the nucleus (Hirayama *et al.* 2003; Deng *et al.* 2006). Systematic overexpression of yeast transcription factors, combined with transcriptome profiling, has been used effectively to identify direct target genes (Chua *et al.* 2006; Vachon *et al.* 2013; Chua 2013). The *prz1OE* strain displays reduced fitness, which is common for transcription factors overexpressed in fission yeast (Vachon *et al.* 2013). This reduced fitness is likely caused by increased promoter occupancy and aberrant expression of target genes. When overexpressed, Prz1 is still largely localized in the cytoplasm (Hirayama *et al.* 2003). Despite this, Prz1 overproduction allows some of the transcription factors to override the normal cytoplasmic retention signals and enter the nucleus, resulting in differential expression in a large number of putative target genes (893 upregulated and 532 downregulated by more than twofold).

In addition, Prz1 activation was observed in the Δ*pmr1* genetic background. The *pmr1*$^+$ gene encodes a Golgi P-type Ca^{2+}/Mn^{2+}-ATPase that functions to reduce abnormally high levels of cytosolic Ca^{2+} (Cortés *et al.* 2004). Therefore, loss of *pmr1*$^+$ is expected to cause elevated cytosolic Ca^{2+} relative to wild type and result in the downstream activation of Prz1. Our results show that nuclear localization of Prz1-GFP was increased in Δ*pmr1* cells compared to unperturbed wild-type cells (Figure 1). Although the Prz1-GFP nuclear localization

in Δ*pmr1* cells was less than wild-type cells treated with Ca^{2+} or tunicamycin, the putative target genes of Prz1 could still be recovered by comparing the transcriptome profiles of Δ*pmr1* and Δ*prz1* strains (Figure 2). Interestingly, synthetic lethality is observed in the Δ*pmr1* Δ*prz1* double mutant (Ryan *et al.* 2012), indicating that Prz1 activity is required in the Δ*pmr1* strain. This observation is intriguing as it suggests that synthetic-lethal interactions could be used to identify genetic backgrounds that contain transcription factors in their active state (genetic activation). Subsequently, the comparison of transcriptomes between the transcription factor deletion strain and a deletion strain with which it shares a synthetic-lethal interaction could conceivably identify direct target genes. We are currently exploring whether this approach is indeed effective in identifying target genes of transcription factors in *S. pombe*.

The annotated functions of the positively regulated 165 putative target genes of Prz1 align well with certain functions of the fungal Crz1 orthologs: ion homeostasis and transport, lipid metabolism, and cell-wall biosynthesis. In *S. pombe*, functional enrichment was detected only for genes implicated in reproduction and cell-wall organization or biogenesis. There were 15 putative target genes of Prz1 that have a role in cell-wall processes, 6 of which have orthologs in *S. cerevisiae*. These genes are implicated in β-glucan synthesis (*bgs1*$^+$/*FKS1* and *rga5*$^+$/*SAC7*), mannosidase activity (SPBC1198.07c/*DFG5*), cell-wall integrity (*pun1*$^+$/*PUN1*), as well as chitin deposition (SPBC19C7.05/*RCR1*) and transglycosylation (SPBC21B10.07/*UTR2*). In contrast, two *S. pombe*-specific target genes (*pvg1*$^+$ and *pvg5*$^+$) were involved in the synthesis of pyruvylated galactose residues that are not found in the cell wall of *S. cerevisiae* (Andreishcheva *et al.* 2004; Yoritsune *et al.* 2013). The upregulation of *omh1*$^+$, which encodes a putative mannosyltransferase, and *pvg1*$^+$ by *prz1*$^+$ overexpression contributes to the zymolyase-resistant phenotype of the *prz1OE* strain (Figure 3; Figure S3).

In addition, 33 target genes implicated in reproduction were identified as positively regulated by Prz1. These genes involved in reproduction are consistent with the decreased mating efficiency observed in *Δprz1* mutants (Sun *et al.* 2013). In *S. cerevisiae*, Crz1 is required for survival in prolonged exposure to mating pheromones (Stathopoulos and Cyert 1997), but pheromone genes have not been identified as targets of Crz1. The function of Prz1 in the mating process is likely different since very few of the target genes have orthologs in *S. cerevisiae* (Table 1). Interestingly, we detected Prz1 promoter occupancy of *ste11*$^+$, which encodes the primary transcriptional activator of the mating response (Sugimoto *et al.* 1991).

Our expanded list of 165 putative target genes upregulated by Prz1 identified a CDRE motif in 91 promoters similar to the 5′-AGCCTC-3′ motif found by Deng *et al.* (2006). This motif is also similar to the *S. cerevisiae* CDRE motif [5′-TG(C/A)GCCNC-3′] (Stathopoulos and Cyert 1997). The apparent conservation in binding specificity of Crz1 and Prz1 indicates the possibility of heterologous complementation. To test this, we overexpressed *CRZ1* with the *nmt1* or *nmt41* promoter in the *Δprz1* strain. In contrast to *prz1*$^+$ overexpression, heterologous overexpression of *CRZ1* did not cause reduced fitness or suppress the calcium sensitivity of the *Δprz1* strain (data not shown). Transcriptome profiling of *nmt1*-driven *CRZ1* in the *Δprz1* strain did not result in differential regulation of the Prz1 target genes. The inability of Crz1 to regulate Prz1 target genes may be due to the absence of binding to the CDRE motifs or required *trans*-acting factors. In contrast, the *C. albicans* Crz1 was able suppress the Ca^{2+} sensitivity of the *S. cerevisiae Δcrz1* strain, as well as drive expression of the CDRE reporter (Karababa *et al.* 2006).

We have also uncovered a novel repressive role of Prz1 in flocculation. Loss of *prz1*$^+$ causes constitutive flocculation in high-density cultures and the induction of both the transcription factor gene *cbf12*$^+$ and the dominant flocculin gene *gsf2*$^+$ (Figure 4, A and B). The constitutive flocculation is dependent on the presence of *cbf12*$^+$ and *gsf2*$^+$ (Figure 4C). Prz1 promoter occupancy was also detected for *cbf12*$^+$ and the *pfl3*$^+$ flocculin gene (Figure 4B). Although CDRE motifs were not detected in the promoters of these genes, five copies of the Ca^{2+}-dependent response element (5′-CAACT-3′) were present in the *cbf12*$^+$ promoter. Prz1 has been shown to bind to this motif in the *ncs1*$^+$ promoter (Hamasaki-Katagiri and Ames 2010). The repressive flocculation function of Prz1 and the Ca^{2+}-dependent response element have not been discovered in other fungal Crz1 orthologs.

Previous studies on Prz1 orthologs have predominantly focused on positively regulated functions and target genes. One exception was 3 genes identified as negatively regulated by *C. glabrata* Crz1 (Chen *et al.* 2012). In *A. fumigatus*, 31 genes were also identified as being downregulated in response to calcium treatment, and some may represent direct targets of CrzA (Soriani *et al.* 2008). The low number of identified negatively regulated target genes of Prz1 orthologs could be the result of limitations in previous studies, which do not contain genome-wide binding data.

The repression of target genes by Prz1 could be the result of *S. pombe*-specific rewiring of the transcriptional regulatory network due to an alteration in the amino acid sequence of the transcription factor. In contrast to *S. cerevisiae* Crz1, *S. pombe* Prz1 does not contain a polyglutamine tract domain that is often associated with transcriptional activation (Hirayama *et al.* 2003). It could be the absence of this domain that provides Prz1 with its transcriptional repressive function. Interestingly, Schaefer *et al.* (2012) observed that considerably fewer *S. pombe* genes (3) compared to *S. cerevisiae* genes (79) contain this domain. In addition, we examined whether the repressed putative targets of Prz1 could be the result of transcriptional interference where a transcribed gene can repress the transcription of the adjacent gene (Martens *et al.* 2004). Among the 92 negatively regulated putative target genes of Prz1, only 13 (14.1%) are located adjacent to a putative activated target gene. This indicates that the vast majority of putative negatively regulated target genes of Prz1 are not a result of transcriptional interference.

One notable example, SPAC513.07, which encodes a flavonol reductase, is negatively regulated by Prz1 in *S. pombe*, but its orthologs are activated by Crz1 in *S. cerevisiae*, *C. albicans*, and *C. glabrata*. This may indicate a rewiring of the transcriptional regulation of this gene between fungal species. The specific mechanism of Prz1-mediated negative regulation remains unknown, and more experimentation will be needed to determine if there is nucleosome remodeling occurring at these sites or some undiscovered cofactor involved.

Our genome-wide analysis of Prz1 has uncovered gene regulation in conserved and species-specific functions among fungal Crz1 orthologs. Preliminary comparison with other Crz1 target genes among different fungi shows substantial rewiring within the transcriptional regulatory network controlling calcineurin-mediated processes. This rewiring is evident even in well-conserved processes such as cell-wall biogenesis, where distinct fungi accomplish a similar function by positive regulation of different target genes. Moreover, this study revealed that the calcineurin-mediated transcriptional regulatory network of *S. pombe* has undergone substantial rewiring to include negative regulation of target genes implicated in species-specific processes such as flocculation.

Acknowledgments

This work was supported by grants from the Natural Sciences and Engineering Research Council of Canada and Canada Foundation for Innovation (to G.C.).

Literature Cited

Andreishcheva, E. N., J. P. Kunkel, T. R. Gemmill, and R. B. Trimble, 2004 Five genes involved in biosynthesis of the pyruvylated Galβ1,3-epitope in *Schizosaccharomyces pombe* N-linked glycans. J. Biol. Chem. 279: 35644–35655.

Araki, Y., H. Wu, H. Kitagaki, T. Akao, H. Takagi *et al.*, 2009 Ethanol stress stimulates the Ca^{2+}-mediated calcineurin/Crz1 pathway in *Saccharomyces cerevisiae*. J. Biosci. Bioeng. 107: 1–6.

Bailey, T. L., and C. Elkan, 1994 Fitting a mixture model by expectation maximization to discover motifs in biopolymers. Proc. Sec. Int. Conf. Intell. Syst. Mol. Biol. 2: 28–36.

Bodvard, K., A. Jörhov, A. Blomberg, M. Molin, and M. Käll, 2013 The yeast transcription factor Crz1 is activated by light in a Ca^{2+}/calcineurin-dependent and PKA-independent manner. PLoS One 8: e53404.

Boyle, E. I., S. Weng, J. Gollub, H. Jin, D. Botstein *et al.*, 2004 GO: TermFinder-open source software for accessing Gene Ontology information and finding significantly enriched Gene Ontology terms associated with a list of genes. Bioinformatics 20: 3710–3715.

Buck, M. J., A. B. Nobel, and J. D. Lieb, 2005 ChIPOTle: a user-friendly tool for the analysis of chIP-chip data. Genome Biol. 6: R97.

Cai, L., C. K. Dalal, and M. B. Elowitz, 2008 Frequency-modulated nuclear localization bursts coordinate gene regulation. Nature 455: 485–490.

Chen, Y.-L., J. H. Konieczka, D. J. Springer, S. E. Bowen, J. Zhang *et al.*, 2012 Convergent evolution of calcineurin pathway roles in thermotolerance and virulence in *Candida glabrata*. G3 (Bethesda) 2: 675–691.

Chua, G., 2013 Systematic genetic analysis of transcription factors to map the fission yeast transcription-regulatory network. Bioc Soc T 41: 1696–1700.

Chua, G., Q. D. Morris, R. Sopko, M. D. Robinson, O. Ryan *et al.*, 2006 Identifying transcription factor functions and targets by phenotypic activation. Proc. Natl. Acad. Sci. USA 103: 12045–12050.

Cisneros-Barroso, E., T. Yance-Chávez, A. Kito, R. Sugiura, A. Gómez-Hierro *et al.*, 2014 Negative feedback regulation of calcineurin-dependent Prz1 transcription factor by the CaMKK-CaMK1 axis in fission yeast. Nucleic Acids Res. 42: 9573–9587.

Clipstone, N. A., and G. R. Crabtree, 1992 Identification of calcineurin as a key signalling enzyme in T-lymphocyte activation. Nature 357: 695–697.

Cortés, J. C. G., R. Katoh-Fukui, K. Moto, J. C. Ribas, and J. Ishiguro, 2004 *Schizosaccharomyces pombe* Pmr1p is essential for cell wall integrity and is required for polarized cell growth and cytokinesis. Eukaryot. Cell 3: 1124–1135.

Deng, L., R. Sugiura, M. Takeuchi, M. Suzuki, H. Ebina *et al.*, 2006 Real-time monitoring of calcineurin activity in living cells: evidence for two distinct Ca^{2+}-dependent pathways in fission yeast. Mol. Biol. Cell 17: 4790–4800.

Dürr, G., J. Strayle, R. Plemper, S. Elbs, S. K. Klee *et al.*, 1998 The medial-Golgi ion pump Pmr1 supplies the yeast secretory pathway with Ca^{2+} and Mn^{2+} required for glycosylation, sorting, and endoplasmic reticulum-associated protein degradation. Mol. Biol. Cell 9: 1149–1162.

Edlind, T., L. Smith, K. Henry, S. Katiyar, and J. Nickels, 2002 Antifungal activity in *Saccharomyces cerevisiae* is modulated by calcium signalling. Mol. Microbiol. 46: 257–268.

Eisen, M. B., P. T. Spellman, P. O. Brown, and D. Botstein, 1999 Cluster analysis and display of genome-wide exopresion patterns. Proc. Natl. Acad. Sci. USA 95: 12930–12933.

Fardeau, V., G. Lelandais, A. Oldfield, H. Salin, S. Lemoine *et al.*, 2007 The central role of PDR1 in the foundation of yeast drug resistance. J. Biol. Chem. 282: 5063–5074.

Ferreira, R. T., A. R. Courelas Silva, C. Pimentel, L. Batista-Nascimento, C. Rodrigues-Pousada *et al.*, 2012 Arsenic stress elicits cytosolic Ca^{2+} bursts and Crz1 activation in *Saccharomyces cerevisiae*. Microbiol 158: 2293–2302.

Graef, I. A., F. Chen, L. Chen, A. Kuo, and G. R. Crabtree, 2001 Signals transduced by Ca^{2+}/calcineurin and NFATc3/c4 pattern the developing vasculature. Cell 105: 863–875.

Hagiwara, D., A. Kondo, T. Fujioka, and K. Abe, 2008 Functional analysis of C_2H_2 zinc finger transcription factor CrzA involved in calcium signaling in *Aspergillus nidulans*. Curr. Genet. 54: 325–338.

Hamasaki-Katagiri, N., and J. B. Ames, 2010 Neuronal calcium sensor-1 (Ncs1p) is up-regulated by calcineurin to promote Ca^{2+} tolerance in fission yeast. J. Biol. Chem. 285: 4405–4414.

Hendey, B., C. B. Klee, and F. R. Maxfield, 1992 Inhibition of neutrophil chemokinesis on vitronectin by inhibitors of calcineurin. Science 258: 296–299.

Hirayama, S., R. Sugiura, Y. Lu, T. Maeda, K. Kawagishi *et al.*, 2003 Zinc finger protein Prz1 regulates Ca^{2+} but not Cl^{-} homeostasis in fission yeast: identification of distinct branches of calcineurin signaling pathway in fission yeast. J. Biol. Chem. 278: 18078–18084.

Hu, Z., P. J. Killion, and V. R. Iyer, 2007 Genetic reconstruction of a functional transcriptional regulatory network. Nat. Genet. 39: 683–687.

Ikeda, Y., T. Ohashi, N. Tanaka, and K. Takegawa, 2009 Identification and characterization of a gene required for α1,2-mannose extension in the O-linked glycan synthesis pathway in *Schizosaccharomyces pombe*. FEMS Yeast Res. 9: 115–125.

Janke, C., M. M. Magiera, N. Rathfelder, C. Taxis, S. Reber *et al.*, 2004 A versatile toolbox for PCR-based tagging of yeast genes: new fluorescent proteins, more markers and promoter substitution cassettes. Yeast 21: 947–962.

Karababa, M., E. Valentino, G. Pardini, A. T. Coste, J. Bille *et al.*, 2006 CRZ1, a target of the calcineurin pathway in *Candida albicans*. Mol. Microbiol. 59: 1429–1451.

Koike, A., T. Kato, R. Sugiura, Y. Ma, Y. Tabata *et al.*, 2012 Genetic screening for regulators of Prz1, a transcriptional factor ccting downstream of calcineurin in fission yeast. J. Biol. Chem. 287: 19294–19303.

Kwon, E. J. G., A. Laderoute, K. Chatfield-Reed, L. Vachon, J. Karagiannis *et al.*, 2012 Deciphering the transcriptional-regulatory network of flocculation in *Schizosaccharomyces pombe*. PLoS Genet. 8: e1003104.

Macian, F., 2005 NFAT proteins: key regulators of T-cell development and function. Nat. Rev. Immunol. 5: 472–484.

Maeda, T., R. Sugiura, A. Kita, M. Saito, L. Deng *et al.*, 2004 Pmr1, a P-type ATPase, and Pdt1, and Nramp homologue, cooperatively regulate cell morphogenesis in fission yeast: the importance of Mn^{2+} homeostasis. Genes Cells 9: 71–82.

Mansuy, I. M., D. G. Winder, T. M. Moallem, M. Osman, M. Mayford *et al.*, 1998 Inducible and reversible gene expression with the rtTA system for the study of memory. Neuron 21: 257–265.

Martens, J. A., L. Laprade, and F. Winston, 2004 Intergenic transcription is required to repress the *Saccharomyces cerevisiae* SER3 gene. Nature 429: 571–574.

Matheos, D. P., T. J. Kingsbury, U. S. Ahsan, and K. W. Cunningham, 1997 Tcn1p/Crz1p, a calcineurin-dependent transcription factor that differentially regulates gene expression in *Saccharomyces cerevisiae*. Genes Dev. 11: 3445–3458.

Molkentin, J. D., J.-R. Lu, C. L. Antos, B. Markham, J. Richardson *et al.*, 1998 A calcineurin-dependent transcriptional pathway for cardiac hypertrophy. Cell 93: 215–228.

Moreno, S., A. Klar, and P. Nurse, 1991 Molecular genetic analysis of fission yeast *Schizosaccharomyces pombe*. Methods Enzymol. 194: 795–823.

Převorovský, M., M. Oravcová, J. Tvarůžková, R. Zach, P. Folk *et al.*, 2015 Fission yeast CSL transcription factors: mapping their target genes and biological roles. PLoS One 10: e0137820.

Ruiz, A., R. Serrano, and J. Ariño, 2008 Direct regulation of genes involved in glucose utilization by the calcium/calcineurin pathway. J. Biol. Chem. 283: 13923–13933.

Ryan, C. J., A. Roguev, K. Patrick, J. Xu, H. Jahari *et al.*, 2012 Hierarchical modularity and the evolution of genetic interactomes across species. Mol. Cell 46: 691–704.

Saldanha, A. J., 2004 Java Treeview: extensible visualization of microarray data. Bioinformatics 20: 3246–3248.

Schaefer, M. H., E. E. Wanker, and M. A. Andrade-Navarro, 2012 Evolution of CAG/polyglutamine repeats in protein-protein interaction networks. Nucleic Acids Res. 40: 4273–4287.

Serrano, R., A. Ruiz, D. Bernal, J. R. Chambers, and J. Ariño, 2002 The transcriptional response to alkaline pH in *Saccharomyces cerevisiae*: evidence for calcium-mediated signalling. Mol. Microbiol. 46: 1319–1333.

Smyth, G. K., 2004 Linear models and empirical Bayes methods for assessing differential expression in microarray experiments. Stat. Appl. Genet. Mol. 3: 1–25.

Smyth, G. K., and T. Speed, 2003 Normalization of cDNA microarray data. Methods 31: 265–273.

Sonnhammer, E. L., and G. Östlund, 2015 InParanoid 8: orthology analysis between 273 proteomes, mostly eukaryotic. Nucleic Acids Res. 43: D234–D239.

Soriani, F. M., I. Malavazi, M. E. Da Silva Ferreira, M. Savoldi, M. R. Von Zeska Kress *et al.*, 2008 Functional characterization of the *Aspergillus fumigatus* CRZ1 homologue, CrzA. Mol. Microbiol. 67: 1274–1291.

Stathopoulos, A. M., and M. S. Cyert, 1997 Calcineurin acts through the CRZ1/TCN1-encoded transcription factor to regulate gene expression in yeast. Genes Dev. 11: 3432–3444.

Stathopoulos-Gerontides, A., J. J. Guo, and M. S. Cyert, 1999 Yeast calcineurin regulates nuclear localization of the Crz1p transcription factor through dephosphorylation. Genes Dev. 13: 798–803.

Sugimoto, A., Y. Iino, T. Maeda, and Y. Watanabe, and M. Yamamoto, 1991 *Schizosaccharomyces pombe* $ste11^{+}$ encodes a transcription factor with an HMG motif that is a critical regulator of sexual development. Genes Dev. 5: 1990–1999.

Sun, L.-L., M. Li, F. Suo, X.-M. Liu, E.-Z. Shen *et al.*, 2013 Global analysis of fission yeast mating genes reveals new autophagy factors. PLoS Genet. 9: e1003715.

Thewes, S., 2014 Calcineurin-Crz1 signaling in lower eukaryotes. Eukaryot. Cell 13: 694–705.

Vachon, L., J. Wood, E. J. G. Kwon, A. Laderoute, K. Chatfield-Reed *et al.*, 2013 Functional characterization of fission yeast transcription factors by overexpression analysis. Genetics 194: 873–884.

Viladevall, L., R. Serrano, A. Ruiz, G. Domenech, J. Giraldo *et al.*, 2004 Characterization of the calcium-mediated response to alkaline stress in *Saccharomyces cerevisiae*. J. Biol. Chem. 279: 43614–43624.

Wang, H., N. Pathan, I. M. Ethell, S. Krajewski, Y. Yamaguchi *et al.*, 1999 Ca^{2+}-induced apoptosis through calcineurin dephosphorylation of BAD. Science 284: 339–343.

Wapinski, H., A. Pfeffer, N. Friedman, and A. Regev, 2007 Automatic genome-wide reconstruction of phylogenetic gene trees. Bioinformatics 23: i549–i558.

Wood, V., M. A. Harris, M. D. McDowall, K. Rutherford, B. W. Vaughan *et al.*, 2012 PomBase: a comprehensive online resource for fission yeast. Nucleic Acids Res. 40: D695–D699.

Yoritsune, K., T. Matsuzawa, T. Ohashi, and K. Takegawa, 2013 The fission yeast Pvg1p has galactose-specific pyruvyltransferase activity. FEBS Lett. 587: 917–921.

Yoshida, T., T. Toda, and M. Yanagida, 1994 A calcineurin-like gene $ppb1^{+}$ in fission yeast: mutant defects in cytokinesis, cell polarity, mating and spindle pole body positioning. J. Cell Sci. 107: 1725–1735.

Yoshimoto, H., K. Saltsman, A. P. Gasch, H. X. Li, N. Ogawa *et al.*, 2002 Genome-wide analysis of gene expression regulated by the calcineurin/Crz1p signaling pathway in *Saccharomyces cerevisiae*. J. Biol. Chem. 277: 31079–31088.

Zakrzewska, A., A. Boorsma, S. Brul, J. K. Hellingwerf, and F. M. Klis, 2005 Transcriptional response of *Saccharomyces cerevisiae* to the plasma membrane-perturbing compound chitosan. Eukaryot. Cell 4: 703–715.

Zhang, Y.-Q., and R. Rao, 2007 Global disruption of cell cycle progression and nutrient response by the antifungal agent amiodarone. J. Biol. Chem. 282: 37844–37853.

Communicating editor: A. P. Mitchell

GENETICS

Supporting Information
www.genetics.org/lookup/suppl/doi:10.1534/genetics.115.184218/-/DC1

Conserved and Diverged Functions of the Calcineurin-Activated Prz1 Transcription Factor in Fission Yeast

Kate Chatfield-Reed, Lianne Vachon, Eun-Joo Gina Kwon, and Gordon Chua

DOI: 10.1534/genetics.115.184218

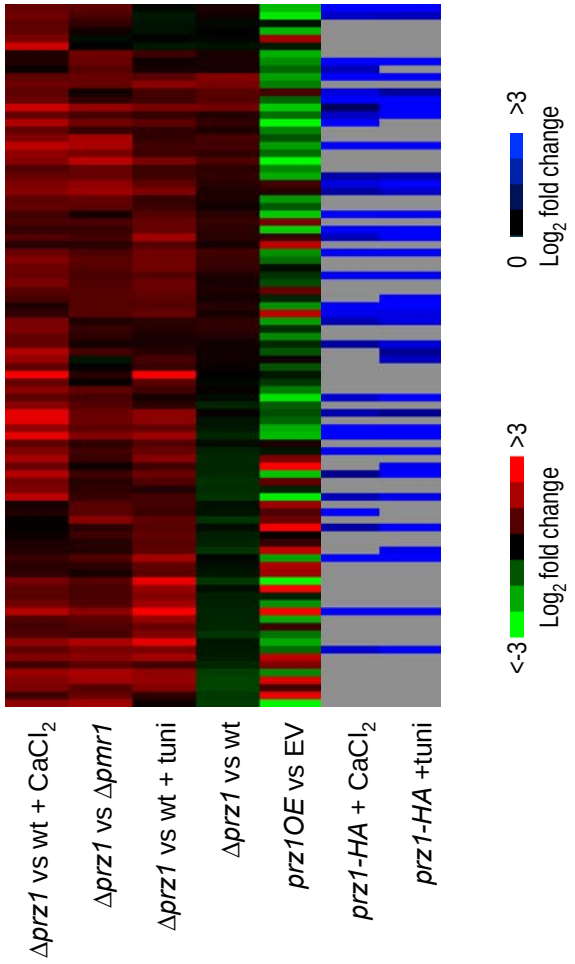

Figure S1 The heat map shows the expression profiles of 92 putative target genes that are negatively-regulated by Prz1. The first four columns of the heat map match the expression data from Figure 2A while the fifth column shows the expression profiles of the same target genes in a *prz1*$^{+}$ overexpression strain compared to the empty vector (EV) control. The next two columns in the heat map show ChIP-chip analysis of a *prz1-HA* strain treated with 0.15 M $CaCl_2$ or 2.5 µg/mL tunicamycin for 0.5 and 1.5 hours, respectively. The colour bars indicate relative expression and ChIP enrichment ratios between experimental and control strains. All microarray expression and ChIP-chip experiments were performed in replicate with dye reversal.

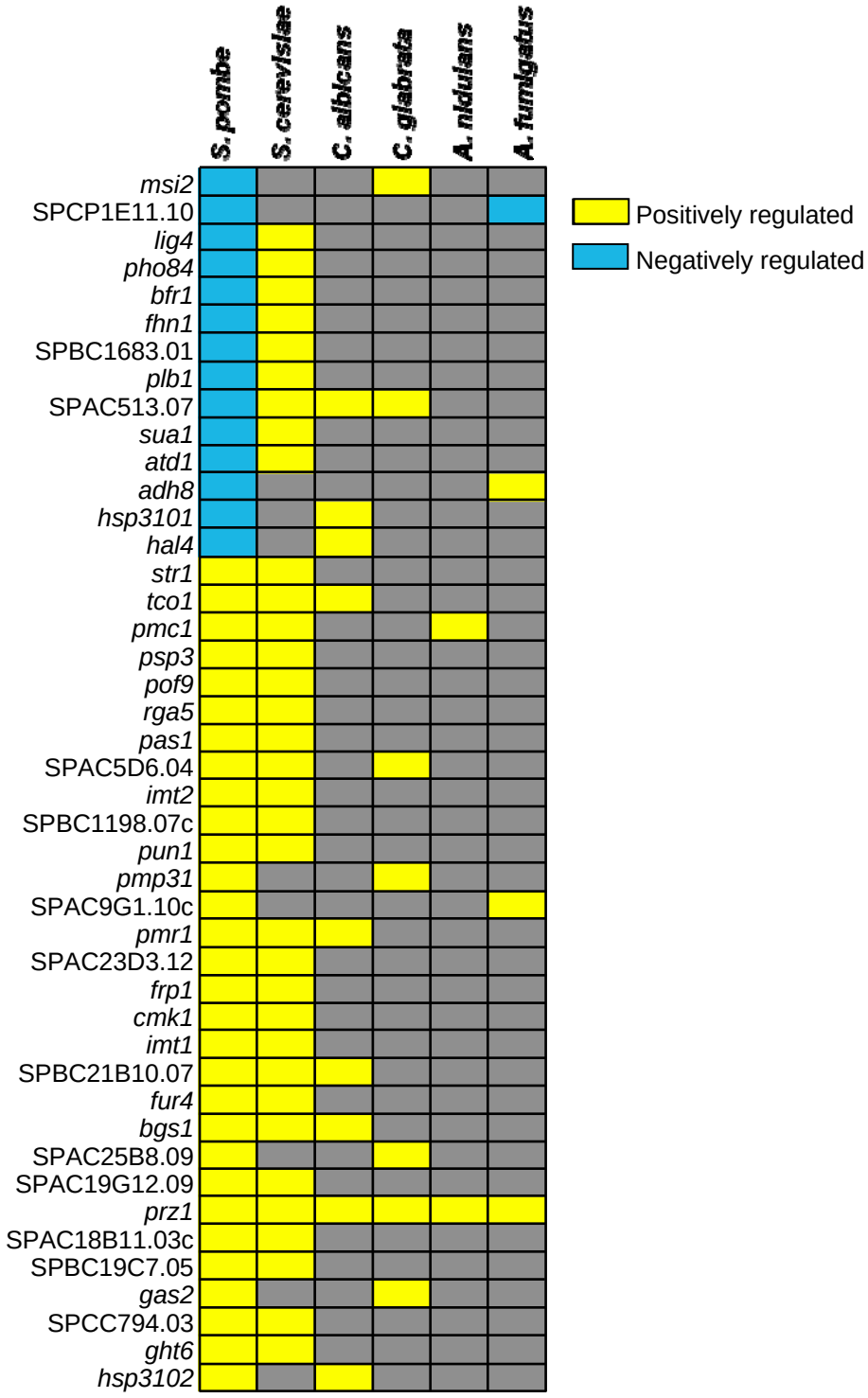
S. pombe
S. cerevisiae
C. albicans
C. glabrata
A. nidulans
A. fumigatus
msi2
SPCP1E11.10
lig4
pho84
bfr1
fhn1
SPBC1683.01
plb1
SPAC513.07
sua1
atd1
adh8
hsp3101
hal4
str1
tco1
pmc1
psp3
pof9
rga5
pas1
SPAC5D6.04
imt2
SPBC1198.07c
pun1
pmp31
SPAC9G1.10c
pmr1
SPAC23D3.12
frp1
cmk1
imt1
SPBC21B10.07
fur4
bgs1
SPAC25B8.09
SPAC19G12.09
prz1
SPAC18B11.03c
SPBC19C7.05
gas2
SPCC794.03
ght6
hsp3102
Positively regulated
Negatively regulated

Figure S2 The orthologs of the 339 differentially regulated genes in *S. pombe* that are regulated by a Prz1 ortholog in other fungal species. The highest number of orthologs is found between *S. pombe* and *S. cerevisiae*. This is likely due to several factors including the closer evolutionary distance. The other factors include the better characterized ortholog list between *S. pombe* and *S. cerevisiae* as well as the greater number of experiments and conditions used to find Crz1 targets in *S. cerevisiae* compared to the other species included.

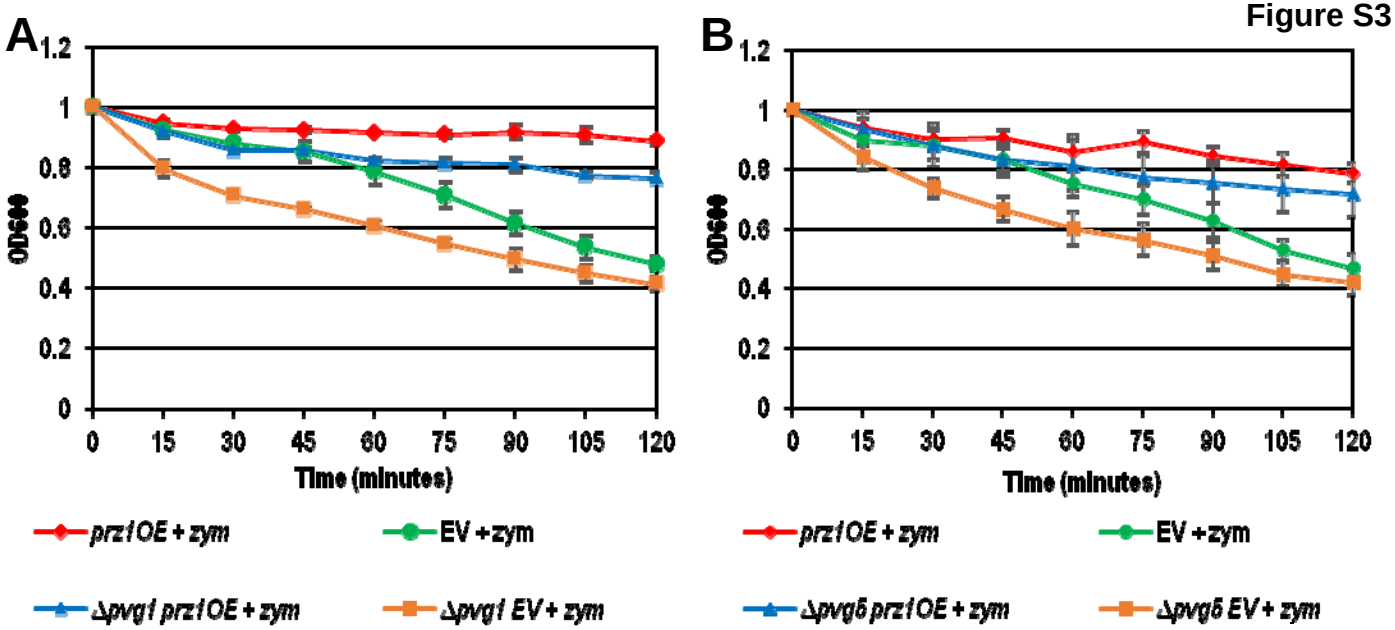

Figure S3 Cell wall degradation assays. (A) The zymolyase-resistant phenotype from overexpression of *prz1*$^{+}$ was abrogated by loss of *pvg1*$^{+}$ ($p=6.8e^{-3}$). (B) The Δ*pvg5* strain does not suppress the zymolyase-resistant phenotype from overexpression of *prz1*$^{+}$. The samples were adjusted to matching cell densities and transferred to test tubes in the presence and absence of 25 U/mL Zymolyase 100T. The samples were shaken at 37°C and OD_{600} readings were taken every 15 minutes to assess the degree of cell wall degradation. The zymolyase experiments were repeated in triplicate and error bars represent the standard error. The *P*-values were determined with ANOVA followed by a two-tailed student *t*-test after two hours of zymolyase treatment.

Table S1: *Schizosaccharomyces pombe* strains used in this study. (.xlsx, 10 KB)

Available for download as a .xlsx file at:

http://www.genetics.org/lookup/suppl/doi:10.1534/genetics.115.184218 /-/DC1/TableS1.xlsx

Table S2: Normalized $\log_2$ ratios with p-values from all of the expression microarray experiments. (.xlsx, 919 KB)

Available for download as a .xlsx file at:

http://www.genetics.org/lookup/suppl/doi:10.1534/genetics.115.184218 /-/DC1/TableS2.xlsx

Table S3: ChIP-chip analysis of *Prz1-HA* with $CaCl_2$ treatment. (.xlsx, 31 KB)

Available for download as a .xlsx file at:

http://www.genetics.org/lookup/suppl/doi:10.1534/genetics.115.184218 /-/DC1/TableS3.xlsx

Table S4: ChIP-chip analysis of *Prz1-HA* with tunicamycin treatment. (.xlsx, 33 KB)

Available for download as a .xlsx file at:

http://www.genetics.org/lookup/suppl/doi:10.1534/genetics.115.184218 /-/DC1/TableS4.xlsx

Table S5: The 165 putative target genes positively regulated by Prz1. (.xlsx, 18 KB)

Available for download as a .xlsx file at:

http://www.genetics.org/lookup/suppl/doi:10.1534/genetics.115.184218 /-/DC1/TableS5.xlsx

Table S6: The 92 putative target genes negatively regulated by Prz1. (.xlsx, 13 KB)

Available for download as a .xlsx file at:

http://www.genetics.org/lookup/suppl/doi:10.1534/genetics.115.184218 /-/DC1/TableS6.xlsx