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Involvement of Yap1p downregulation in calcineurin-mediated G_2 cell-cycle regulation in Saccharomyces cerevisiae

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ABSTRACT

The Ca^{2+}-activated pathways in Saccharomyces cerevisiae induce a delay in the onset of mitosis through the activation of Swe1p, a negative regulatory kinase that inhibits the Cdc28p/Clb complex. We isolated the YAP1 gene as a multicopy suppressor of the calcium sensitivity due to the loss of ZDS1, a negative regulator of SWE1 and CLN2 gene expression. YAP1 deletion on a zds1Δ background exacerbated the Ca^{2+}-related phenotype. Yap1p was degraded in a calcineurin-dependent manner when cells were exposed to calcium. In yap1Δ cells, the expression level of the RPN4 gene encoding a transcription factor for the subunits of the ubiquitin-proteasome system was diminished. The deletion of YAP1 gene or RPN4 gene led to the accumulation of Swe1p and Cln2p. Yap1p was a substrate of calcineurin in vivo. The calcineurin-mediated Yap1p degradation seems to be a mechanism that assures a G_2 delay in response to a stress that causes the activation of the calcium signaling pathway(s).

INTRODUCTION

Calcium-triggered signaling mechanisms are used by virtually all eukaryotic organisms to regulate a wide variety of cellular processes, including gene expression. Transient increases in cytosolic calcium results in the activation of diverse enzymes, including calcineurin, a highly conserved Ca^{2+}/calmodulin-dependent serine/threonine protein phosphatase (Aramburu *et al*, 2000).

In the yeast Saccharomyces cerevisiae, calcineurin has been implicated in stress-induced gene expression, ion homeostasis, and viability after exposure to mating pheromone (Aramburu *et al*, 2000; Cyert, 2001). More recently, we demonstrated that calcineurin and the Mpk1p-Mck1p pathway, in a coordinated fashion, activate Swe1p, a negative regulatory kinase of the Cdc28p/Clb complex (Booher *et al*, 1993), leading the cell to a cell-cycle delay in the G_2 phase (Mizunuma *et al*, 2001). The activation of these pathways is thought to occur in response to membrane stretching (Mizunuma *et al*, 1998).

The effect of calcium on cell-cycle regulation is clearly observed on a zds1Δ background lacking the negative regulator for SWE1 and CLN2 transcription (Ma *et al*, 1996; Mizunuma *et al*, 2004). Previously, we showed that the cell cycle of the zds1Δ strain cultivated in medium containing a high concentration of $CaCl_2$ was delayed in G_2 phase and that this strain displayed polarized bud growth due to the activation of the cellular calcium signaling pathways (Mizunuma *et al*, 1998). To identify the proteins that bear Zds1p-like function in the calcium-induced G_2 cell-cycle regulation, we screened for the genes whose overexpression could suppress the calcium phenotypes of the zds1Δ strain. By this screening, the YAP1 gene was obtained as a suppressor. Here, we show that the Swe1p and Cln2p levels were negatively regulated indirectly by Yap1p. Upon exposure of cells to exogenous calcium, Yap1p was degraded, which led to the transcriptional downregulation of the RPN4 gene encoding a transcription factor that modulates the expression levels of the genes involved in the

ubiquitin-proteasome system. The calcineurin-mediated Yap1p degradation, which leads to the Swe1p and Cln2p upregulation, was suggested to be a mechanism to assure a G_2 delay in response to a stress that causes the activation of calcineurin.

RESULTS

Identification of YAP1 gene as a high-copy suppressor of calcium sensitivity of ZDS1-null strain, which lacks a negative regulator of SWE1 and CLN2 gene expression

To investigate the detailed mechanism for the calcium-mediated cell-cycle regulation, we performed a screening for the genes whose overexpression could suppress the calcium sensitivity of the zds1Δ strain. The suppressor plasmids were classified into 19 genes, including the ZDS1 gene and its homolog ZDS2 gene (Bi & Pringle, 1996; Yu *et al*, 1996). After omitting these plasmids, a representative plasmid was chosen from each group, and they were designated as pSUZ1~17 (suppressor of zds1). By subcloning of the pSUZ14 plasmid, the YAP1 gene, which encodes a mammalian AP-1-like protein (Moye-Rowley *et al*, 1989) was identified as the gene responsible for suppression. As shown in Fig 1, the growth of the zds1Δ strain in the medium containing a high concentration of $CaCl_2$ was severely inhibited, exhibiting a G_2 delay and highly polarized bud growth. Overexpression of the YAP1 gene partially suppressed all of the calcium-induced phenotypes. So we further investigated the mechanism by which Yap1p modulated the calcium-mediated cell-cycle regulation.

Deletion of YAP1 gene exacerbated calcium sensitivity of zds1Δ strain

To verify the contribution of Yap1p in suppressing the various calcium-induced phenotypes of the

zds1Δ strain, we examined the effect of a YAP1 deletion mutation on these phenotypes (Fig 2A-C). The yap1Δ mutation by itself did not lead to a significant increase in the sensitivity of the cells to $CaCl_2$. However, the growth defect of the zds1Δ strain on plates containing $CaCl_2$ was exacerbated by the additional mutation yap1Δ (Fig 2A). Morphologically, the yap1Δ zds1Δ double deletion cells, in comparison with the zds1Δ cells, displayed a slightly more elongated shape in YPD medium (without added $CaCl_2$), and showed a more pronouncedly elongated morphology than the zds1Δ cells in the presence of $CaCl_2$ (Fig 2B). FACS analysis of the cellular DNA content demonstrated that the yap1Δ zds1Δ double deletion strain, in comparison with the respective single deletion strains, suffered from a severer defect in G_2 progression in the presence of exogenous $CaCl_2$ (Fig 2C). The additive effect of the yap1Δ and zds1Δ mutations on various calcium-induced phenotypes indicated that Yap1p and Zds1p may share a common biological function in parallel pathways in calcium signaling.

Yap1p was degraded in a calcineurin-dependent manner when cells were exposed to calcium

The results of the genetic experiments indicated that Yap1p was involved in the regulation of the G_2 delay and polarized bud growth induced by calcium. To examine if the YAP1 level itself was altered by exogenous calcium, we first investigated the effect of calcium on the YAP1 mRNA level by Northern blot analysis. However, the YAP1 mRNA level of the wild-type strain was not altered by calcium (Fig 3A). We then examined by Western blot analysis whether the level of Yap1p was altered by calcium. For this purpose, we constructed the strain carrying a chromosomally integrated construct for Myc-epitope-tagged Yap1p at the C-terminus under the control of its own promoter. The Yap1p-Myc level was remarkably diminished by calcium (Fig 3B). To distinguish whether Yap1p was downregulated by calcium at the translational or post-translational level, we examined the effect of calcium on the stability of Yap1p-Myc under the conditions in which de novo protein synthesis was

shut off by cycloheximide (CHX). The degradation of Yap1p-Myc in the presence of CHX was accelerated by calcium (Fig 3C, D). To examine if calcineurin was involved in the calcium-induced Yap1p-Myc downregulation, we determined the Yap1p-Myc level in the cells lacking the calcineurin regulatory subunit (cnb1Δ) under similar conditions. The Yap1p-Myc level of the cnb1Δ cells, in contrast to that of the wild-type cells, was not altered by calcium, suggesting that the calcium-induced Yap1p-Myc degradation was strictly dependent on calcineurin (Fig 3C, D).

Yap1p downregulation was accompanied by a decrease in RPN4 mRNA level and an increase in Swe1p and Cln2p levels

Because the effects of the overexpression and deletion of the YAP1 gene in calcium signaling resembled those of the ZDS1 gene, we next asked if the YAP1 gene was involved in the regulation of SWE1 and CLN2 transcription. However, the effect of the YAP1 deletion on the expression levels of these genes was negligible, as determined by Northern blot analysis (Fig 4A).

Next, to examine if Yap1p functioned at the post-transcriptional level, we compared the Swe1p and Cln2p levels in the wild-type and yap1Δ strains carrying chromosomally integrated constructs for Myc-tagged Swe1p and HA-tagged Cln2p (Fig 4B). The levels of Swe1p-Myc and Cln2p-HA in the yap1Δ strain, compared with those in the wild-type strain, were significantly elevated. Contrary to the effect of the YAP1 deletion, overexpression of the YAP1 gene led to decreased levels of Swe1p and Cln2p (Fig. 4C). These results indicate that Yap1p negatively regulated these levels.

The Yap1p degradation, which was triggered by the activation of calcium signaling, led to the upregulation of Swe1p and Cln2p. Then, what might be the mechanism underlying this upregulation? Recently, Yap1p, together with the Pdr1p and Pdr3p transcription factors, was implicated in the transcriptional activation of the RPN4 gene encoding a transcription factor that

modulates the expression levels of the genes involved in the ubiquitin-proteasome system during the oxidative stress response (Owsianik *et al*, 2002). We reasoned that the Yap1p degradation may lead to the downregulation of Rpn4p expression and that the decreased activity of the proteasome system may cause the upregulation of Swe1p, which may normally be degraded by the ubiquitin-proteasome system (Kaiser *et al*, 1998). To examine this possibility, we first compared the RPN4 mRNA levels in the wild-type and yap1Δ cells. As expected, the RPN4 mRNA level in the yap1Δ cells, in comparison with that in wild-type cells, was diminished (Fig 5A).

To investigate if the downregulation of the RPN4 mRNA level indeed leads to the decreased activity of the proteasome, we compared the activities of the ubiquitin-proteasome of wild-type and yap1Δ cells by measuring the levels of the ubiquitin-β-galactosidase conjugate protein, a well-characterized proteasome substrate (Johnson *et al*, 1995). As seen in Fig 5B, wild-type cells treated with exogenous calcium for 2 h showed a diminished activity of degrading the substrate protein and its degradation was Yap1p dependent. Consistent with the idea that the degradation of Swe1p and Cln2p is dependent on the proteasome-mediated proteolysis, the Swe1p-Myc and Cln2p-HA levels of the rpn4Δ strains was higher than those of the wild-type strain (Fig 5C). These results suggest that the calcium-induced downregulation of the RPN4 expression level caused by the Yap1p degradation may have contributed to the promotion of the G_2 delay and polarized bud growth through the upregulation of Swe1p and Cln2p in response to Ca^{2+} signaling.

Yap1p is a phosphorylated protein dephosphorylated by calcineurin

Genetic data indicated that the Yap1p downregulation occurred in a manner strictly dependent on calcineurin, raising a possibility that Yap1p may be a substrate for calcineurin. We first examined by the co-immunoprecipitation experiment if calcineurin and Yap1p physically interacted in vivo. As seen

in Fig 6A, Myc-tagged Cnb1p and HA-tagged Yap1p were co-precipitated in a reciprocal manner, suggesting that calcineurin and Yap1p indeed did so.

Since Yap1p phosphorylation has not been well characterized previously, we first examined if Yap1p could be phosphorylated or not in vivo. When the Yap1p-Myc immunoprecipitate was treated with calf intestinal alkaline phosphatase (CIP), a band that migrated faster than that treated in the presence of a phosphatase inhibitor was observed, showing that Yap1p had been phosphorylated (Fig 6B). We further examined the possibility that Yap1p was a substrate of calcineurin in vivo. When cell extracts were prepared from the wild-type cells grown in the presence of exogenous calcium, the intensity of the Yap1p-Myc bands was diminished with progression of the incubation time in calcium medium, reflecting its calcineurin-dependent degradation. The change in the Yap1p intensity was accompanied by the preferential loss of the slow migrating species from the broad band seen at time 0. By contrast, no significant changes in the intensity and the mobility of Yap1p-Myc were observed in the cnb1Δ cells. These results indicate that the generation of the faster migrating species occurred in a calcineurin-dependent manner. The CIP-treated Yap1p-Myc migrated faster than Yap1p-Myc generated by calcium in vivo in wild-type cells, suggesting that Yap1p contained additional phosphorylated residue(s) that were not removable by calcineurin. Moreover, we found that Yap1p was a direct substrate of calcineurin in vitro (Supplementary Fig 1). These results demonstrate that Yap1p was a direct substrate of calcineurin.

DISCUSSION

Here, we discovered a novel function of Yap1p in the calcineurin-mediated G_2 cell-cycle regulation (Fig 7). Yap1p has been well characterized as a central regulator of responses to oxidative stress (Balzi

& Goffeau, 1994; Hirata *et al*, 1994; Kuge & Jones, 1994; Wu & Moye-Rowley, 1994; Wemmie *et al*, 1994; Kuge *et al*, 1997). Presently we found a novel Yap1p function in calcium signaling. The calcium sensitivity phenotypes of the zds1Δ strain were suppressed by Yap1p overexpression, and conversely, exacerbated by the lack of Yap1p. Our genetic data indicated Yap1p to be a negative regulator for the calcium signaling that is linked to the Swe1p- and Cln2p-mediated regulation of cell-cycle and morphology (Fig 1 and 2). The Yap1p degradation caused the accumulation of Swe1p and Cln2p through the downregulation of the ubiquitin-proteasome system, which was mediated by the inhibition of the RPN4 expression. Supporting this possibility, the activity of the proteasome system as determined by the level of the ubiqutin-conjugated β-galactosidase was decreased by exogenous calcium by a manner dependent on Yap1p degradation. The Yap1p degradation did not seem to be mediated by the proteasome system, since nin1-1 mutation (Kominami *et al*, 1995) did not affect the rate of Yap1p degradation (data not shown). On the other hand, the degradation of Hsl1p, a protein kinase that negatively regulates the Swe1p kinase, is induced by calcium via the ubiqutin-proteasome pathway (Mizunuma et al, 2001). The degradation of Hsl1p occurs rapidly after exposure to calcium, with a half life of <10 min compared to that of Yap1p (~30 min) at 37°C. It was also noted that the rate of Yap1p degradation in the presence of calcium at 37°C was more rapid than that seen at 28°C, suggesting that the calcium-induced Yap1p degradation proceeded more rapidly at high temperatures. Therefore, the inhibition of the RPN4 expression caused by the Yap1 degradation seems to be a slow process, which seems to be required to assure G_2 delay in response to stress.

In the Ca^{2+}-mediated G_2 cell-cycle regulation, calcineurin was shown to upregulate Swe1p through 2 distinct but cooperative pathways; i.e., one involving the activation of SWE1 transcription and the other, the destabilization of Hsl1p (Mizunuma et al, 2001). In the present study, we revealed an additional role of calcineurin, i.e., promotion of Swe1p accumulation through the degradation of the

Yap1p transcription factor. Thus, the upregulation of Swe1p by calcium signaling seems to be regulated multilaterally to ensure Swe1p activation by at least 3 distinct calcineurin-mediated mechanisms that operate on Swe1p at different levels; i.e., transcriptional, posttranslational and degradation.

We showed that Yap1p was phosphorylated by an as yet unidentified kinase(s) and dephosphorylated by calcineurin, thus identifying Yap1p as a substrate of calcineurin (Fig 6). The Yap1p dephosphorylation by calcineurin appeared to be required for its degradation in the downregulation of the expression levels of the Yap1p-dependent genes including the RPN4 gene. In the 5' non-coding region of the RPN4 gene, a Yap1p-response element (YRE; TTACTAA) is located at position -373 relative to the ATG translation initiation codon (Wu and Moye-Rowley, 1994). However, it was previously shown that the effect of the YAP1 deletion on the activation of the ubiquitin-proteasome system was seen only in the cells lacking the Pdr1p transcription factor (Owsianik *et al*, 2002). Nevertheless, a decrease in the levels of RPN4 mRNA and proteasome activity was seen on the yap1Δ PDR1 background (Fig 5A and B). These different observations may be explained by the difference in the strain background. Alternatively, it may be possible that Pdr1p is also degraded in a manner similar to Yap1p.

METHODS

Methods are shown in Supplementary information.

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FIGURE LEGENDS

Fig 1 Overexpression of YAP1 gene suppresses various calcium sensitivity phenotypes of zds1Δ strain.

(A) Cells of wild-type (WT) and zds1Δ strains transformed with empty plasmid (pYI1) or pYI1-YAP1 plasmid were spotted on YPD plates with or without 250 mM $CaCl_2$ and incubated for 2 days at 28°C. (B) Cell morphology after 6 h of incubation in YPD containing 50 mM $CaCl_2$ at 28°C. (C) FACS profile of propidium iodide (PI)-stained cells prepared as described in (B) (1C, 1 DNA copy; 2C, 2 DNA copies).

Fig 2 Calcium sensitivity phenotypes of zds1Δ strain are exacerbated by deletion of YAP1 gene.

(A) Cells of WT, yap1Δ, zds1Δ, and zds1Δ yap1Δ strains were spotted on YPD plates supplemented or not with 200 mM $CaCl_2$ at 28°C (2 days). (B) Cell morphology after 6 h of incubation in YPD with or without 100 mM $CaCl_2$ at 28°C. (C) FACS profiles of PI-stained cells. The cells were prepared as described in (B).

Fig 3 Yap1p level is downregulated by calcium post-translationally in a calcineurin-dependent manner.

(A) YAP1 and ACT1 mRNA levels in WT strain were determined by Northern blotting. Early log-phase growing cells were suspended in YPD containing 100 mM $CaCl_2$ at 37°C. (B) The bands of Yap1p-Myc and Cdc28p were detected by Western blotting using cell cultures similar to those indicated in (A). (C) Early log-phase growing cells of WT and cnb1Δ strains were suspended in YPD plus 100 μg/ml cycloheximide (CHX) supplemented or not with 100 mM $CaCl_2$ and incubated at 37°C. Yap1p-Myc and Cdc28p were detected by Western blotting. (D) The amount of Yap1p shown in (C) was quantified by using Image SXM and plotted.

Fig 4 Swe1p and Cln2p levels are elevated in yap1Δ strain.

(A) SWE1, CLN2, and ACT1 mRNA levels in WT and yap1Δ strains were determined by Northern blotting. Samples were prepared from early log-phase growing cells at 28°C. (B) Swe1p-Myc, Cln2p-HA, and Cdc28p were detected by Western blotting using cell cultures similar to those indicated in (A). (C) Early log-phase growing cells of WT transformed with empty plasmid (vec.) or pYI1-YAP1 plasmid (YAP1) were suspended in YPD containing 100 mM $CaCl_2$, incubated for 3 h, and then used for Western blotting.

Fig 5 Yap1p is required for activation of RPN4 gene expression and the latter is required for proteasome activity and elimination of Swe1p.

(A) RPN4 and ACT1 mRNA levels in WT and yap1Δ cells were determined by Northern blotting. Samples were prepared from early log-phase growing cells at 28°C. (B) Early log-phase growing cells of the indicated strains transformed with plasmid carrying a gene encoding Ub-Pro-β-galactosidase (Ub-P-βgal) were suspended in YPG with or without 100 mM $CaCl_2$ and incubated at 37°C for 2 h. The Ub-P-βgal and Cdc28p were detected by Western blotting. (C) Swe1p-Myc, Cln2p-HA, and Cdc28p in WT and rpn4Δ cells were detected by Western blotting. Samples were prepared from early log-phase growing cells at 28°C.

Fig 6 Phospho-Yap1p is a substrate of calcineurin.

(A) cnb1Δ yap1Δ strain cotransformed with CTF-CNB1-Myc and YCp50-YAP1-HA was grown in YPD medium at 28ºC until early log-phase. Cell extracts were proccessed for immunoprecipitation with α-HA antibody or α-Myc antibody. Total protein and IP fraction proteins were detected by

Western blot analysis. (B) Early log-phase growing cells of WT or cnb1Δ strain were suspended in YPD with or without 100 mM $CaCl_2$ at 37°C, and samples were taken at the indicated times. Cell extracts of cnb1Δ cells were treated with λ-phosphatase (CIP) for 30 min with or without sodium orthovanadate (Inhibitor). Yap1p-Myc was detected by Western blotting.

Fig 7 Model of a novel function of Yap1p in calcineurin-mediated G_2 cell-cycle regulation.

Supplementary information

METHODS

Strains and Media.

All yeast strains were derivatives of strain W303. The strains used were the following: DHT22-1b (MAT**a** trp1 leu2 ade2 ura3 his3 can1-100 W303-1A a gift from Dr. R. Rothstein), YAT1 (MAT**a** zds1::TRP1; Mizunuma *et al*, 1998), YHY3 (MAT**a** yap1::HIS3), YHY7 (MAT**a** yap1::HIS3 zds1::TRP1), YHY129 (MAT**a** rpn4::kanMX4), YHY93 (MAT**a** swe1::HIS3::SWE1-9xMyc CLN2-3xHA), YHY97 (MAT**a** yap1::HIS3 swe1::HIS3::SWE1-9xMyc CLN2-3xHA), YHY130 (MAT**a** rpn4::kanMX4 swe1::HIS3::SWE1-9xMyc CLN2-3xHA), YHY30 (MAT**a** YAP1-13xMyc), YHY31 (MAT**a** cnb1::HIS3 YAP1-13xMyc), and YK109 (nin1-1, a gift from Dr. A. Toh-e). The rpn4Δ strain was constructed by gene replacement. Genomic DNA was isolated from the rpn4::kanMX4 strain on a BY4741 background (Invitrogen). The RPN4 locus was amplified by PCR using primers 5'-GAAGAACTCCGCTTTTTAGTTGAAC-3' and 5'-TTCCATTTTGTGTGAGGTTTTCTTC-3'. The amplified fragment was used to transform the DHT22-1b strain. The strain with a chromosomally integrated gene for the construction of Yap1p with a 13xMyc epitope at its C terminus was prepared as follows: PCR was performed using primers 5'-TGGCAAAGGCAAAATGTTCAGAAAGA GGGGTTGTCATCAATGCAGAAGACGTTCAATTAGCTTTGAATAAGCATATGAACCGGATCC CCGGGTTAATTAA-3' and 5'-CAAGGTAAGTTAAAAAAGTTTAATTGTAACATTATAGAAAAA GTTCTTTCGGTTACCCAGTTTTCCATAAAGTTCCCGCTGAATTCGAGCTCGTTTAAAC-3' and pFA6a-13Myc- kanMX6 as a template. The amplified fragment was used to transform the DHT22-1b strain. Media used in the present study were as described previously (Mizunuma *et al*, 1998; Mizunuma *et al*, 2001).

Isolation of multicopy suppressors of zds1Δ strain.

For screening, the zds1 deletion strain (zds1::TRP1) was transformed with a yeast genomic DNA library constructed in the high-copy number plasmid YEp24, and the transformed cells (8.4×10^4) were spread on SD minus uracil plates. The transformants were picked up and inoculated onto YPD plates supplemented with 300 mM $CaCl_2$, a concentration that virtually inhibited the growth of the zds1Δ strain. From each of 145 calcium-resistant transformants, plasmids were recovered after transformation into E. coli. Of these, 123 plasmids reproducibly suppressed the calcium-sensitive phenotype when they were re-introduced into the zds1Δ strain. By partial DNA sequencing of the genomic DNA insert, these plasmids were classified into 19 groups according to the genome fragment contained in the plasmid.

Construction of plasmids.

pHY1 (pYI1-YAP1) contained the genomic fragment of the YAP1 gene (Sau3AI- Sau3AI, 4.4 kb) in high-copy vector pYI1.

Flow cytometry and cell microscopy.

Approximately 1×10^7 cells from a yeast culture were harvested by centrifugation and resuspended in 300 μl of 0.2 M Tris-HCl (pH 7.5). Cells were fixed by the gradual addition of 700 μl of cold ethanol with vortexing, and then incubated overnight at -20°C. The fixed cells were washed with the same buffer containing RNase A (1 mg/ml) for 3 h at 30°C. The cells were then stained with 100 μl of propidium iodide (50 μg/ml) solution in 4 mM sodium citrate, 10 mM NaCl, and 0.1% Nonidet P-40 for 15 min on ice and subsequently analyzed by using a FACSCalibur (Becton Dickinson).

Northern Blot Analysis.

The SWE1, CLN2, YAP1, RPN4 and ACT1 probes were generated by random-primed labeling of a 0.7-kb BglII fragment of SWE1, a 1.3-kb NcoI-XhoI fragment of CLN2, a 1.7-kb NdeI-BamHI fragment of YAP1, a 1.7-kb PCR product of RPN4 and a 1.1-kb XhoI-KpnI fragment of ACT1, respectively, with [α-^{32}P]dCTP by use of a multiprime DNA labeling kit (Amersham Pharmacia Biosciences). The RPN4 fragment was amplified by PCR by using primers 5'-GAAGAACTCCGCTTTTTAGTTGAAC-3' and 5'- TTCCATTTTGTGTGAGGTTTTCTTC-3'. ACT1 was used as an internal loading control.

Western blot analysis.

Cells were washed with TEG buffer (50 mM Tris-HCl [pH 7.5], 10 mM EDTA, 5 mM EGTA, 25 mM NaCl, 0.05% Tween 20, and 10% glycerol) containing 1 mM dithiothreitol, 1 mM PMSF, phosphatase inhibitors (10 mM NaF and 1 mM sodium orthovanadate), and protease inhibitor mixture (α-complete, Roche Molecular Biochemicals) and harvested by centrifugation. Pellets were frozen in liquid N_2 and stored at -80°C until used. Frozen pellets were resuspended in 100 µl of 2x sample buffer and lysed by vigorous vortexing for 20 min with an equal volume of glass beads at 4°C. After removal of the beads by centrifugation at 10,000 rpm for 1 min at 4°C, the lysates were boiled for 3 min. Proteins were resolved by SDS-PAGE for 2 h at 30 mA and analyzed by immunoblotting using either anti-Myc antibody (9E10, BAbCO), anti-HA antibody (HA-11, BAbCO), anti-βGal antibody (Promega) or anti-PSTAIRE antibody (this recognizes Cdc28p and Pho85p; Santa Cruz Biotechnology). For all immunoblots, horseradish peroxidase-conjugated second antibodies were used, and blots were visualized by use of the ECL detection system (Amersham Biosciences). Cdc28p protein was used as

an internal loading control.

Assay for Ub-Pro-β-galactosidase protein level.

Early log-phase growing cells of wild-type, yap1Δ, and rpn4Δ strains transformed with plasmid carrying a gene encoding Ub-Pro-β-galactosidase (Ub-P-gal) were grown in SD-Uracil medium at 28ºC until early log-phase. The cells were suspended in YPGalactose medium and incubated for 2 h at 37ºC to induce Ub-Pro-β-galactosidase protein. Then, they were incubated or not with 100 mM $CaCl_2$ for another 2 h at 37°C. Ub-Pro-β-galactosidase and Cdc28p were detected by Western blotting.

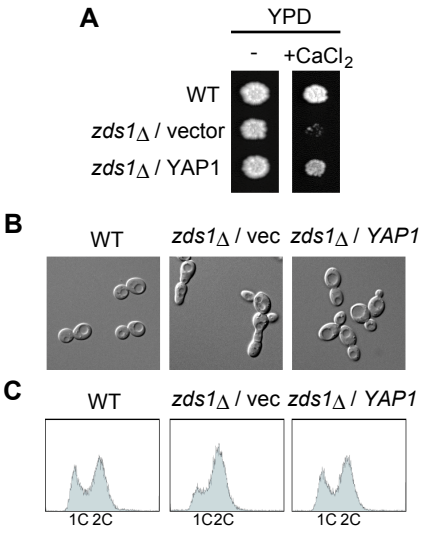
***In vitro* phosphatase assay.**

YHY31 cells were grown to early log phase in YPD. The cells were washed and resuspended in 100 μl of TEG buffer containing 1 mM dithiothreitol, phosphatase inhibitors (10 mM NaF and 1 mM sodium orthovanadate), and protease inhibitor mixture (α-complete, Roche Molecular Biochemicals). The cells were lysed by vigorous vortexing for 20 min with an equal volume of glass beads at 4°C. After removal of the beads by centrifugation at 10,000 rpm for 1 min at 4°C, the supernatants were incubated, with rotation, for 2 h at 4°C with 500 μl TEG buffer containing 30 μl protein G beads (Dynal) and 5μl of anti-Myc antibody (9E10, BAbCO). The immunoprecipitates were washed twice in TEG buffer containing protease inhibitors, phosphatase inhibitors and 0.05% Tween 20, twice in TEG buffer containing protease inhibitors only, and twice in CP buffer (50 mM Tris-HCl [pH 7.5], 1 mM $MgCl_2$, 1 mM dithiothreitol) containing protease inhibitors. Phosphatase assays were performed in a total of 150 μl in CP buffer containing 9.6 U of recombinant human calcineurin (Sigma) and 498 U of recombinant human calmodulin (Sigma) per assay. Where indicated, $CaCl_2$ was added to a final concentration of 20 mM and EGTA to 100 mM. Phosphatase assays were conducted at 30°C for 90

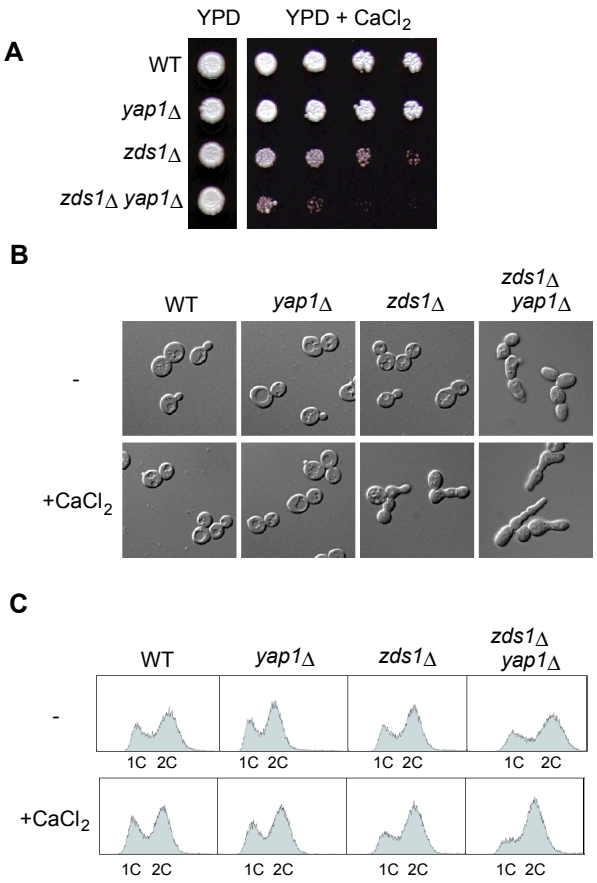
min; then the supernatant was then removed, and 50 μl of 2x sample buffer was added to the protein beads. After having been boiled for 5 min, samples were run on a 6% gel and blotted as described above. Blots were visualized by using the ECL detection system.

Supplementary Figure 1

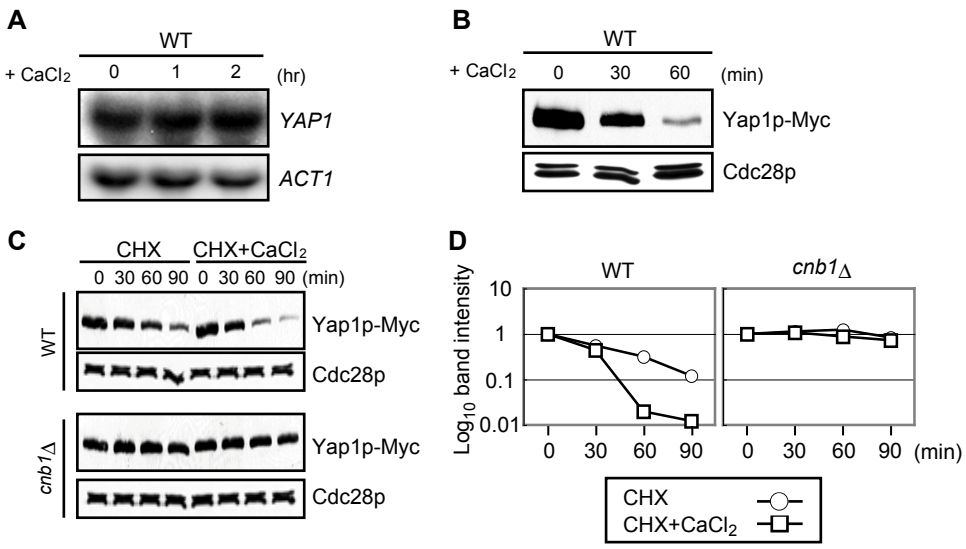
Calcineurin dephosphorylated Yap1 in vitro. Extracts were prepared from cnb1Δ cells. Immunoprecipitated Yap1p-Myc was treated as described in "Methods" with recombinant calcineurin (CN), calmodulin (CaM), $CaCl_2$ and EGTA. The samples were subjected to SDS-PAGE, and Yap1p-Myc was then detected by Western blotting.



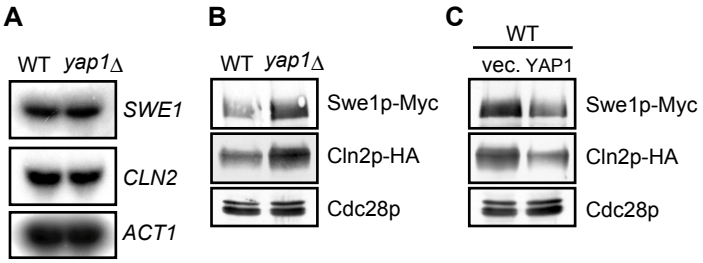
Yokoyama *et al* Fig. 1



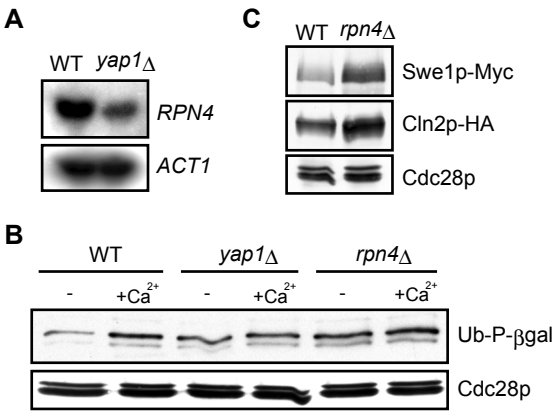
Yokoyama *et al* Fig. 2



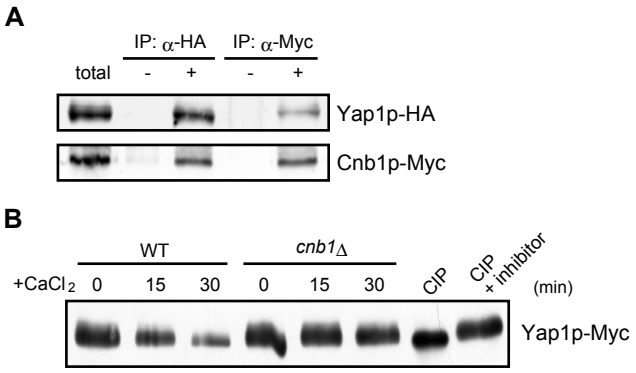
Yokoyama *et al* Fig. 3



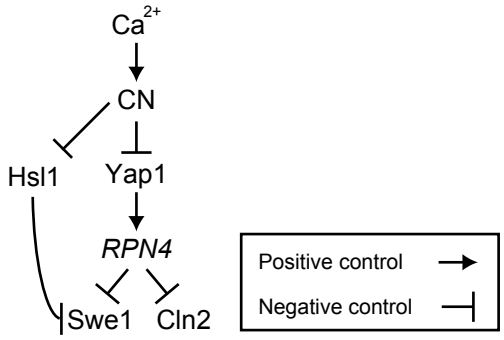
Yokoyama *et al* Fig. 4



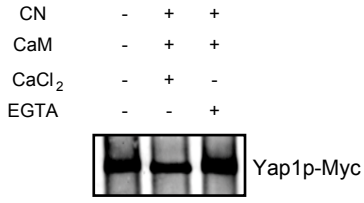
Yokoyama *et al* Fig. 5



Yokoyama *et al* Fig. 6



Yokoyama *et al* Fig. 7



Yokoyama *et al* Supplementary Fig. S1