Ptc6 Is Required for Proper Rapamycin-Induced Down-Regulation of the Genes Coding for Ribosomal and rRNA Processing Proteins in \textit{S. cerevisiae}

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Abstract

Ptc6 is one of the seven components (Ptc1-Ptc7) of the protein phosphatase 2C family in the yeast \textit{Saccharomyces cerevisiae}. In contrast to other type 2C phosphatases, the cellular role of this isoform is poorly understood. We present here a comprehensive characterization of this gene product. Cells lacking Ptc6 are sensitive to zinc ions, and somewhat tolerant to cell-wall damaging agents and to Li\textsuperscript{+}. Ptc6 mutants are sensitive to rapamycin, albeit to lesser extent than ptc1 cells. This phenotype is not rescued by overexpression of \textit{PTC1} and mutation of \textit{ptc6} does not reproduce the characteristic genetic interactions of the \textit{ptc1} mutation with components of the TOR pathway, thus suggesting different cellular roles for both isoforms. We show here that the rapamycin-sensitive phenotype of \textit{ptc6} cells is unrelated to the reported role of Ptc6 in controlling pyruvate dehydrogenase activity. Lack of Ptc6 results in substantial attenuation of the transcriptional response to rapamycin, particularly in the subset of repressed genes encoding ribosomal proteins or involved in rRNA processing. In contrast, repressed genes involved in translation are Ptc6-independent. These effects cannot be attributed to the regulation of the Sch9 kinase, but they could involve modulation of the binding of the Ifh1 co-activator to specific gene promoters.

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Introduction

Type 2C Ser/Thr protein phosphatases (PP2Cs) are a group of monomeric enzymes highly conserved throughout evolution. The classification of these proteins according to their primary structure shows that in fungi there are five major groups of PP2Cs [1]. In the budding yeast \textit{Saccharomyces cerevisiae} the PP2C family is composed of seven members (Ptc1-7) that include representatives of all structural groups previously described. The last member incorporated to the Ptc family was \textit{YCR079w} (\textit{PTC6}), which was presumed for many years to encode a type 2C enzyme [2,3], but whose phosphatase activity was only recently demonstrated [4]. It is known that the \textit{PTC7} gene can produce 2 different polypeptides by differential splicing [5]. As occurs in higher eukaryotes, yeast PP2Cs were initially associated to the regulation of cell growth and stress signaling. Our current knowledge, however, suggests that PP2C functions are much more diverse (for a review see [1] and references therein). While the subcellular localization of Ptc1-4 is cytoplasmatic or nuclear [6,7], Ptc5, Ptc6 and the spliced version of Ptc7 are located in the mitochondria [5,8–11]. There is some controversy, however, about the localization of Ptc6 within this organelle because it has been proposed that it is localized to either the mitochondrial intermembrane space or the mitochondrial matrix [11,12].

In spite of the growing body of knowledge, our understanding of the function(s) and regulatory mechanisms for each specific PP2C isoform is still rather limited, and this is particularly true for the mitochondrially-located isoforms. For instance, only one cellular target for Ptc6 has been described so far. Both, Ptc6 and Ptc5, seem to dephosphorylate Ser313 of Pda1, a component of the E1\textsubscript{a} subunit of the pyruvate dehydrogenase (PDH) complex that catalyzes the oxidative decarboxylation of pyruvate to form acetyl-CoA thus connecting glycolysis and the tricarboxylic acid cycle [13]. In fact, the PDH complex activity in \textit{ptc6} or \textit{ptc5} strains is greatly reduced [10,13]. Moreover, \textit{ptc6} mutants are unable to degrade aconitase in a Pep4-dependent fashion and have impaired mitochondrial transport into the vacuole after prolonged stationary phase, suggesting that Ptc6 plays a role in the mitochondrial degradation process known as mitophagy (see [14] and references therein). As a consequence, Ptc6 is necessary for survival of lactate-growing stationary phase cells [11]. The role of Ptc6 in mitophagy is probably exerted through Rgs3, the transcription factor that mediates the RTG (retrograde signaling pathway) response [15]. Due to this function in mitophagy, Ptc6 has received the Aup1 (for autophagy-related protein phosphatase) alias [11].

Remarkably, among the \textit{ptc} family mutants, only \textit{ptc1} and \textit{ptc6} are sensitive to rapamycin (an inhibitor of the activity of Tor kinases) and caffeine (a compound that has been related with the
cell wall integrity pathway and that can also act as an inhibitor of Tor kinases [4,16–19]. On the other hand, overexpression of \( PT\text{C6} \) renders cells tolerant to rapamycin [4]. The TOR (target of rapamycin) pathway is a conserved signaling network (for a review, see [20] and references therein) important for cell growth control that involves the phosphatidylinositol kinase-related protein kinase (PIKK) Tor1 and Tor2. These kinases are found in two functionally and structurally distinct multiprotein complexes: TORC1 and TORC2, each of which signals through a different set of effector pathways. TORC1 is rapamycin-sensitive, whereas TORC2 is rapamycin insensitive [21]. TORC1 activates cell growth by positively regulating diverse anabolic processes, such as transcription, protein synthesis, ribosome biogenesis, nutrient transport, and mitochondrial metabolism, whereas it represses several catabolic pathways, such as mRNA degradation, ubiquitin-dependent proteolysis, autophagy and apoptosis. However, the molecular mechanisms by which TORC1 signals to these diverse processes in both yeast and mammals are still open to discussion. In particular, only a few substrates of either TORC1 or its direct effectors such as the AGC kinase Scl9 [22] or the Tap42/Tip41-PP2A/Sirt4 system (see [20] and references therein) are known.

The rapamycin-sensitive phenotype of the \( ptc1 \) mutant lead us to recently uncover a role for Ptc1 in normal signaling through the TORC1 pathway, possibly by regulating a step upstream of Sit4/PP2A/Sit4 system (see [20] and references therein) [19]. Consequently, we also decided to investigate the nature of the possible functional connection between Ptc6 and this pathway. Our results suggest that the role of Ptc1 and Ptc6 in maintaining the normal function of TORC1 pathway is different. Genetic studies also indicate that the rapamycin-sensitive phenotype derived by the lack of Ptc6 is not mediated by its role on the regulation of the PDH complex or the mitophagy process. Remarkably, transcriptomic analyses show that mutation of \( PT\text{C6} \) significantly attenuates the transcriptional changes caused by rapamycin, mainly at the level of repressed genes. In this study we propose that the inability to repress transcription of certain genes in response to rapamycin may be the cause of the phenotypes observed in Ptc6-deficient cells.

Materials and Methods

Yeast and Escherichia coli growth conditions

Yeast cells were incubated at 28°C in YPD medium (1% yeast extract, 2% peptone and 2% glucose) or in synthetic medium [23] containing 2% glucose and lacking the appropriate selection requirements. The Low Ammonium medium is synthetic medium containing 2% glucose and supplemented with 10 mM ammonium sulphate, 40 mg/l methionine, 20 mg/l histidine and 100 mg/l leucine.

\( E.\) coli DH5\( \alpha \) cells were used as plasmid DNA host and were grown at 37°C in LB broth supplemented with 50 \( \mu \)g/ml ampicillin, when required. Bacterial and yeast cells were transformed using standard methods. Standard recombinant DNA techniques were performed as described elsewhere.

The sensitivity of yeast cells to diverse stressing agents was evaluated by growth on agar plates (drop tests) as described in [24]. Sensitivity of each strain to rapamycin was evaluated in liquid cultures as previously described [25] and represented as relative growth respect the untreated strain.

Gene disruptions and plasmid construction

The \( S.\) cerevisiae strains used in this work are listed in the Table 1. Single \( \text{kanMX} \) deletion mutants in the BY4741 background were generated in the context of the Saccharomyces Genome Deletion Project [26]. Replacement of the \( PT\text{C6} \) coding region by the \( \text{nat1} \) marker from Streptomyces noursei was accomplished as follows: the 1.40 kbp DNA fragment containing the \( \text{nat1} \) gene, flanked by genomic sequences corresponding to -40/+5 and +1352/+1352 relative to the \( PT\text{C6} \) ATG codon, was amplified from the plasmid pAG25 [27] with the oligonucleotides 3'-\( PT\text{C6}-\)disr_\( \text{nat} \) and 3'-\( PT\text{C6}-\)disr_\( \text{nat} \) (Table S1). The \( ptc6::\text{nat1} \) disruption cassette was transformed in the appropriate strains and positive clones were selected in the presence of 100 \( \mu \)g/ml neomycin (Werner BioAgents). Double mutants \( ptc2\) \( \text{ptc6} \) and \( ptc3\) \( \text{ptc6} \) were constructed by introducing the \( ptc6::\text{nat1} \) disruption cassette in the \( ptc2::\text{kanMX} \) and \( ptc3::\text{kanMX} \) mutants from the BY4741 deletion bank.

The plasmids containing Lac\( \text{Z} \) translational fusions with the promoters of \( GAP1,\) \( GLN1,\) \( GDH1 \) and \( M\text{EPI} \) have been previously described in [19]. The construction of plasmids \( \text{YEp195-PTC1} \) and \( \text{YEp195-PTC1}_{\text{D\text{O\text{G}}IN}} \) was reported earlier in [28] and [19], respectively. Plasmid \( p\text{JU676} \) (pRS416-SCH9-5HA) was a gener-

Table 1. Yeast strains used in this work.

| Name | Relevant genotype | Source/Reference |
|------|------------------|-----------------|
| BY4741 | MATa his3Δ1 leu2Δ1 met15Δ1 ura3Δ1 | [26] |
| AG571 | BY4741 \( ptc6::\text{nat1} \) | This work |
| MAR14 | BY4741 \( ptc1::\text{nat1} \) | [52] |
| CCV190 | BY4741 \( ptc2::\text{kanMX4} \) \( ptc6::\text{nat1} \) | This work |
| CCV191 | BY4741 \( ptc3::\text{kanMX4} \) \( ptc6::\text{nat1} \) | This work |
| AG560 | BY4741 \( ptc6::\text{kanMX4} \) \( ptc1::\text{nat1} \) | This work |
| CCV22 | BY4741 \( ptc3::\text{kanMX4} \) \( ptc6::\text{nat1} \) | This work |
| CCV24 | BY4741 \( \text{tcp}1::\text{kanMX4} \) \( ptc6::\text{nat1} \) | This work |
| AG572 | BY4741 \( \text{tcp}1::\text{kanMX4} \) \( ptc6::\text{nat1} \) | This work |
| AG573 | BY4741 \( \text{tcp}4::\text{kanMX4} \) \( ptc6::\text{nat1} \) | This work |
| AG574 | BY4741 \( \text{sit}4::\text{kanMX4} \) \( ptc6::\text{nat1} \) | This work |
| CCV25 | BY4741 \( \text{tcp}5::\text{kanMX4} \) \( ptc6::\text{nat1} \) | This work |
| CCV26 | BY4741 \( \text{tcp}6::\text{kanMX4} \) \( ptc6::\text{nat1} \) | This work |
| AG575 | BY4741 \( \text{pdz2::kanMX4} \) \( ptc6::\text{nat1} \) | This work |
| AG576 | BY4741 \( \text{pkp1::kanMX4} \) \( ptc6::\text{nat1} \) | This work |
| AG577 | BY4741 \( \text{pdb1::kanMX4} \) \( ptc6::\text{nat1} \) | This work |
| CCV29 | BY4741 \( \text{pep4::kanMX4} \) \( ptc6::\text{nat1} \) | This work |
| CCV30 | BY4741 \( \text{agt1::kanMX4} \) \( ptc6::\text{nat1} \) | This work |
| YM70 | W303-1A Ifh1-myc13 | [45] |
| AG582 | W303-1A Ifh1-myc13 \( ptc6::\text{nat1} \) | This work |

The single \( \text{kanMX} \) deletion mutants in the BY4741 background that were generated in the context of the Saccharomyces Genome Deletion Project are not listed here. doi:10.1371/journal.pone.0064470.t001
ous gift from R. Loewith (Department of Molecular Biology Sciences, University of Geneva, Switzerland).

RNA purification, cDNA synthesis and DNA microarray analysis

For RNA purification, 30 ml of yeast cultures of wild type BY4741 and its derivatives mutant strains were grown at 28°C in YPD medium until A600 0.6–0.8 and, when required, treated with 200 ng/ml rapamycin or drug vehicle alone (90% ethanol and 10% Tween P20) for 1 hour. Cells were harvested by centrifugation and washed with cold water. Dried pellets were kept at −80°C until RNA purification. Total RNA was extracted using the RiboPure-Yeast kit (Ambion) following the manufacturer’s instructions. RNA quality was assessed by electrophoresis in denaturing 0.8% agarose gel and quantified by measuring A260 in a BioPhotometer (Eppendorf). Transcriptional analyses using the GenePix Pro 6.0 software. Spots with either a diameter smaller than 400 μm or fluorescence intensities for Cy3 (indocarbocyanine) and Cy5 (indodicarbocyanine) lower than 150 units, were not considered for further analysis.

Three different sets of microarray experiments were performed. In the first set of experiments we compared the expression profiles of ptc6 mutant cells with that of wild type cells by performing two independent experiments (biological replicates), each in duplicate (dyes were swapped to avoid dye-specific bias). In the second series of experiments, we compared the transcriptomic profiles of ptc1 ptc6 double mutant cells with that of wild type cells. Two independent experiments were performed, each in duplicate. For these two sets of experiments we only considered for further analysis genes with data in at least two out four spots. In the last set of experiments we compared the transcriptomic profiles of WT, ptc6, and ptc1 ptc6 cells in the presence and the absence of rapamycin. In this case, data from duplicate experiments were combined, and the mean was calculated. A given gene was considered to be induced or repressed when the expression ratio was higher than 2.0 or lower than 0.50, respectively. The GEPAS3.0 software, now implemented in the Babelomics tool (http://babelomics.bioinfo.cipf.es/), was used to pre-process the data [33]. The MIPS Functional Catalogue Database [34], at http://mips.helmholtz-muenchen.de/proj/lucatDB/search_main_frame.html, and Gene Ontology Enrichment tool available at YeastMine [35] (http://yeastmine.yeastgenome.org), were used for the functional distribution of gene lists.

Different levels of dependence on Ptc6 were defined as “totally dependent” (TD), “strongly dependent” (SD), “weakly dependent” (WD) and independent, according to the expression of up- or down-regulated genes after rapamycin treatment in ptc6 cells in comparison with wild type cells, as previously reported [36]. The data discussed in this publication have been deposited in NCBI’s Gene Expression Omnibus database [37] and are accessible through GEO SuperSeries accession number GSE38260.

Chromatin immunoprecipitation assays

Chromatin cross-linking and immunoprecipitation were carried out based on previously described methods [38] with several modifications. Forty ml cultures were grown up to OD660 0.6–0.8 on YPD medium, and cells were exposed to 200 ng/ml rapamycin for 5, 15, 30 and 45 min. Then, cells were treated with 1.1 ml of 37% formaldehyde (1% final concentration) for 15 min at 24°C and quenched by addition of 2 ml of 2.5 M glycerol for 5 min at 24°C. Cells were collected by centrifugation and washed twice with 10 ml of ice-cold HBS (50 mM HEPES-KOH, pH 7.5, 140 mM NaCl) and once with 1.5 ml of Lysis buffer (50 mM HEPES-KOH, pH 7.5, 140 mM NaCl, 1 mM EDTA, 1% Nonidet P 40, 0.1% sodium deoxycholate). The pellet was resuspended in 300 μl of Lysis buffer with 1 mM phenylmethylsulfonyl fluoride (PMSF) and complete protease inhibitor mixture (Roche Applied Science). One volume of zirconia-silica 0.5 mm beads (BioSpec) was added and cells were broken at 4°C by vigorous shaking (5 times for 25 s each at setting 5.5, with intervals of 1 min on ice) in a Fast Prep cell breaker (FastPrep 24, MP Biomedicals). The chromatin was sheared using a Bioruptor Plus UCD-300 sonication device (Diagenode) (high intensity, ten cycles of 30 s sonication interspersed with 60 s pause). The cleared lysate (whole cell extract, WCE) was recovered by centrifugation at 9300 x g for 5 min at 4°C. For chromatin immunoprecipitation, 50 μl of Protein G-Sepharose (GE Healthcare) was coupled to 2.5 μg of anti e-myc monoclonal antibody (Covance). The anti-myc-Protein G-Sepharose complexes were incubated overnight with 200 μl of WCE (4 mg) at 4°C in a rotator. Sepharose-protein complexes were transferred to 96-well filter plates (MultiScreen, Millipore) and extensively washed at 4°C with 250 μl of the indicated solutions as follows: Four times with lysis buffer for 1 min, four times with lysis buffer containing 500 mM NaCl, twice with washing buffer with 10 mM Tris-HCl, pH 7.5, 1 mM EDTA, 250 mM LiCl, 0.5% Nonidet P-40, 0.5% sodium deoxycholate), and twice with TE (10 mM Tris-HCl, pH 7.5, 1 mM EDTA). Washes were discarded by centrifugation at 180°C in a rotator. Protein-DNA complexes were recovered from beads by incubation with 80 μl of elution buffer (50 mM Tris-HCl, pH 8.0, 10 mM EDTA, 1% SDS) at 65°C for 20 min. The supernatant was removed (60 μl), 240 μl of elution buffer were added, and samples were incubated overnight at 65°C. The eluted DNA was purified with phenol-chloroform, precipitated with isopropanol, and dissolved in 30 μl (immunoprecipitated samples) or 30 μl of TE (WCE samples), and stored at −20°C. For PCR assays, 30 ng of the immunoprecipitated DNA was used. Oligonucleotides for PCR were designed to amplify 50- to 100-bp fragments (Table S1).

Western blot and chemical fragmentation analysis

The Sch9 detection experiments were performed essentially as described in [22]. Briefly, yeast strains were grown on YPD to OD660 0.5–0.6 at 28°C. Nine ml of cultures were mixed with TCA (final concentration 6%) and put on ice for at least 10 min before cells were pelleted at 1680xg for 2 min. The pellet was washed twice with 1 ml cold acetone and dried at 37°C. One-hundred μl of urea buffer (50 mM Tris pH 7.5, 5 mM EDTA, 6 M urea, 1% SDS, 1 mM PMSF) was added to the pellet and cell lysis was...
performed with zirconia-silica 0.5 mm beads (BioSpec) by vigorous shaking in a Fast Prep cell breaker (FastPrep 24, MP Biomedicals) as described above. Then, samples were heated for 10 min at 65°C. For NTCB (2-nitro-5-thiocyanatobenzoic acid) cleavage, 30 µl of 0.5 M CHES (pH 10.5) and 20 µl of NTCB (7.5 mM in H2O) were added and samples were incubated overnight at 24°C. Then, one volume of 2× sample buffer was added. Samples were fractionated by SDS-PAGE in 7.5% polyacrylamide gels and transferred to Immobilon PVDF membranes (Millipore). Membranes were incubated overnight with anti-β-actin antibody (Covance) at 1:20000 dilution followed by the secondary HRP-conjugated anti-mouse IgG antibody (GE Healthcare) at 1:20000 dilution. The immunocomplexes were visualized using ECL Western blotting detection kit (GE Healthcare) at 1:20000 dilution. The immunocomplexes were visualized using ECL Western blotting detection kit (GE Healthcare). Chemiluminescence was detected using a LAS-3000 equipment (Fuji).

For Ac1 detection, whole cell lysates (10 µl of culture) were prepared by resuspending the cells in 200 µl of extraction buffer (50 mM Tris-Cl pH 7.5, 150 mM NaCl, 0.1% Triton X-100, 1 mM dithiothreitol, 10% glycerol, 2 mM phenylmethysulfonyl fluoride and Complete inhibitor mixture (Roche Applied Science)). One volume of zirconia-silica 0.5 mm beads (BioSpec) was added and cells were broken at 4°C by vigorous shaking as above. After sedimentation at 500 g for 10 min at 4°C, the cleared lysate was recovered and the protein concentration was determined by Bradford assay. Total proteins (40 µg) were fractionated by SDS-PAGE in 10% polyacrylamide gels and transferred to Immobilon PVDF membranes (Millipore). Membranes were incubated overnight with anti-Aco1 antibody (a generous gift of Dr. S. Atriañ, Universitat de Barcelona) at 1:10000 dilution followed by the secondary HRP-conjugated anti-rabbit IgG antibody (GE Healthcare) at 1:20000 dilution. The immunocomplexes were visualized using ECL Western blotting detection kit (GE Healthcare). Chemiluminescence was detected using a LAS-3000 equipment (Fuji).

Other techniques

Total RNA was prepared as described above. Semi-quantitative RT-PCRs were performed using 100 ng of total RNA (except for MEPI amplifications, where 200 ng were used) and the Ready-To-Go RT-PCR Beads kit (GE Healthcare). Specific pairs of oligonucleotides were used (Table S1) to determine the levels of GAP1 and ACT1 (25 amplification cycles) and MEPI (30 amplification cycles). The PCR products were visualized in 2% agarose gels.

For quantitative RT-PCR, cDNA was synthesized from 1.5 µg of each RNA with the Superscript III First-Strand Synthesis System (Life Technologies). Five ng of each cDNA were used as a template, together with the pairs of oligonucleotides specified in the Table S1 and the Power SYBR green PCR Master mix (Applied Biosystems). Amplification reactions were carried out in a StepOnePlus Real-Time PCR System (Applied Biosystems). Fold-changes were calculated using the 2−ΔΔCt method.

Evaluation of the promoter activity of diverse NCR-sensitive genes in response to rapamycin or to Low Ammonium Medium was performed using LacZ-reporters as described in [19].

Vacuolar staining and visualization was carried out using the lipophilic styryl dye FM4-64 (Molecular Probes) as described in [39].

Msn2 subcellular localization was performed using the pMSN2-GFP plasmid (a generous gift of F. Estruch, University of Valencia) as described in [19].

Results

Functional characterization of the ptc6 mutant

Because of the striking rapamycin-sensitive phenotype of ptc6 cells, we considered necessary to carry out a comprehensive analysis of phenotypes derived from this mutation, and compare the phenotypes with those of the ptc1 mutant, the only other ptc mutant that has been reported as sensitive to rapamycin [19]. We have found that cells lacking ptc6, in contrast to ptc1 mutants, are not sensitive to high temperature (37°C), alkaline pH, or high amounts of calcium, copper or iron (not shown) and can grow on glycerol and ethanol as carbon sources (not shown). Similarly, the vacuolar structure of ptc6 cells appears to be normal, whereas it is highly fragmented in the ptc1 mutant (Figure 1A). The ptc6 mutation renders cells slightly sensitive to zinc, albeit to less extent than that of PTC1. Remarkably, the ptc6 mutant is somewhat tolerant to cell-wall damaging agents such as calcofluor white or Congo Red, as well as to Li+ ions (Figure 1B) and, in fact, lack of Ptc6 improves to some extent the deficient growth caused by the ptc1 mutation under these conditions.

To gain insight into the possible cellular roles of Ptc6, we investigated by microchip analysis the changes in the global transcriptional profile caused by the ptc6 mutation in cells growing under standard conditions. Disappointingly, we observe that lack of PTC6 had almost no effect on the expression profile. The level of mRNA of only two genes, excluding PTC6 itself, was found decreased (FIT3 and TIS11) and, although mRNA levels of several genes involved in the lysine biosynthesis (LYS1, LYS20, LYS21, LYS112 and LYS19) were slightly increased, they did not reach the defined threshold (2-fold increase). In any case, when we compared the reported expression profile of the ptc1-5 mutants [29], obtained using the same DNA microarray platform and yeast genetic background, together with that of ptc6 cells, it became evident that the expression profile of ptc6 cells is quite different of that obtained for the ptc1-5 mutants (Fig. 1C). We also evaluated the expression profile of ptc1 ptc6 cells. Simultaneous deletion of PTC1 and PTC6 genes provoked more transcriptional changes than those observed previously for the single deletion of the PTC1 gene (Fig. 1D). We found that the levels of mRNA for 80 genes were upregulated, whereas 14 genes, including PTC1 and PTC6, were down-regulated in ptc1 ptc6 double mutant cells, when compared to the wild type strain (see Tables S2 and S3 for the complete list of genes).

A substantial number of genes considered up-regulated in ptc1 ptc6 cells (30 genes or 37.5% of the induced) were either related to the cell wall function or were found induced by cell-wall stress [40], as previously described for the ptc1 mutant [29]. In fact, 18 out 20 genes whose expression was up-regulated at least two-fold in ptc1 mutant cells [29] with valid data for the ptc1 ptc6 mutant, were also up-regulated in ptc1 ptc6 cells. The expression of the two other genes was either found consistently elevated (NCA3) or not induced at all (PHO89) in ptc1 ptc6 cells.

Changes in the expression levels were quantitatively higher in the ptc1 ptc6 double mutant than in ptc1 cells. The average of the values of induction (in log2) for the set of 18 genes found simultaneously up-regulated (more than 2-fold) in both strains was higher in ptc1 ptc6 (1.93, equivalent to 3.82-fold) than in ptc1 cells (1.45, equivalent to 2.74-fold). This is also true when only the set of genes involved in cell wall integrity was considered.

Among the 60 genes specifically induced in ptc1 ptc6 but not in ptc1 or ptc6 cells it is worth noting the presence of a set of 17 genes involved in carbon-compound and carbohydrate metabolism (p-value: 3.88E-06), six of them (TSL3, GLC3, GFP2, GSC2, PGM2 and GDB1) were involved in the metabolism of energy reserves (p-
value: 1.51E-05). We also found 7 genes (ARN2, PDR5, FIT3, MEP2, ARN1, FIT1 and FIT2) involved in ion transport (p-value: 5.71E-04).

Taken together, the comparison of the phenotypic and transcriptomic data presented here, as well as the previously reported results [29] suggest that the cellular functions of Ptc6 are substantially different from those of Ptc1 and also from Ptc2-Ptc5. Ptc6 and Ptc1 participate in the TOR signaling pathway by different mechanisms

As previously described, the ptc1 strain is more sensitive to rapamycin than ptc6 cells. We show here that the double mutant ptc1 ptc6 was even more sensitive to this compound than ptc1 cells (Figure 2A). Interestingly, the rapamycin-sensitive phenotype of ptc1 is not altered at all by further deletion of PTC2 or PTC3, which encode the closest structural relatives to Ptc1 (data not shown). The observation that the mutation of PTC6 in a ptc1 background is specific and additive suggests that Ptc1 and Ptc6 phosphatases could interact with the TOR pathway at different levels. It has been reported that certain spo mutants fail to recover from rapamycin-induced arrest [41]. Since ptc1 shows diverse phenotypes alike to spo mutants [29] we tested the ability to resume growth in rapamycin-treated ptc1 cells after the drug is removed.

We observed that, indeed, the ptc1 and ptc1 ptc6 strains do not recover from the drug treatment (Fig. 2B). In contrast, ptc6 mutants recover nearly as a wild type strain, indicating that, in spite of the common rapamycin-sensitive phenotype, only Ptc1 is essential for resumption of growth after treatment with the drug. This result also supports the notion that Ptc1 and Ptc6 interact with the TOR pathway in a different manner.

It is reasonable to assume that if Ptc1 would be affecting the Ptc6 target(s) in the TOR pathway, its over-expression could rescue the hypersensitivity to rapamycin of the ptc6 mutant. As shown in Figure 2C, overexpression of native Ptc1 rescues the ptc1 phenotype and, as previously reported [19], is even able to confer some tolerance to rapamycin, whereas a catalytically impaired version of the phosphatase (D58N) does not. In contrast, overexpression of Ptc1 does not increase at all the tolerance of the ptc6 mutant to the drug.

Our previous results indicated that Ptc1 acts on the TOR pathway by regulating Tip41 or Sit4 function, since the deletion of PTC1 in sit4 or tip41 cells did not alter the phenotype displayed by these mutants [19]. We now deleted the PTC6 gene in strains carrying mutations in diverse non-essential components of the pathway (Fig. 3A), and the sensitivity to rapamycin was tested measuring the growth in liquid cultures. As observed in Fig. 3B
deletion of PT6 increased the rapamycin hypersensitive phenotype of tor1 cells, similarly to that observed for the deletion of PTC1 [19]. However, contrary to the previously observed for the ptc1 mutants, deletion of the PT6 gene in the sit4 or tip41 backgrounds decreased the tolerance to the drug. Therefore, our data suggest that, in contrast to what happens in the case of ptc1, the sit4 and tip41 mutations are not epistatic to the ptc6 mutation.

Therefore, taking together these results indicate that both Ptc6 and Ptc1 are involved in the normal functioning of the TOR signaling pathway but they are affecting different mechanisms.

Rapamycin-induced mitophagy is blocked in ptc6 mutants growing on glucose

One of the events controlled by the TOR signaling pathway, together with Ras-PKA signaling and the general stress response pathways, is mitophagy, a vacuole-dependent mitochondrial degradation process [42]. Several circumstances have been described that lead to yeast mitophagy, including those that inhibit the TOR pathway, such as entry in stationary phase, nitrogen starvation or treatment with rapamycin [11]. By following the degradation of the mitochondrial protein aconitase (Aco1), a well-established method to monitor mitophagy [11], we confirmed that rapamycin treatment in wild type cells exponentially growing in YPD medium induced mitophagy, since the protein was not detectable after 3 h of treatment with the compound (Fig. 4A). In contrast, when ptc6 mutant cells were exposed to the same concentration of rapamycin, Aco1 was still detectable for at least 6 h, indicating that rapamycin-induced mitophagy in wild type cells is, at least in part, dependent of Ptc6. This behavior is similar to that of cells lacking one of the main vacuolar hydrolases (the proteinase A, encoded by the PEP4 gene), which have been shown unable to degrade Aco1 when growing on lactate upon rapamycin incubation [43]. Thus, cells lacking Ptc6 have an impaired mitophagy phenotype when treated with rapamycin in medium containing glucose as carbon source. Therefore, our results extend those reported by Tal and coworkers showing that Ptc6 was required for efficient mitophagy in prolonged stationary-phase incubation in medium containing lactate, a non-fermentable carbon source [11]. Ptc6 is also required for the delay in Aco1 degradation observed in wild type cells treated with rapamycin in a non-fermentable carbon source such as glycerol (Fig. 4A). We next asked whether the ptc6 mutant would be similarly sensitive to rapamycin irrespective of the carbon source. Interestingly, as shown in Figure 4B, wild type and ptc6 cells display a similar sensitivity to rapamycin, in spite that the wild type strain still shows some degree of mitophagy and the ptc6 mutant does not (Fig. 4A), thus indicating that mitophagy and the rapamycin-sensitive phenotype could be dissociated. This notion is further reinforced by the observation that there are strains, such as the uhl1 mutant, that are unable to induce mitophagy in lactate-grown cells challenged with rapamycin, but are tolerant to rapamycin irrespectively of the carbon source (43), Figure 4C).

In fact, deletion of the PT6 gene decreases tolerance to rapamycin of the uhl1 mutant (Figure 4C).

The rapamycin-sensitive phenotype of the ptc6 mutant is independent of the lack of PDH activity

The only cellular target for Ptc6 described so far is Pda1, which is also dephosphorylated by Ptc5. As shown in Figure 5A, five proteins form the three structural components of the PDH complex. In addition, two protein phosphatases (Pc6 and Ptc5) and two protein kinases (Pkp1 and Pkp2) are regulatory elements, responsible for the increase and decrease of PDH activity, respectively. Interestingly, Gey and coworkers demonstrated that lack of Ptc6 causes a dramatic drop in the PDH activity and it has been reported that deletion Pda1 [13] or Pdb1 [18] results in sensitivity to rapamycin. Therefore, it could be speculated that a link between the rapamycin-sensitive phenotype of ptc6 cells and the role of the phosphatase in activating PDH activity could exist.

To test this possibility we evaluated the sensitivity to rapamycin of mutants in genes involved in regulation of PDH activity in the presence and in the absence of the PT6 gene. As shown in Figure 5B, deletion of PT6 in the ptc5 background (which is not rapamycin-sensitive) decreases tolerance to the drug to achieve ptc6 levels. Remarkably, we observe that the pkip1 strain, which does not show a decrease in PDH activity [13,18], is also hypersensitive to rapamycin and that deletion of PT6 further decreases the tolerance to the drug. Similarly, lack of Ptc6 decreases tolerance to rapamycin in cells lacking structural components of the PDH complex, such as in the pdb1, lat1 or lpd1 strains (Figure 5C).

Therefore, deletion of PT6 decreases rapamycin tolerance irrespectively of an increase or decrease of PDH activity, thus suggesting that decreased PDH activity is not the cause of the hypersensitivity to rapamycin described for the ptc6 mutant. Because the slightly increased sensitivity to rapamycin observed in mutant cells lacking either structural components of the PDH complex or the Pkip1 gene, we cannot discard a role of these components in the pathway regulated by rapamycin.

The rapamycin-induced transcriptional response is attenuated in ptc6 mutant cells

Short-term treatment of wild type cells with rapamycin results in strong remodeling of gene expression. To identify the effect of ptc6
mutation in the global expression pattern after rapamycin treatment, we performed microarray experiments comparing the rapamycin-induced transcriptional response of wild type and \textit{ptc6} mutants. Considering only the genes with valid data in both experiments (3332 genes) we found that rapamycin caused changes in the expression levels of 1115 genes (33.4%) in wild type cells (476 up-regulated and 639 down-regulated). In \textit{ptc6} mutant cells, rapamycin changed the levels of 884 transcripts (26.5%), being the number of up-regulated and down-regulated genes of 375 and 509, respectively (Fig. 6A, upper panel). In the case of the \textit{ptc1 ptc6} mutants, from the total number of genes with valid data (3355), the rapamycin-induced genes were 417 (12.4%) and the down-regulated ones were 564 (16.8%) (Fig. 6A, lower panel). These figures suggest that lack of Ptc6 may cause an attenuation of the transcriptional response to rapamycin. The alterations provoked by the \textit{ptc1} and \textit{ptc6} mutations in the response induced by rapamycin were verified in a set of four rapamycin-responsive, nitrogen catabolite repression (NCR) regulated genes (\textit{GAP1, GLN1, GDH1} and \textit{MEP1}), required for adaptation to non-preferred nitrogen sources and whose expression is controlled by the Gln3 transcription factor. As shown in Fig. 6B, when cells were challenged with rapamycin, it became evident that the response of these genes to the drug was attenuated in Ptc6-deficient cells, although somewhat less than in \textit{ptc1} cells. The transcription attenuation of the \textit{GAP1} and \textit{MEP1} genes in response to rapamycin in the \textit{ptc6} strain was also validated by RT-PCR (not shown).

To explore the extent of the transcriptional attenuation in the response to rapamycin caused by the \textit{ptc6} mutation, we plotted the transcriptional changes (log2 values) for wild type (X axis) and \textit{ptc6} (Y axis) after rapamycin treatment, and the value of the slope of the fitted line obtained by simple linear regression was taken as an “attenuation index” (Figure S1). Therefore, if the global responses to the drug in both strains were similar, the expected slope value would be close to unity, whereas a weakened response in the \textit{ptc6} cells would decrease this index. The values of the slope for the genes considered up- and down-regulated by rapamycin in \textit{ptc6} cells respect wild type cells were 0.8186 and 0.7424, respectively [19]. We also performed a similar calculation with the \textit{ptc1 ptc6} cells, and the corresponding slopes were 0.6671 and 0.8869 (Figure S1). Thus, changes in the transcriptional profiles in response to rapamycin were attenuated in all three mutants analyzed, being this attenuation more relevant for the down-regulated genes in the \textit{ptc6} strain, and for the up-regulated genes in the \textit{ptc1 ptc6} strain.

One of the cellular responses to rapamycin is the translocation to the nucleus of the Msn2 transcription factor that collaborated in the regulation of the general stress response. We have already described that lack of Ptc1 prevents the nuclear translocation of Msn2 in response to rapamycin [19]. When similar experiments were performed with \textit{ptc6} mutant cells, we observed only a moderate decrease in the number of cells with nuclear localization.

Figure 3. Epistatic analysis of \textit{ptc6} and mutations affecting the TOR pathway. A) A simplified model of signaling through the TOR pathway, focused on the regulation of NCR genes and displaying the proposed role for Ptc1 [19]. B) Growth in the presence of the indicated concentrations of rapamycin of diverse mutants in genes involved in the TOR pathway in combination with deletion of \textit{PTC6}. Relative growth is represented as a percentage respect the growth of each strain in YPD without rapamycin. Six experiments were performed and the mean ± SEM is represented.

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of Msn2, when compared to wild type cells (Figure S2A). Consistently, when the attenuation in the expression change in ptc6 cells of genes known to be under the regulation of Msn2/ Msn4 was compared with that of genes independent of these transcriptional factors, no significant differences were observed (Figure S2B). Cells lacking both genes, PTC1 and PTC6, however, resulted in a stronger prevention of the nuclear localization that the observed for ptc1 cells.

Pt6 is important for the rapamycin-induced down-regulation of the genes involved in ribosome biogenesis

We next investigated if the ptc6 mutation affected specific gene families whose expression was altered by rapamycin-treatment. We have found that the expression level of 476 genes was up-regulated by rapamycin in wild type cells, when genes with valid data for the ptc6 strain were considered. The most relevant category among these genes was the metabolism of aminoacids (67 genes, p-value: 7.63E-22). Only 86 genes were, at some extent, dependent of Pt6. The additional 390 genes were up-regulated in a Pt6-independent manner. Among them, the family of genes involved in the metabolism of aminoacids was the most relevant (55 genes, p-value: 7.43E-18).

Concerning the 639 genes whose expression was found down-regulated by rapamycin in wild type cells we observed, in agreement with previous reports, a very strong predominance of genes related to protein synthesis (217 genes, p-value 4.98E-102), particularly those involved in ribosomal biogenesis and translation (Table S4).

We also observed a significant enrichment in genes related to transcription (144 genes, p-value 3.09E-5), predominantly those associated to rRNA processing (70 genes, p-value 3.87E-22). When the level of dependence on Pt6 for transcriptional down-regulation was examined, we found 290 genes (45.4%) exhibiting some degree of dependence (9 totally, 83 strong and 198 weakly dependent on Pt6). Interestingly, while globally considered, genes related to protein synthesis did not exhibit a particular trend regarding Pt6-dependence, the down-regulation of the subset corresponding to ribosome biogenesis showed a tendency to be Pt6-dependent, in particular those coding for ribosomal proteins (p-value of 4.68E-41 for the Pt6-dependent vs 6.14E-24 for the

Figure 4. Rapamycin-induced mitophagy is dependent on Pt6. A) Wild type BY4741 and isogenic ptc6 and pep4 derivatives were grown in YPD or YP Glycerol (2%) medium until OD660 1.0 and then rapamycin (200 ng/ml) was added. Samples were taken at the indicated times and 40 μg of total protein from each sample were analyzed by immunoblotting with anti-aconitase and anti-actin antibodies. Ponceau staining (Pon S) is shown as loading control. Quantification of band intensities was performed by the GelAnalyzer software (http://www.gelanalyzer.com) and shown as Aco1/ actin signals ratio. B) Wild-type strain BY4741 and the isogenic ptc6 mutant were spotted onto plates containing glucose (YPD) or glycerol (YPGly) as carbon source, and the indicated concentrations of rapamycin. Growth was monitored after 3 (YPD plates) or 4 days (YPGly plates) of incubation at 28° C. C) The indicated strains were grown in the presence of the different concentrations of rapamycin as described in the legend of Fig. 3B. doi:10.1371/journal.pone.0064470.g004
In general, the degree of dependence for this gene family was weak (Table S4). In contrast, rapamycin-repressed genes related to translation turned out to be largely independent of Ptc6. When genes involved in transcription were considered, we observed a predominance of Ptc6-dependent genes (Table S4). Remarkably, this tendency is not general for all functional subcategories, but it is particularly strong for genes related to rRNA processing (53 genes, p-value 5.34E-27, vs 17 genes, p-value 4.92E-2, for independent genes). Attenuation of repression of genes within this category in response to rapamycin was, in many cases, largely dependent on the presence of Ptc6, with predominance of TD- or SD- dependent genes (Table S4).

Ptc6 is not required for rapamycin-induced dephosphorylation of Sch9

It is known that TORC1 directly phosphorylates Sch9, a member of the AGC family of protein kinases, thus triggering expression of genes involved in ribosome biogenesis [22]. Interestingly, comparison of the response profile to rapamycin of cells carrying a constitutively active version of Sch9, that mimics a TORC1-phosphorylated form (SCH9<sup>202E</sup> allele) with that of the native protein reveals an attenuation of the response to drug that is qualitatively and quantitatively similar to that observed here for ptc6 mutants. Thus, when the “attenuation index” described above was calculated for all genes repressed after 90 min of exposure to rapamycin, a value of 0.706 was obtained, in close agreement with that obtained for ptc6 cells (0.742). The similarity extends to the differential level of dependence when gene families were considered. Repression of genes coding for proteins involved in rRNA processing showed an important grade of dependence for both Ptc6 and Sch9. These dependences were weaker in both cases for the set of genes related to the ribosomal protein synthesis (Figure 7A). Down-regulation of the genes of the regulon Ribi was also found attenuated in both mutants (Figure S3).

This raised the possibility that Ptc6 might exert its function in the TOR pathway by directly or indirectly dephosphorylating and inactivating Sch9. To test this, we examined the phosphorylation state of Sch9 in wild type cells and ptc6 mutants in response to
However, our results indicate that the phosphorylation state of Sch9 remains unchanged irrespective of the presence or absence of Ptc6, suggesting that the phosphatase is not responsible for the control of the kinase.

Down-regulation of the genes controlled by Ifh1 in response to rapamycin requires Ptc6

According to our microarray data, ptc6 mutant cells showed a weakened down-regulation of the gene expression caused by the inhibition of TOR for genes encoding cytosolic RP (ribosomal proteins). These results were also validated by quantitative RT-PCR (Figure S4) and semiquantitative RT-PCR (not shown) for three RP and a member of the Ribi regulon. Therefore, we hypothesized that Ptc6 might be involved in the regulation of expression of these genes. Ifh1 is a co-activator of the Forkhead-like Fhl1 transcription factor that is recruited to the promoters of the ribosomal protein encoding genes during optimal growth conditions by Fhl1 and is absent when transcription is repressed.
Rapamycin treatment is one of the known situations that decrease the binding of Ifh1 to RP promoters [45]. Therefore, it was conceivable that Ptc6 might influence binding of Ifh1 to its target promoters. To test this hypothesis we examined the binding of a myc-tagged version of Ifh1 to the promoters of diverse RP genes after rapamycin treatment in both wild type and ptc6 cells by chromatin-immunoprecipitation (ChIP) followed by PCR assays. For all three RP genes analyzed, we detected a marked decrease (50–60%) of Ifh1 binding to the promoters after 5 min of rapamycin treatment in wild type cells and this effect was even more prominent at longer times (60% of binding after 45 min). In ptc6 cells, however, Ifh1 binding was clearly higher for the promoters of RPL16A and RPL37A, particularly after 30 minutes of exposition to rapamycin (Figure 8). It is worth noting that RPL16A was found to be strongly dependent on Ptc6 according to our microarray data, whereas for RPL30 the effect of lack of the phosphatase was only marginal. Although no microarray data for RPL37A was available, quantitative RT-PCR results (Figure S4) indicate a significant effect of the ptc6 mutation on RPL37A mRNA accumulation. This suggests that the attenuation of the repression of the genes encoding RP described for the ptc6 mutant cells could be due, at least in part, to a defect in releasing Ifh1 from their promoters.

Discussion

We have accomplished an extensive characterization of the phenotypes shown by Ptc6-deficient cells, identifying novel phenotypes for the ptc6 mutant cells not reported in a previous work that characterized mutants in non-essential catalytic subunits of protein phosphatases [46]. The transcriptional profiling of the ptc6 mutants is more similar to that of the ptc1 strain than to other ptc-deficient cells, such as ptc5 (Figure 1C). This is somewhat surprising, since it has been proposed that ptc5 and ptc6 share the same subcellular location (mitochondria) and a regulatory function on the PDH complex [13]. In spite of the similarity of their transcriptional profiles, most ptc6 phenotypes differ from those displayed by ptc1 mutant. For example, ptc1 cells were hypersensitive to alkaline pH as well as to high calcium concentrations [29], phenotypes that are landmarks of deficient vacuolar function [47]. Indeed, cells lacking PTC1 displayed highly fragmented vacuoles. In contrast, ptc6 cells were not sensitive to either high pH or calcium and, accordingly, their vacuolar morphology was normal.
Another relevant difference between *ptc6* and *ptc1* strains is that *ptc6* cells are tolerant to both, cell-wall stressors and excess of LiCl, while *ptc1* cells are sensitive. However, *ptc1* and *ptc6* cells do share some phenotypes, such as hypersensitivity to rapamycin, suggesting that Ptc6 may be involved in the TOR pathway. Nevertheless, several lines of evidence suggest that both enzymes impact the TOR pathway at different levels: i) the rapamycin-sensitive phenotypes of the *ptc1* and *ptc6* mutations are additive. ii) overexpression of *PTC1* cannot rescue the rapamycin-sensitive phenotype of the *ptc6* strain, iii) exposure to high concentrations of rapamycin causes an irreversible halt in growth in *ptc1* mutants, whereas Ptc6-deficient cells can survive when the drug is removed from the medium, and iv) the *ptc6* mutation does not show the epistatic relationships with relevant components of the TOR pathway displayed by the *ptc1* mutation [19].

Early work on Ptc6 [13] suggested that the rapamycin-sensitive phenotype of the *ptc6* mutant could derive from the proposed role of the phosphatase in activating PDH activity. However, we cannot find a relationship between the effect of a given mutation on PDH activity and the effect of such mutation on rapamycin sensitivity. For instance, using liquid cultures we observe that lack of *ptc6*, which should inhibit Pda1, causes a stronger rapamycin-sensitive phenotype than deletion of the *PDA1* gene (not shown). Similarly, deletion of both *PTC5* on the *ptc6* background, which should fully eliminate the ability to dephosphorylate the PDH complex, does not result in increased sensitivity to rapamycin. More importantly, lack of *PKP1*, encoding a Pda1 kinase, also results in sensitivity to rapamycin which is further aggravated by lack of Ptc6 (Figure 5B). Therefore, our results do not support the hypothesis linking the sensitivity to rapamycin of the *ptc6* mutant and the role of this phosphatase in the regulation of PDH activity. Similarly, our data also demonstrate that whereas the rapamycin-sensitive phenotype of the *ptc6* mutant is dependent on the carbon source, it cannot be linked to the occurrence of mitophagy upon exposure to rapamycin (Figure 4).

We observed that the expression of genes coding for proteins involved in both the ribosome biogenesis and the rRNA processing were down-regulated in a Ptc6 dependent manner (Table S4). It is known that repression of these sets of genes after inhibition of the TOR pathway may be under the control of Sch9 [22,48] and that Sch9 is activated by phosphorylation [22]. Therefore, it could be hypothesized that Ptc6 might directly or indirectly promote Sch9 dephosphorylation and deactivation. However, we did not detect changes in the phosphorylation state of Sch9 in the absence of Ptc6 and, consequently, we conclude that Ptc6 must have targets other than Sch9. This would be in agreement with the fact that, whereas it has been reported that Sch9 mediates TORC1 regulation of transcription initiation [22], we find that rapamycin-induced repression of most genes related to this function is largely independent of the presence of Ptc6 (Table S4). Similarly, whereas Sch9 is not involved in the expression of Gln3-regulated genes [22], we observe attenuated expression of this kind of genes.
in ptc6 cells (Figure 6B), indicating the existence of alternative Ptc6 cellular targets.

Expression of ribosomal protein- and pre-rRNA-processing-encoding genes is also under the control of the Ifh1 forkhead transcription factor [44,49]. When the TOR pathway is active, the coactivator Ifh1 binds to Ifh1, thus promoting expression of Ifh1-regulated genes. Inactivation of the TOR pathway results in Yak1-mediated phosphorylation of the Crf1 corepressor, promoting its binding to Ifh1, displacement of Ifh1, and switching off transcription of the Ifh1-regulated genes [49]. We observe that in cells lacking Ptc6, rapamycin-induced release of Ifh1 from Ifh1-regulated promoters is delayed or abolished. Since failure to effectively release Ifh1 from its target promoters would interfere with transcriptional switch off, this might contribute to explain, at least in part, the attenuation of rapamycin-induced repression of genes involved in ribosome biogenesis. The mechanisms for this effect would be open to conjecture. One possibility is that Ptc6 may regulate the phosphorylation state of Crf1. If so, Ptc6 could not act as a Crf1 phosphatase, since lack of Ptc6 would lead to hyperphosphorylation of Crf1 and this would lead to the potentiation of the repressor effects of rapamycin on target genes expression. It would be possible, however, that Ptc6 could negatively regulate the input of the TOR pathway on Yak1 activation. It must be noted that in vivo phosphorylation of Ifh1 has been reported in high-throughput studies. Interestingly, it has been recently shown that Ifh1 can be phosphorylated in vitro by Yak1 [50]. Therefore, the hypothetic regulation of Yak1 activity by Ptc6 could also impact on Ifh1 itself. In addition, it has been shown that Ifh1 is in a complex with casein kinase 2 (CK2), Utp22 and Rrp7 (CURI complex) are implicated in the processing of pre-rRNA and that CK2 phosphorylates in vitro Ifh1 [44]. Therefore, CK2-mediated phosphorylation of Ifh1 could also be a target for Ptc6 function.

Supporting Information

Figure S1 Linear regression analyses were used to estimate the transcriptional attenuation caused by the lack of ptc6 or ptc1 and ptc6. Linear regression analysis of the plotted values for the changes in the level of expression triggered by rapamycin in wild type and in the indicated mutant strains. The obtained equation is indicated for each case. A) Set of 476 genes up-regulated by rapamycin in wild type cells plotted against their expression value in the ptc6 mutant. B) Set of 639 genes down-regulated by rapamycin in wild type cells plotted against their expression value in the ptc6 mutant. C) Set of 494 genes up-regulated by rapamycin in wild type cells plotted against their expression value in the ptc1 ptc6 mutant. D) Set of 619 genes down-regulated by rapamycin in wild type cells plotted against their expression value in the ptc1 ptc6 mutant. (TIF)

Figure S2 Lack of Ptc6 moderately affects rapamycin-induced down-regulation of the Msn2/Msn4-controlled genes. A) Intracellular localization of Msn2-GFP at the indicated times after addition of rapamycin to the cultures of WT, ptc1, ptc6 and ptc1 ptc6 cells. Cells from each strain were distributed into three categories according to the intracellular localization of Msn2-GFP: cytosolic (black bars), cytosolic and nuclear (grey bars) and nuclear (white bars). B) Plots of the log2 values for the changes in the level of expression consequence of the treatment with rapamycin in both WT (dots) and ptc6 strains (closed triangles) for the 150 most up-regulated genes (top panel) and 150 most down-regulated (bottom panel) genes in the WT strain. The expression values for the genes documented targets of Msn2 or Msn4 described in YEASTRACT [53] plus those identified elsewhere [54] in ptc6 cells are denoted as open squares. (TIF)

Figure S3 Comparison of transcriptional changes in ptc6 mutants and cells expressing a constitutive active version of Sch9. Representation of the averages of the changes in the level of expression (in log2) induced by rapamycin for the genes included in the specified categories that constitute the Ribi regulon [55]. Left panel shows data corresponding to yeast (W303 background) expressing normal and constitutively active Sch9 (Sch9DDDD) after 90 min treatment with rapamycin obtained from the GEO database (series reference GSE7660) [22]. Right panel shows data from the experiments described in this work. (TIF)

Figure S4 ptc6 mutant cells exhibit an attenuation in the repression of genes involved in the ribosome biogenesis caused by rapamycin. Wild type BY4741 and its isogenic ptc6 derivatives were grown in YPD and treated with rapamycin as described in Figure 4A. Samples were collected at different times and total RNA was prepared. Quantitative RT-PCR were performed by duplicate using specific oligonucleotides, as described in the Materials and Methods section, and the levels of expression of the RNA for the specified genes in wild type BY4741 (empty bars) and ptc6 mutant cells (filled bars), after actin normalization, are represented as a percentage respect the quantity of RNA in untreated cells. Error bars represent the standard deviation. (TIF)

Table S1 Oligonucleotides used in this study. (DOCX)
Table S2 Genes up-regulated in ptc1 ptc6 cells. (DOCX)
Table S3 Genes down-regulated in ptc1 ptc6 cells. (DOCX)
Table S4 Major functional categories of genes down-regulated by rapamycin. The set of genes in each category (according to the MIPS Functional Catalogue Database) is classified as affected (dependent) or unaffected (independent) by the absence of Ptc6. Ptc6-dependent genes are further classified into totally plus strongly dependent (TD+SD) and weakly dependent (WD). (XLS)

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Author Contributions

Conceived and designed the experiments: JA AC AG. Performed the experiments: AG AC CC. Analyzed the data: AG AG. Wrote the paper: JA AC AG.
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