Abstract

Background: An excess of caffeine is cytotoxic to all eukaryotic cell types. We aim to study how cells become tolerant to a toxic dose of this drug, and the relationship between caffeine and oxidative stress pathways.

Methodology/Principal Findings: We searched for Schizosaccharomyces pombe mutants with inhibited growth on caffeine-containing plates. We screened a collection of 2,700 haploid mutant cells, of which 98 were sensitive to caffeine. The genes mutated in these sensitive clones were involved in a number of cellular roles including the H2O2-induced Pap1 and Sty1 stress pathways, the integrity and calcineurin pathways, cell morphology and chromatin remodeling. We have investigated the role of the oxidative stress pathways in sensing and promoting survival to caffeine. The Pap1 and the Sty1 pathways are both required for normal tolerance to caffeine, but only the Sty1 pathway is activated by the drug. Cells lacking Pap1 are sensitive to caffeine due to the decreased expression of the efflux pump Hba2. Indeed, hba2 cells are sensitive to caffeine, and constitutive activation of the Pap1 pathway enhances resistance to caffeine in an Hba2-dependent manner.

Conclusions/Significance: With our caffeine-sensitive, genome-wide screen of an S. pombe deletion collection, we have demonstrated the importance of some oxidative stress pathway components on wild-type tolerance to the drug.

Introduction

The methylxanthine derivative caffeine is an analogue of purine bases which has been involved in a variety of cellular processes in eukaryotic cells, including mammals, plants and fungi. Caffeine has shown a wide array of pharmacological and biological effects that interfere with DNA repair and recombination pathways, delay cell cycle progression and modulate intracellular calcium homeostasis. However, the manner in which caffeine triggers these pleiotropic effects is still largely unknown. Many groups have used genetically tractable organisms to study the biological and toxic effects of caffeine. Thus, in Saccharomyces cerevisiae, caffeine has been reported to affect cell cycle progression [1,2] and cell morphology and integrity [3]. In Schizosaccharomyces pombe, caffeine has been demonstrated to inhibit repair mechanisms [4,5], and to interfere with both meiotic [6] and UV-induced mitotic [7] recombination. Furthermore, caffeine is known to be an inhibitor of cAMP phosphodiesterase in different eukaryotic cell types [8].

Most of the reports which use unicellular eukaryotes to unravel the effects of caffeine are based on the isolation of strains which display enhanced resistance to cytotoxic levels of the drug, either by a chromosomal mutation [9,10] or by over-expression from a multicopy plasmid [11]. In order to become tolerant to a toxic drug, over-expression or modification of a target molecule would allow cells to withstand a higher concentration. Also, amplification of repair or scavenger activities could improve survival. Finally, cells with altered import (reduced) or export (increased) of the drug would display higher tolerance to caffeine.

In S. pombe, the Sipiczki laboratory isolated a number of caffeine-resistant mutants which defined single loci. Thus, the caf1–21, caf2–3, caf3–89, caf4–83 and caf5 mutants displayed pleiotropic, albeit slightly different, phenotypes to the cells: caffeine resistance, increased sensitivity to UV-irradiation, a reduction in fertility, lengthening of the cell cycle and some chromatin remodeling. We have investigated the role of the oxidative stress pathways in sensing and promoting survival to caffeine. The Pap1 and the Sty1 pathways are both required for normal tolerance to caffeine, but only the Sty1 pathway is activated by the drug. Cells lacking Pap1 are sensitive to caffeine due to the decreased expression of the efflux pump Hba2. Indeed, hba2 cells are sensitive to caffeine, and constitutive activation of the Pap1 pathway enhances resistance to caffeine in an Hba2-dependent manner.


Editor: Marcelo Bonini, National Institutes of Health (NIH)/National Institute of Environmental Health Sciences (NIHES), United States of America

Received April 17, 2009; Accepted July 9, 2009; Published August 12, 2009

Copyright: © 2009 Calvo et al. This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

Funding: This work was supported by Direccion General de Investigacion of Spain Grant BFU2006-02610, and by the Spanish program Consolider-Ingenio 2010 Grant CSD 2007-0020, to E.H. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

* E-mail: elena.hidalgo@upf.edu

¤ Current address: Department of Biochemistry, Cell and Molecular Biology of Plants, Estación Experimental del Zaidín, Granada, Spain

† These authors contributed equally to this work.
*cafl–3* carries a loss-of-function mutation at *crm1*; Crm1 is the nuclear exporter of Pap1, and the defective *cafl–3* allele leads to constitutive nuclear localization of Pap1 [9,14]. Hba1 is a cofactor of the Crm1-mediated export of Pap1, and the *cafl–21* mutation contains an early stop codon in the open-reading frame [15]. *cafl–9* has a gain-of-function of the *papl* gene: the encoded Pap1 protein has constitutive nuclear localization [11]. *caf4–83* carries a loss of function mutation at *trr1*; the lack of the thioredoxin reductase *Trr1* leads to the constitutive oxidation and therefore nuclear localization of Pap1 [13]. Lastly, over-expression of the *cafl* locus mutation has been described to enhance the protein levels of an ABC transporter, Ca5 [11]; the expression of this transporter is dependent on Pap1 [16].

Using microbes as model systems, several groups have isolated genes related to oxidative stress pathways in the search for mutants with increased resistance to unrelated drugs (multidrug resistant phenotype). This could be due to a natural induction of the stress pathway by the drugs, since they could trigger reactive oxygen species production. Alternatively, many oxidative stress regulons include ATP-binding cassette (ABC)-family transporters among the genes induced upon stress, which may act as efflux pumps to extrude the drugs from the intracellular compartment. In *S. pombe*, there are two alternative oxidative stress pathways; the Pap1-dependent one responds to moderate concentrations of *H₂O₂*, and the MAP kinase Sty1 pathway becomes activated not only upon toxic doses of *H₂O₂* but also in response to heat shock, osmotic stress and other situations which compromise cell viability (for a review, see [17]). Only over-expression of the Pap1, but not Sty1, pathway has arisen as beneficial in overcoming high doses of caffeine [9,10].

We decided to initiate an alternative approach to gain insights into the molecular targets of caffeine, and to study in depth its relationship with oxidative stress pathways. We searched for mutants from *S. pombe* with inhibited growth on caffeine-containing agar plates, using a deletion collection of about 2,700 haploid mutant cells, of which 98 were sensitive to the drug. The genes mutated in these sensitive clones were involved in a number of cellular roles including the stress, the integrity and the calcineurin pathways. Also, genes involved in the establishment of cell morphology, chromatin remodeling and protein traffic were identified as essential to maintain a wild-type tolerance to caffeine. We confirmed the sensitivity of most clones by sequential dilutions on solid plates, and investigated the role of the Pap1 and Sty1 stress pathways with regard to caffeine toxicity.

**Results**

**Genome-wide screen of caffeine-sensitive mutants**

Different concentrations of caffeine in liquid media can either partially or completely inhibit the growth of *S. pombe*, in a similar way as *H₂O₂* does (Figure 1) [18]. A comparable cytotoxic effect can be observed when caffeine is added to solid plates. In order to paint a global picture of the cellular mechanisms used by *S. pombe* to cope with toxic doses of caffeine, we carried out a genome-wide isolation of mutants displaying growth defects in the presence of 10 mM caffeine. We spread a collection of about 2,700 haploid mutants, and searched for cells with impaired growth on YE plates containing the drug. We obtained 98 putative isolates. The sensitivity to caffeine of 59 of those strains was confirmed by sequential spotting (see below).

With our screen, a number of mutants were isolated whose sensitivity to caffeine had already been established, but many other were new. We grouped the mutants by functional categories, and analyzed each one of them by sequential spots on solid plates containing caffeine (see Materials and Methods). Since most mutations enhancing resistance to the drug had been described as leading to constitutive activation of the oxidative stress-dependent Pap1 pathway (see Introduction), we also plated our putative caffeine-sensitive strains in *H₂O₂*-containing plates. Only those mutants confirmed to be sensitive to caffeine by sequential spotting are shown in Supplementary Table S1. We have also included in this table some *S. pombe* mutants that were not positive in the initial screen (either because the genome-wide plating was less sensitive than the sequential spots, or because those particular mutants were not present in the deletion collection), but were subsequently checked (and came up as positives) in the spot assays. Among the strains isolated in the initial screen, both *Atr1* and *Apr1* cells displayed a strong sensitive phenotype, and were used thereafter, together with a wild-type strain, as controls in all the spot assays we performed.

**Involvement of the Pap1 pathway in cellular tolerance to caffeine**

Deletion of the gene coding for the Pap1 transcription factor rendered cells sensitive to caffeine (Figure 2D). Pap1, which becomes oxidized by moderate doses of *H₂O₂* and thereafter accumulates at the nucleus to trigger an anti-oxidant gene response (Figure 2A) [13], did not become oxidized (Figure 2B) nor did it accumulate at the nucleus (Figure 2C) by any dose of caffeine we tested. Thus, the *papl* gene is essential for normal tolerance to caffeine, and other proteins related to the pathway, such as the Pap1-regulated Srx1 and Trx1, are essential as well (Supplementary Figure S1). Conversely, activation of the pathway, e.g. by deletion of the thioredoxin reductase *Trr1*, enhances the resistance to caffeine (Figure 2D), as described earlier [14].

Since AP1-like transcription factors have been described to promote caffeine resistance by up-regulating efflux pumps [19], we analyzed whether lack of the ABC-transporter Hba2/Bfr1 and/or Ca5, whose genes are under the control of the transcription factor *Apr1* [20], would abolish the caffeine-resistant phenotype of *Apr1* cells. Indeed, *Apr1* cells lacking both *hba2* and *ca5* genes were very sensitive to caffeine (Figure 2D). In fact, cells lacking Hba2 were almost as sensitive to caffeine as *Apr1* cells (Figure 2D), indicating that Hba2 is the major caffeine exporter in *S. pombe*. It has been reported, and we have confirmed it by Northern blot analysis (data not shown), that hba2 basal transcription is 3-fold lower in *Apr1* than in wild-type cells [20], which explains the sensitivity to caffeine of cells lacking Pap1.
Activation of the MAP kinase Sty1 stress pathway by caffeine

Components of the general stress pathway, centered on the MAP kinase Sty1 (Figure 3A), were sensitive to caffeine and to H$_2$O$_2$ (Figure 3B). Pcr1, a b-ZIP transcription factor known to heterodimerize with Atf1 to induce the Sty1-dependent gene response, is dispensable for the cellular response to caffeine (Figure 3B), as described above for other stresses [21]. It is worth pointing out that the screens performed by others to isolate genes whose mutations increased resistance to multidrug or even to components of the Sty1 pathway. Consistently, we tested that constitutive activation of the pathway, either by expression of a constitutively activated MAP kinase kinase Wis1DD or by deletion of the MAP kinase phosphatase Pyp1, enhanced resistance to toxic doses of H$_2$O$_2$ but did not increase or just slightly improved the tolerance to caffeine (Figure 3C).

The Sty1 pathway is activated in response to a whole variety of stress signals (for a review, see [17]). We determined that caffeine also induced phosphorylation of the Sty1 MAP kinase (Figure 3D), which triggered a rapid translocation of Sty1-GFP from the cytosol to the nucleus (Figure 3E) and the phosphorylation and accumulation of its main substrate, the transcription factor Atf1 (Figure 3D). Therefore, caffeine activates the main global anti-stress response pathway known to be required for survival upon compromised environmental situations.

Once established that both stress pathways, Pap1 and Sty1, are essential to maintain normal sensitivity to caffeine, we centered our attention on three genes isolated in our screen which regulate protein levels, and which may therefore exert their effects through regulation of Pap1, Sty1 or its main transcription factor, Atf1: moe1 (may regulate translation or protein stability) [22,23], csn1 (involved in the signalosome) [24] and pof3 (F-box protein, may specifically regulate protein levels) [25]. As shown in figure 4A, only deletion of moe1 confers sensitivity to both caffeine and H$_2$O$_2$. Concomitantly, only cells lacking Moe1 have altered levels of Atf1.
and display a deficient activation of Sty1 (Figure 4B and 4C). The participation of the Moe1-interacting partner Int6 in wild-type tolerance to caffeine and its relationship with Atf1 protein levels has been recently assessed [26,27]. Further work will be required to determine the role of Moe1 in Sty1 phosphorylation.

**Other cellular processes**

In our screen we isolated other mutations in genes coding for activities related to processes previously connected to caffeine, and that validates the results obtained. Thus, we found that several strains carrying deletions in genes coding for proteins of the cell integrity pathway whose central component is the MAP kinase Pmk1 (Figure 5A) were sensitive to caffeine, as described earlier [28,29], but not to 

\[ \text{H}_2\text{O}_2 \] (Figure 5B). Similarly, strains carrying deletions in genes involved in cell polarity, cell wall biosynthesis or cytokinesis, which are related to the cell integrity pathway, are also over-represented (Supplementary Figure S2).

A role for caffeine in the regulation of *S. cerevisiae* calcium homeostasis has been described earlier [30]. Mutations in components of the calcineurin pathway also led to caffeine
sensitivity in our screen (Figure 5C) suggesting that caffeine also inhibits extracellular Ca\(^{2+}\) uptake in fission yeast.

Other cell functions linked to caffeine toxicity have been impaired recombination and DNA damaging [31]. In agreement with those studies, several deletions in genes coding for activities related to recombination and/or repair have been isolated in the screen, such as \(\text{rad}3\), \(\text{ssb}3\), \(\text{rad}54\) and \(\text{rad}51\) (Figure 6A). Similarly, intracellular protein traffic is one of the major functional categories, with many gene deletions conferring lower tolerance to caffeine (vacuole protein sorting, Golgi or ER function, etc.) (Supplementary Figure S3). Probably this traffic is required to eliminate the caffeine. On the other hand, a pathway traditionally linked to caffeine tolerance is the protein kinase A. However, the sensitivity to caffeine of \(S.\ pombe\) cells lacking Pka1 was only slightly higher, if any, of that of a wild-type strain (Supplementary Figure S4). Furthermore, strains bearing mutations in other components of the pathway did not display any growth inhibition by caffeine (Supplementary Figure S4).

Other genes isolated in the screen and that therefore have functions related to the cell response to caffeine might be indirectly affecting pathways required to counteract the effects of caffeine or required to facilitate its degradation. Thus, general regulators of mRNA abundance, such as the chromatin remodeler \(\text{SPAC}25A8.01\text{c}\), members of the Ccr4 complex, or the histone acetyl transferase Sin3 are present in this global list (Figure 6B). Several genes related to general metabolic pathways also altered the tolerance to caffeine (Supplementary Figure S5), as well as genes known to regulate the meiotic or mitotic cell cycles (Supplementary Figure S6).

**Discussion**

Caffeine, which elicits well-documented cytotoxic effects to eukaryotic cells, has been proposed to target and inactivate many cellular activities (Fig. 7). Several genetic approaches had been undertaken to identify those targets, frequently based on the isolation of caffeine-resistant microbial cells, and very often constitutive activation of oxidative stress pathways had been connected to caffeine tolerance. With our caffeine-sensitive, genome-wide screen of an \(S.\ pombe\) deletion collection, we have demonstrated the importance of some oxidative stress pathway components on wild-type tolerance to the drug. Furthermore, we have demonstrated with a parallel screen on \(H_2O_2\)-containing plates that some, but not all, of the caffeine-sensitive mutants also display defects in the presence of \(H_2O_2\). Thus, cells lacking components of the Pap1 and Sty1 pathways, the intracellular protein transport system, cell polarity machinery, DNA recombination/repair systems, and chromatin remodelling regulators are both sensitive to caffeine and to \(H_2O_2\) (Supplementary Table S1).

We do not, however, believe that any of the toxic effects of caffeine is mediated through direct generation of reactive oxygen species,
since the sensitive Pap1 pathway is not induced at any concentration of the drug (we have tested caffeine concentrations ranging from 0.05 to 30 mM, and none of them activate Pap1; data not shown). The global stress response pathway, centered on the MAP kinase Sty1, does become activated by caffeine. However, this pathway is not only triggered by H2O2, but also by any type of environmental stress which compromises cell viability, and caffeine does so. Up-regulation of the Sty1 pathway had never been isolated as a genetic component of resistance to caffeine, and that is consistent with our results: inactivation of the pathway by the deletion of some components increases sensitivity to the drug, but hyper-activation of the pathway through the lack of the Sty1 phosphatase Pyp1, or through expression of a constitutively active Wis1 kinase, does not significantly enhance the tolerance to caffeine.

In contrast, lack of Pap1 triggers sensitivity and up-regulation of Pap1 induces resistance to caffeine. We show here that such an effect is mainly due to a downstream target of Pap1, the gene coding for the efflux pump Hba2. The development of multidrug resistance in microorganisms may be due to a number of mechanisms. The most documented one is enhanced extrusion of drugs mediated by efflux pump Hba2. The development of multidrug resistance in microorganisms may be due to a number of mechanisms. The most documented one is enhanced extrusion of drugs mediated by efflux pump Hba2. The development of multidrug resistance in microorganisms may be due to a number of mechanisms. The most documented one is enhanced extrusion of drugs mediated by efflux pump Hba2. The development of multidrug resistance in microorganisms may be due to a number of mechanisms. The most documented one is enhanced extrusion of drugs mediated by efflux pump Hba2. The development of multidrug resistance in microorganisms may be due to a number of mechanisms. The most documented one is enhanced extrusion of drugs mediated by efflux pump Hba2.

Figure 5. The cell integrity and the calcineurin pathways are required for normal tolerance to caffeine. (A) Scheme of the activation of the MAP kinase Pmk1 by cell wall damage. Other upstream and downstream components of the pathway are indicated. The calcineurin components are also indicated. (B, C) Survival to caffeine or H2O2 exposure at the indicated concentrations of strains harboring mutations in genes coding for components of the Pmk1 (B) or Cnb1 (C) pathways. Survival of the deletion collection strains 666 (WT), Δpap1, Δshk2, Δpck1, Δmkh1, Δpek1, Δpmp1, Δsty1, Δcnb1 and Δprz1 was analyzed by sequential spotting, as described in Fig. 2D.

doi:10.1371/journal.pone.0006619.g005
locus of *Escherichia coli* [32], the *mexGHI-ompD* four-gene operon of *Pseudomonas aeruginosa*, which encodes a multidrug efflux pump system involved in quorum-sensing signal homeostasis and which may be activated by superoxide [33], or the export pumps for glutathione S-conjugates, which have been cloned from mammals, yeast, plants, and nematodes [for a review, see [34]]. In the case of the Pap1 regulon, up-regulation of the pathway increases the expression of both Hba2 and Caf5 [20], and these efflux pumps induce a multidrug resistant phenotype. Our data indicate that Hba2 is the major efflux pump for caffeine, since deletion of its gene causes sensitivity to the drug (Figure 2D). However, Caf5 is also able to partially extrude caffeine, since the triple Δ*hba2 Δcaf5* strain displays stronger sensitivity to the drug than Δ*hba2* cells (Figure 2D). It is worth noting that over-expression of the Pap1 homolog YAP1 of *S. cerevisiae* also confers resistance to several drugs [35], and that such a phenotype is dependent on the presence of two efflux pumps, FLR1 and YCF1, whose expression is under the control of YAP1 [36]. However, deletion of the YAP1 gene does not result in sensitivity to cycloheximide [37] or diazaborine [36], indicating that either YAP1 in *S. cerevisiae* is not such a strong determinant of multidrug resistance as Pap1 is in *S. pombe*, or that the basal levels of FLR1/YCF1 transcripts are unchanged in Δ*yap1* cells (hba2 basal transcription is 3-fold lower in Δ*pap1* cells than in wild-type cells) [20].

The demonstration of linkage between a gene deletion and a phenotype is only a first step that might unveil details of a whole cellular response to an environmental stress. With our screen, we have further explored additional cellular pathways involved in caffeine resistance and we have identified genes belonging to pathways participating in *S. cerevisiae* survival to caffeine (Fig. 7). These genes validate our screen and corroborate the biological significance of conserved processes between the two distant yeasts. Thus, it was not surprising to isolate genes coding for the cell integrity MAP kinase pathway (Figure 5A & B) as well as cell morphology genes related to that pathway (Supplementary Figure S2). For several microorganisms, caffeine is currently used as a phenotypic criterion to evaluate the function of cell wall integrity pathways [38]. Similarly, it has been described that *S. cerevisiae* uptake of the extracellular Ca\(^{2+}\) is inhibited by caffeine [30], and, according to our results, that is probably the case in fission yeast (Figure 5C).

It was also predictable to find intracellular protein traffic as one of the major functional categories, with many caffeine-sensitive gene deletions (Supplementary Figure S3). Caffeine acts as a competitive inhibitor for adenosine and its presence likely causes an artificial metabolic stress to the cells. Probably traffic to the vacuole is required to eliminate the caffeine in *S. pombe* as in *S. cerevisiae* [39]. Additionally, some metabolic pathways might be required to counteract the caffeine competitive inhibition effect.

Caffeine was the first drug reported to override checkpoints and several reports described caffeine inhibition of Rad3 [31], and Rad-related kinases ATM or ATR in mammalian cells [40–42]. Importantly enough, three genes known to be involved in replication, recombination and/or repair (rad3, rad51 and *rhp54*) were isolated as essential for normal tolerance to both caffeine and H\(_2\)O\(_2\) (Figure 6A), highlighting the importance of DNA homeostasis in the response to both insults.

---

**Figure 6.** Several regulators of chromatin remodeling (A) and DNA repair/recombination pathways (B) are required for normal tolerance to caffeine. We analyzed by sequential spotting as described in Fig. 2D the survival to caffeine or H\(_2\)O\(_2\) exposure at the indicated concentrations of the deletion collection strains 666 (WT), Δ*pap1*, Δ*rad3*, Δ*ssb3*, Δ*rad54*, Δ*rad51*, Δ*C25A8.01c*, Δ*accr4*, Δ*caf1* and Δ*sin3.

doi:10.1371/journal.pone.0006619.g006

---
A surprising result from our screen concerns the cAMP-signalling pathway traditionally involved in caffeine tolerance, with cAMP phosphodiesterase being perhaps the best known protein target inactivated by the drug [8]. However, we have not detected a significant alteration of tolerance to caffeine in any of the mutants of this pathway that we have tested [Supplementary Figure S4]. Similarly, the TORC1 kinase has recently been described as the growth-limiting target of caffeine [43,44], but the homologous Tor2 kinase is essential and therefore its deletion mutant was not present in the collection, and other components of the pathway were not isolated in our screen. These results suggest, but do not demonstrate, that \textit{S. pombe} cAMP phosphodiesterase is not an essential caffeine target while other signalling pathways important to \textit{S. pombe} survival are affected by this drug.

\textbf{Materials and Methods}

\textbf{Yeast strains and growth conditions}

We used the strains 972 (\textit{h}+), JA364 (\textit{h}+ \textit{ura4-D18}), JA365 (\textit{h}+ \textit{ura4-D18}), AV18 (\textit{h}+ \textit{sty1::kanMX6}) [45], AV25 (\textit{h}+ \textit{pap1::kanMX6}) [45], EHH14 (\textit{h}+ \textit{his2} \textit{ura4-D18} \textit{pap1::ura4-D18} \textit{leu1–32} \textit{natMX6}::GFP-pap1:1leu1) [46], KS2088 (\textit{h}+ \textit{ura4-D18} \textit{his2} \textit{ura4-D18} \textit{leu1–32} \textit{sty1::kanMX6}::ura4-D18) [47], AV15 (\textit{h}+ \textit{attf1::kanMX6}) [45], EAH38 (\textit{h}+ \textit{leu1–32} \textit{sty1::kanMX6}) [48] and EHH5 (\textit{h}+ \textit{leu1–32} \textit{sty1::GFP::kanMX6}) [45]. To construct \textit{S. pombe} strains with specific loci deleted, we transformed wild-type strains (either 972 or JA364) with linear fragments containing open reading frame (\textit{ORF}):

- \textit{kanMX6} or \textit{ORF::natMX6}, obtained by PCR amplification using \textit{ORF}-specific primers and plasmids pFA6a-\textit{kanMX6} [49] or pFA6a-\textit{natMX6} [50] as templates, and we obtained strains NG28 (\textit{h}+ \textit{hba2::natMX6}) NG29 (\textit{h}+ \textit{cafa::kanMX6}), MJ2 (\textit{h}+ \textit{trf1::kanMX6} \textit{ura4-D18} \textit{leu1–32}), NG35 (\textit{h}+ \textit{hba2::natMX6} \textit{ura4-D18}), NG34 (\textit{h}+ \textit{hba2::natMX6} \textit{cafa::kanMX6} \textit{ura4-D18}) and NG41 (\textit{h}+ \textit{cafa::kanMX6} \textit{ura4-D18}), NG24 (\textit{h}+ \textit{cafa::ura4-D18}) was isolated after crossing \textit{Delta4} (\textit{h}+ \textit{cafa::ura4-D18} \textit{ade6–704} \textit{leu1–32}) [14] with JA365 (\textit{h}+ \textit{ura4-D18}). To obtain NG42 (\textit{h}+ \textit{hba2::natMX6} \textit{cafa::ura4-D18}), we crossed NG35 with NG24. NG37 (\textit{h}+ \textit{hba2::natMX6} \textit{cafa::kanMX6} \textit{ura4-D18}) was isolated after crossing the double mutant NG34 with NG24. We isolated NG25 (\textit{h}+ \textit{cafa::ura4-D18}) after crossing NG24 with JA364. To obtain NG39 (\textit{h}+ \textit{cafa::kanMX6} \textit{ura4-D18}) we crossed NG41 with NG25. Cells were grown in standard media [minimal media or rich media (YE)] [51], with or without caffeine or \textit{H}_2\textit{O}_2 at the indicated concentrations.

\textbf{Growth curves}

To measure cellular growth we used an assay based on automatic measurements of optical densities (OD) of small (100 \textmu{}l) liquid cell cultures, which allowed us to plot comparable growth curves for each treatment. Basically, we grew cells in YE media to an \textit{OD}_{600} of 0.3 at 30°C under continuous shaking in Erlenmeyer flasks. Then, we diluted the cultures in YE media to an \textit{OD}_{600} of 0.025 and cells continued growing in the same conditions till they reached an...
Caffeine and oxidative stress are required for normal tolerance to caffeine. We analyzed by sequential spotting (as described in Fig. 2D) the survival to caffeine or H$_2$O$_2$ exposure at the indicated concentrations of the deletion collection strains 666 (WT), $Apo$1, $A$tyl1, and $A$tx2 (coding for the mitochondrial thiorodoxin). Found at: doi:10.1371/journal.pone.0006619.s002 (3.53 MB TIF)

Figure S2 Several regulators of cell polarity or cell wall biosynthesis are required for normal tolerance to caffeine. We analyzed by sequential spotting (as described in Fig. 2D) the survival to caffeine or H$_2$O$_2$ exposure at the indicated concentrations of the deletion collection strains 666 (WT), $Apo$1, $A$tyl1, $A$myo1, $A$coq10, $A$rsv1, $A$cut8, $A$aps1, $A$git3, $A$hol1, and $A$erd2. Found at: doi:10.1371/journal.pone.0006619.s003 (6.71 MB TIF)

Figure S3 Several components of the Pap1 pathway are required for normal tolerance to caffeine. We analyzed by sequential spotting (as described in Fig. 2D) the survival to caffeine or H$_2$O$_2$ exposure at the indicated concentrations of the deletion collection strains 666 (WT), $Apo$1, $A$tyl1, $A$tyl2, $A$ps32, $A$ps54, $A$ps104, $A$ps105, $A$ps106, $A$ps108, $A$ps34, $A$ps115, and $A$ps116. Found at: doi:10.1371/journal.pone.0006619.s004 (6.59 MB TIF)

Figure S4 The protein kinase A pathway is not required for normal tolerance to caffeine. We analyzed by sequential spotting (as described in Fig. 2D) the survival to caffeine or H$_2$O$_2$ exposure at the indicated concentrations of the deletion collection strains 666 (WT), $Apo$1, $A$tyl1, $A$ps1, $A$ps2, $A$ps32, $A$ps36, and $A$ps116. Found at: doi:10.1371/journal.pone.0006619.s005 (3.77 MB TIF)

Figure S5 Several genes coding for enzymes related to metabolic pathways (A) and for mitochondrial components (B) are required for normal tolerance to caffeine. We analyzed by sequential spotting (as described in Fig. 2D) the survival to caffeine or H$_2$O$_2$ exposure at the indicated concentrations of the deletion collection strains 666 (WT), $Apo$1, $A$tyl1, $A$ps1, $A$ps2, $A$ps104, $A$ps105, $A$ps106, $A$ps108, $A$ps34. Found at: doi:10.1371/journal.pone.0006619.s006 (6.58 MB TIF)

Figure S6 Several regulators of the mitotic or meiotic cell cycles are required for normal tolerance to caffeine. We analyzed by sequential spotting (as described in Fig. 2D) the survival to caffeine or H$_2$O$_2$ exposure at the indicated concentrations of the deletion collection strains 666 (WT), $Apo$1, $A$tyl1, $A$mfn2, $A$mfn3, $A$ps104, $A$ps105, $A$ps106, $A$ps108, $A$ps116, $A$mfn4, $A$mfn5, $A$mfn6, $A$mfn7, and $A$mfn10. Found at: doi:10.1371/journal.pone.0006619.s007 (4.31 MB TIF)

Acknowledgments
We thank members of the laboratory for helpful discussions. We thank Zigi Benko for providing strain caf4-::ura4+ ($A$mfl1) and Paul Russell and Miguel Rodriguez-Gabriel for strain KR088 (wis1DD).

Author Contributions
Conceived and designed the experiments: JA EH. Performed the experiments: IAC NG IIB SGS. Analyzed the data: IAC NG IIB SGS PP JA EH. Contributed reagents/materials/analysis tools: KLH DUK MS AZ. Wrote the paper: EH.

References


