Gene dosage effects in yeast support broader roles for the LOG1, HAM1 and DUT1 genes in detoxification of nucleotide analogues

Mattias Carlsson, Guo-Zhen Hu, Hans Ronne*

Department of Forest Mycology and Plant Pathology, Swedish University of Agricultural Sciences, Uppsala, Sweden

* Hans.Ronne@slu.se

Abstract

Purine and pyrimidine analogues have important uses in chemotherapies against cancer, and a better understanding of the mechanisms that cause resistance to these drugs is therefore of importance in cancer treatment. In the yeast Saccharomyces cerevisiae, overexpression of the HAM1 gene encoding inosine triphosphate pyrophosphatase confers resistance to both the purine analogue 6-N-hydroxylaminopurine (HAP) and the pyrimidine analogue 5-fluorouracil (5-FU) (Carlsson et al., 2013, PLoS One 8, e52094). To find out more about the mechanisms of resistance to nucleotide analogues, and possible interdependencies between purine and pyrimidine analogue resistance mechanisms, we screened a plasmid library in yeast for genes that confer HAP resistance when overexpressed. We cloned four such genes: ADE4, DUT1, APT2, and ATR1. We further looked for genetic interactions between these genes and genes previously found to confer resistance to 5-FU. We found that HMS1, LOG1 (YJL055W), HAM1, and ATR1 confer resistance to both 5-FU and HAP, whereas ADE4, DUT1 and APT2 are specific for HAP resistance, and CPA1 and CPA2 specific for 5-FU resistance. Possible mechanisms for 5-FU and HAP detoxification are discussed based on the observed genetic interactions. Based on the effect of LOG1 against both 5-FU and HAP toxicity, we propose that the original function of the LOG (LONELY GUY) family of proteins likely was to degrade non-canonical nucleotides, and that their role in cytokinin production is a later development in some organisms.

Introduction

Antimetabolite drugs such as purine and pyrimidine analogues play an important role in chemotherapy against cancer. However, tumours may acquire resistance to such drugs by clonal selection of resistant cancer cells. A better understanding of the mechanisms of action of anticancer drugs and in particular the ways by which drug resistance can arise is therefore of importance both for cancer treatment and for the development of new more efficient anticancer drugs. Since nucleotide metabolism is evolutionarily conserved, these mechanisms can be
studied in model organisms such as *Saccharomyces cerevisiae* (baker’s yeast) where advanced methods for molecular genetics are available.

The first antimetabolite drug that was developed to specifically target cancer cells was the pyrimidine analogue 5-fluorouracil, 5-FU [1]. 5-FU targets several cellular mechanisms through its activated metabolites FdUMP, FUTP andFdUTP [2–3]. FdUMP inhibits thymidylate synthase (TYMS), and this is a major mechanism behind both the toxicity and the anticancer effect of 5-FU [4–8]. However, effects on RNA metabolism also play an important role in 5-FU toxicity [9–12]. Such effects result from inhibition of RNA modifications such as methylation and pseudouridylation [11,13], which in turn cause disturbed exosome processing of polyadenylated rRNA [14], interference with spliceosome function [15], and destabilization of tRNAs [12].

We previously carried out a screen in yeast for genes that confer resistance to 5-FU when overexpressed from a high copy number plasmid [16]. Among the cloned resistance genes we found HAM1, which encodes inosine triphosphate pyrophosphatase, an enzyme that dephosphorylates non-canonical purine nucleoside triphosphates ITP, diTP and XTP and thus prevents their incorporation into DNA and RNA [17–18]. This finding suggested that the Ham1 protein may have a broader specificity than originally thought, targeting non-canonical pyrimidines in addition to purines [16]. It also raised questions about interactions between the purine and pyrimidine metabolic pathways and the role of such interactions in acquired resistance to nucleotide analogues.

To look further into these questions, we decided to carry out a screen for yeast genes that confer resistance to 5-FU when overexpressed from a high copy number plasmid. For this screen we chose to use 6-N-hydroxylaminopurine (HAP), the drug that was originally used to identify HAM1 as a gene that causes hypersensitivity to purine analogues when disrupted [17]. HAP is a cytotoxic and hemolytic purine analogue that is similar to adenine but with a hydroxylamine group instead of an amino group attached to the number 6 carbon. In contrast to other purine analogues such as azathioprine and mercaptopurine [19–20] it is not used in medicine since it is hemolytic at concentrations below what could have therapeutic potential [21]. However, it has been used in research to study purine analogue toxicity in both human tumor cell lines and in the yeast *Saccharomyces cerevisiae* [17,22–25].

The screen was carried out both in a wild type strain and in a ham1 knockout mutant that is hypersensitive to HAP. We found four new yeast genes that confer resistance to HAP when overexpressed: ADE4, DUT1, APT2, and ATR1. We proceeded to test these genes for their effects on 5-FU resistance. We also looked for genetic interactions between the genes isolated in the 5-FU and HAP resistance screens as well as interactions with other genes involved in purine metabolism. Based on our findings, we discuss possible roles of the cloned genes in the metabolism and detoxification of HAP, 5-FU and other nucleotide analogues. In particular, we propose that the original function of the widely distributed LOG (LONELY GUY) family of proteins was to facilitate the removal of non-canonical nucleotides from the nucleotide pool, working downstream of Ham1p and other nucleotide phosphatases, and that their role in cytokinin production in plants and some microorganisms [26–28] is a later development that occurred in these organisms.

### Materials and methods

#### Yeast strains and plasmids

Yeast deletion strains in the BY4742 haploid and the isogenic BY4743 diploid background were obtained from Euroscarf (http://www.uni-frankfurt.de/fb15/mikro/euroscarf). The open
reading frame in each deletion strain has been replaced by the KanMX selection cassette [29]. The plasmids pCPA1, pCPA2, pHMS1, pYJL055w, pHAM1 have been described previously [16]. Plasmid pYJL055w is referred to as pLOG1 in the present paper in order to make the genetic nomenclature consistent.

**PCR cloning**

Plasmid pAPT1 was constructed by PCR amplification of a fragment spanning from 430 bp upstream to 150 bp downstream of the APT1 ORF, with primers adding a SacI site at the 3’ end: 5’-GAG CTC GCA CTC CAG AAA CAA CAG CA-3’, and a BamHI site at the 5’ end: 5’-GGA TCC TGT GGC ACA AAG CAG AAA AG-3’. The PCR fragment was TA-cloned into pCR2.1 (Invitrogen, US) and subsequently subcloned between the SacI and BamHI sites in the polylinker of the shuttle vector pHR81 [30]. Plasmid pATR2 was constructed by PCR amplification of a fragment spanning from 356 bp upstream to 210 bp downstream of the ATR2 ORF, with primers adding SacI sites at both ends: 5’-GAG CTC ACA GGG GTG CGC ATA AAT AG-3’ and 5’-GAG CTC CTT GCG CAA ATG AAG AAC AA-3’. The PCR fragment was TA-cloned into pCR2.1, and subsequently subcloned into the SacI site in the polylinker of pHR81.

**Growth media and chemicals**

Rich media (YPD) and synthetic complete media (SC) or dropout media based on SC were prepared as previously described [31]. The synthetic media contained either 2% glucose or 2% galactose as carbon source. 6-N-Hydroxylaminopurine (HAP) and 5-fluorocytosine (5-FC) were obtained from Apollo Scientific (Manchester, UK). 5-Fluorouracil (5-FU) and 6-azauracil (6-AzaU) were obtained from Sigma-Aldrich (Stockholm, Sweden). 8-azaguanine (8-AzaG) was obtained from Accel Pharmatech (East Brunswick, US). Boric acid was obtained from Fluka Chemie (Buchs, Switzerland).

**Shuttle plasmid library screen**

The BY4742 wild type and the ham1 knockout strain were transformed with a yeast genomic library made in the 2 μm URA3 LEU2-d vector pHR81 [30]. The copy number of 2 μm plasmids is 5–50 molecules per cell, and will adjust if the insert is selected for or against. It is therefore possible to recover also weak dosage suppressors in a library screen which may require a higher copy number to be effective. To select for HAP resistance, the wild type transformants were plated on SC galactose media without uracil and adenine containing 50 μg/ml HAP, and the ham1 transformants were plated on SC glucose media without uracil and adenine containing 50 μg/ml HAP. Early emerging colonies and colonies with greater size were picked to grids on drug free media. In order to verify that the picked transformants were HAP resistant, they were sequentially replicated twice to the same HAP containing media that they were initially selected on. Plasmids were rescued from confirmed HAP resistant colonies, retransformed into the wild type BY4742, and retested for HAP resistance. The genes responsible for HAP resistance were mapped by deletions and PCR subcloning (Fig 1), followed by testing of the resulting plasmids after retransformation into yeast. We estimate that in total we screened approximately 120 000 transformants.

**Yeast growth and spot assays**

To assay drug sensitivity, transformants were grown overnight at 30 °C in SC galactose or glucose media without uracil. These overnight precultures were diluted into fresh media to a final
OD$_{600}$ of 0.1 and grown to late exponential phase. Cultures were diluted to OD$_{600}$ 0.1 in water before spotting. For the overexpression and disruption assays 10-fold serial dilutions were made. A 2.5 μl aliquot of each dilution was spotted onto control plates and drug plates. The drug concentrations used were higher than in the initial screens since we wanted to highlight
differences between different suppressor plasmids. Growth was monitored daily starting after two days at 30 °C.

Results
Cloning of genes that confer resistance to HAP when overexpressed

In order to identify yeast genes that confer resistance to purine nucleotide analogues when overexpressed we screened a yeast genomic DNA library in the high copy number shuttle vector pHR81 [30] for plasmids that confer resistance to HAP when overexpressed. The screen was carried out both in a wild type strain and in a ham1 knockout mutant that is hypersensitive to HAP in order to facilitate the recovery of weaker resistance genes. Transformants in the wild type strain were screened on HAP-containing media with galactose as a carbon source since we found that galactose increases the sensitivity to HAP, whereas transformants in the hypersensitive ham1 mutant were screened on HAP-containing media with glucose as a carbon source. The reason for this difference remains to be determined, but it is not unusual to see phenotypic differences between cells grown on glucose and other carbon sources, since glucose repression is a global regulatory response that alters the expression of a large part of the yeast genome [32].

In total, we screened approximately 120,000 transformants, which corresponds to a 30-fold coverage of the yeast genome, assuming an average insert size of 4 kbp. After rescue of the plasmids back into E. coli, retransformation into yeast for confirmation of the HAP resistance phenotype, and mapping of the HAP resistance within the plasmid inserts, we identified four genes, ADE4, DUT1, APT2, and ATR1, that confer resistance to HAP when overexpressed (Fig 1). ADE4, DUT1 and APT2 were cloned from the ham1 strain, whereas ATR1 was cloned from the wild type. In addition, the HAM1 gene was isolated four times from the ham1 strain and once from the wild type. The resistance conferred by each cloned gene to HAP is shown in Fig 2A. As discussed below, some of the genes also confer resistance to 5-FU (Fig 2B).

ADE4 encodes 5-phosphoribosyl-1-pyrophosphate amidotransferase, which catalyzes the first step in the de novo synthesis of purine nucleotides. Overexpression of ADE4 causes increased synthesis of purine nucleotides and has been shown to mediate resistance to the DNA-crosslinker cisplatin, which is used as an anticancer drug [33]. We found that ADE4 overexpression confers resistance to HAP (Fig 2A) but not to 5-FU (Fig 2B), which is consistent with a model where increased de novo synthesis of purines suppresses the toxicity of HAP by diluting the drug with freshly synthesized purines.

DUT1 is an essential gene encoding deoxyuridine triphosphate diphosphatase (dUTPase), an enzyme required both for de novo synthesis of thymidylate, and for genome stability by preventing incorporation of uridylate into DNA [34]. DUT1 is also known to be important for the resistance to antifolates such as aminopterin and the anticancer drug methotrexate [35]. Since Dut1p is thought to act primarily on dUTP, a pyrimidine nucleotide, one might expect it to confer resistance to 5-FU when overexpressed. However, we found that whereas DUT1 clearly confers resistance to HAP (Fig 2A), its effect, if any, on 5-FU resistance is barely detectable (Fig 2B, galactose).

APT2 is a duplicated copy of the APT1 gene encoding adenine phosphoribosyltransferase (APRT). APT1 and APT2 were identified as one of 549 gene pairs that remain as evidence of an ancient whole genome duplication in the Saccharomyces lineage [36]. The retention of both genes suggests that they may have acquired unique and different functions. However, Atp2p lacks APRT activity when expressed in E.coli and a disruption of the APT2 gene has no apparent phenotype in yeast [37]. It is thus not clear what enzymatic activity, if any, that Atp2p possesses. Nevertheless, we found that the APT2 gene confers resistance to HAP (Fig 2A) but not...
Fig 2. Resistance to HAP and 5-FU due to overexpression of different genes. The genes isolated in our HAP resistance screen were tested for resistance to HAP (A) and 5-FU (B). No drug controls are shown in panel C. Also included were the genes isolated in our previous screen for 5-FU resistance [16]. Cells transformed with the empty vector pH881 [30] were included as a negative control. Transformants were grown in liquid medium to late exponential phase, serially diluted, and then spotted onto uracil- and adenine-less SC galactose (Gal-UA) plates, uracil-less glucose (Glu-U) or uracil-less galactose (Gal-U) plates with or without HAP or 5-FU at the indicated concentrations. Plates were incubated for 3 days except for the second plate containing HAP, which was incubated for 5 days (5d) in order to show the weak effect of HMS1 overexpression.

https://doi.org/10.1371/journal.pone.0196840.g002
to 5-FU (Fig 2B) when overexpressed. We also tested a PCR clone of APT1 (Fig 3). As expected, it did not confer resistance to HAP, but instead had a slightly poorer growth on HAP. However, it did instead confer some resistance to 5-FU at low concentrations.

ATR1, finally, encodes a multidrug efflux pump that belongs to the major facilitator superfamily (MFS). Atr1p was originally named from the fact that it is required for resistance to the heterocyclic amine aminotriazole, and ATR1 expression has been found to increase during DNA-replication stress [38–39]. We found that overexpression of ATR1 confers strong resistance to both HAP and 5-FU (Fig 2A and 2B). ATR1 has an unnamed paralogue, YMR279C, which similarly to ATR1 was shown to confer resistance to boric acid [40]. We therefore also tested overexpression of a PCR-cloned copy of that gene (Fig 4). We found that YMR279C confers resistance to boric acid, HAP and 5-FU when overexpressed, though not as strongly as ATR1. We conclude from this that YMR279C has a similar multidrug efflux pump activity as ATR1. We therefore propose the name ATR2 for the open reading frame YMR279C.

Some 5-FU resistance genes also mediate resistance to HAP when overexpressed

Our finding that one of the HAP resistance genes, ATR1, also confers resistance to 5-FU prompted us to test if any of our previously identified 5-FU resistance genes [16] would confer...
resistance to HAP. As shown in Fig 2, we found that overexpression of HAM1 and LOG1 (YJL055W) confers a comparatively strong resistance to HAP and that overexpression of HMS1 confers a very weak but still detectable HAP resistance (Fig 2A). In contrast, overexpression of the carbamoyl phosphate synthetase subunits CPA1 or CPA2 had no apparent effect on the resistance to HAP. Two of the genes that we previously found to confer 5-FU resistance, HMS1 and LOG1[16], did not appear in our screen even though they do confer HAP resistance when overexpressed (Fig 2A). This might suggest that the screen was not exhaustive, though we did recover HAM1 five times. However, HMS1 has a very weak effect, and may therefore have escaped detection in our screen.

To assess the strength of the different resistance genes, we examined the size of single cell clones at the appropriate dilution in the presence of HAP or 5-FU, since colony size is a sensitive measure of growth rate in yeast. We found that HAM1 and ATR1 have the strongest effect on HAP resistance, followed by LOG1 and DUT1, and then by ADE4, APT2 and HMS1 in decreasing order of resistance (Fig 2A). This was determined on galactose media since HAP resistance is much easier to score on galactose than on glucose. As for 5-FU resistance, we found that CPA1 and ATR1 have the strongest effect, followed by HAM1, and then by HMS1, CPA2 and LOG1 in decreasing order of resistance. This was determined on glucose (Fig 2B). However, on galactose we found that LOG1 has a much stronger effect, comparable to that of HAM1, and that DUT1 also had a barely but still detectable effect (Fig 2B). The reason why LOG1 is much more efficient in conferring resistance against 5-FU on galactose remains to be determined. None of the other 5-FU resistance genes showed a similar effect, so it is likely not due to differences in the uptake or metabolism of 5-FU on galactose compared to glucose. A likely explanation for this effect is that LOG1 is moderately (2–3 fold) repressed by glucose [41].

In the case of HAM1, a likely explanation for the resistance to HAP is suggested by its known enzymatic activity. Ham1p has been found to be a nucleoside triphosphate pyrophosphatase, which does not seem to discriminate between deoxyribonucleotides and ribonucleotides. It has the highest specificity against deaminated purines, e.g. (d)XTP and (d)ITP, amongst the nucleotides assayed, but also showed some residual activity against dATP and dCTP [18]. This suggests that the molecular mechanism by which overexpression of HAM1 confers resistance to HAP, and likely also to 5-FU, is its pyrophosphatase activity.

The function of the YJL055W gene is not known. However, a BLAST search [42] with the Yjl055wp amino acid sequence as probe identified the plant LOG (LONELY GUY) family of proteins as its closest homologues, with E-values of 4e-56 for the rice LOG protein and 2e-52 for the Arabidopsis LOG1 protein. YJL055W is the only yeast gene encoding a protein with strong similarity to the plant LOG proteins. This suggests that YJL055W is an ortholog of the plant LOG genes, and we will therefore refer to it as the yeast LOG1 gene. The plant LOG proteins were originally identified as enzymes that activate adenylate-type pre-cytokinins, a group of plant hormones, by cleaving off the N6-modified adenine nucleobase from the precursor cytokinin nucleoside monophosphate [26,43]. It is therefore conceivable that yeast Log1p may catalyze a similar reaction in the degradation of non-canonical nucleotides (see Discussion).

Sensitivity of knockout mutants lacking resistance genes to HAP and 5-FU

Overexpression and loss of a gene frequently has opposite effects on the affected phenotypes. In order to determine if this is true in our case, we proceeded to test knockout mutants lacking each of the resistance genes for sensitivity to both HAP and 5-FU. These experiments were carried out using strains transformed with the empty vector pHR81 to facilitate comparisons with the overexpression experiments and avoid complications due to the fact that the genetic
background used, BY4742, is *ura3* and thus deficient for pyrimidine biosynthesis, which in turn affects 5-FU sensitivity [16]. The pHR81 plasmid carries the *URA3* marker which restores a functional pyrimidine biosynthesis in these strains. Since the *DUT1* gene is essential, the effect of a haploid knockout mutant could not be tested, but we instead included a heterozygous *dut1/DUT1* strain and a wild type diploid control in order to examine the effect of haploinsufficiency.

The sensitivity of different strains to 150 μg/ml HAP is shown in Fig 5A. We found that the *ham1* and *atr1* knockouts are highly sensitive to HAP at this concentration. The sensitivity of the *ham1* knockout is consistent with previous findings that *ham1* strains are sensitive to purine analogues [17,23] and with the known role of Ham1p in dephosphorylation of HAPTP. The sensitivity of the *atr1* knockout to HAP suggests that the Atr1p multidrug efflux pump contributes to HAP detoxification under normal conditions, and not only when overexpressed. We further found that the *log1* and *apt2* knockouts are sensitive to 150 μg/ml HAP, though not as strongly as the *ham1* and *atr1* knockouts (Fig 5A). A sensitivity of the *log1* knockout to purine analogues has been noted previously [23], and is consistent with a proposed role of Log1p in dephosphorylation of nucleoside monophosphates (see Discussion). The sensitivity of the *apt2* knockout to HAP has not been described previously. It suggests that Apt2p contributes to HAP detoxification under normal conditions, and not only when overexpressed. In contrast to these observations, the *cpa1*, *cpa2* and *hms1* strains were not sensitive to HAP (Fig 5A). Nor was the *dut1/DUT1* heterozygote more sensitive to HAP than the wild type diploid. Finally, we note that the sensitivity of the *ade4* strain to HAP could not be assessed since it does not grow in the absence of adenine, a competitive inhibitor of HAP toxicity which must be omitted in order to score sensitivity to HAP. In fact, the *ade4* knockout grows weakly in the presence but not the absence of HAP (Fig 5A). A likely explanation is that deamination of HAP to hypoxanthine permits the *ade4* strain to grow in the absence of adenine.

The sensitivity of different strains to 4 μg/ml 5-FU is shown in Fig 5B (on glucose) and 3C (on galactose). As we previously noted [16], the *cpa1* and *cpa2* knockouts are highly sensitive to 5-FU, but also the *atr1* knockout, which is consistent with a role for the Atr1p multidrug efflux pump in detoxification of 5-FU under normal conditions and not only when overexpressed. Consistent with our previous observations [16], the *log1* knockout was weakly sensitive to 5-FU. Finally, we note that the wild type diploid is more sensitive to 5-FU than the haploid strains, and that this sensitivity is further increased in the *dut1/DUT1* heterozygote. This is a striking effect since diploid strains normally grow much better than the haploids, as evident from the no drug control (Fig 5B). It suggests that diploids are more sensitive to 5-FU than haploids, and also that Dut1p is important for resistance to 5-FU under normal conditions.

### Cross-dependencies between genes that mediate resistance to HAP or 5-FU

We next tested for genetic interactions and cross-dependencies between the different drug resistance genes by transforming each resistance plasmid into yeast strains where one of the other genes had been knocked out (Figs 6 and 7). The most pronounced genetic interactions affecting HAP sensitivity were seen in the *ham1* knockout. Thus, overexpression of *ADE4*, *APT2* and *HMS1* failed to cause HAP resistance in the *ham1* strain, and the effects of *ATR1* and *LOG1* overexpression were significantly reduced (Fig 6). This suggests that these genes to some extent are dependent on *HAM1* for their resistance phenotype, though it could be argued that the increased HAP sensitivity of the *ham1* knockout would make it difficult to detect the effect of other genes, particularly in the case of *HMS1* which has a rather weak effect. Perhaps more surprisingly, the ability of *DUT1* to confer HAP resistance when overexpressed does not
Fig 5. Sensitivity to HAP and 5-FU due to disruption of different resistance genes. Yeast strains disrupted for the genes isolated in our HAP resistance screen and in our previous screen for 5-FU resistance [16] were tested for increased sensitivity to HAP (A) and 5-FU (B and C). All strains were transformed with the empty vector pHR81 in order to complement the ura3 mutation in the genetic background and thus restore a functional pyrimidine biosynthesis. The + sign stands for the haploid wild type control strain BY4742, +/+ stands for the diploid wild type control strain BY4743, and +/dut1 for the diploid DUT1/dut1 heterozygote. Transformants were grown in liquid medium to late exponential phase, serially diluted, and then spotted onto uracil- and adenine-less SC galactose plates (Gal-UA) plates, uracil-less glucose (Glu-U) or uracil-less galactose (Gal-U) plates with or without HAP or 5-FU at the indicated concentrations. The no drug control plates were photographed after 3 days and the drug plates after 5 days.

https://doi.org/10.1371/journal.pone.0196840.g005
seem to be affected by the ham1 knockout (Fig 6). We conclude that the HAP resistance conferred by DUT1 overexpression is strong enough to fully compensate for the increased sensitivity of the ham1 strain. This somewhat surprising since Ham1p targets both HAPTP and dHAPTP whereas Dut1p is thought to be specific for deoxyribonucleotides. It suggests that the main cytotoxic effect of HAP is mediated by dHAPTP (see Discussion). We further note that ADE4 overexpression was less efficient in conferring HAP resistance also in the hms1 and log1 knockouts. APT2 overexpression, finally, was also less efficient in conferring HAP resistance in the log1 knockout (Fig 6).

Resistance to 5-FU was tested on glucose media and also on galactose media for LOG1, since we found that the effect of LOG1 on 5-FU resistance is much stronger on galactose
whereas other effects are more easily seen on glucose. We saw that CPA1 depends on CPA2 and CPA2 depends on CPA1 (Fig 7), consistent with our previous findings [16]. Interestingly, HMS1 overexpression depends on ATR1 for its ability to confer 5-FU resistance, and HAM1 depends partially on LOG1 and DUT1, and also more weakly on CPA1 and CPA2 (Fig 7). Furthermore, HMS1 also seems to be dependent on CPA1 and CPA2 (Fig 7). However, it should be noted that the weak 5-FU resistance conferred by HMS1 overexpression could be hard to detect in the cpa1 and cpa2 strains, which are quite sensitive to 5-FU. Surprisingly, CPA1 and CPA2 were also partially dependent on APT2 (Fig 7). This finding was unexpected since APT2 was cloned due to its effect on HAP resistance, and since its sequence similarity to APT1 suggests a role purine metabolism. It is conceivable that this effect may reflect some kind of cross-

Fig 7. Cross-dependencies between different genes for the ability to confer resistance to 5-FU when overexpressed. Each plasmid was transformed into yeast knockout strains where one of the other resistance genes had been deleted. Overexpression plasmids (see Fig 1) are shown at the top, yeast strains at the left, and drug concentrations at the bottom in the figure. Transformants were grown in liquid medium to late exponential phase, diluted, and then spotted onto uracil-less SC glucose (Glu-U) plates with or without 4 μg/ml 5-FU. Also shown to the right are results obtained on uracil-less SC galactose (Gal-U) plates for the LOG1 gene and the pHR81 vector control. The + sign stands for the haploid wild type control strain BY4742. +/+ stands for the diploid wild type control strain BY4743, and +/dut1 for the diploid DUT1/dut1 heterozygote. The control vector is pHR81 [30]. The no drug control plate was photographed after 3 days and the 5-FU plate after 5 days.

https://doi.org/10.1371/journal.pone.0196840.g007
talk between purine and pyrimidine metabolism, but other explanations are also possible. The observed cross-dependencies for resistance to HAP and 5-FU are summarized in Fig 8.

Dependencies of resistance genes on other genes involved in purine metabolism

We also tested if knockouts of other genes involved in the purine metabolism would affect the ability of the cloned genes to confer resistance to either HAP (Fig 6) or 5-FU (Fig 7). To this end, we transformed all resistance plasmids into the purine deaminase strains \textit{amd1} (AMP deaminase), and \textit{aah1} (adenine deaminase) to test if the HAP resistance conferred by overexpression of different genes was dependent on the ability to remove, through deamination, the hydroxylamine group of HAP, a reaction that might be performed by adenine deaminases [44]. Finally, we transformed all resistance plasmids into the \textit{ade1} and \textit{ade2} strains to test if resistance was dependent on a functional purine biosynthesis pathway. This experiment was prompted by our finding that the 5-FU resistance conferred by overexpression of \textit{CPA1} and \textit{CPA2} is dependent on the pyrimidine biosynthesis pathway [16].

As shown in Fig 6, we found that the sensitivity to HAP was not significantly affected in the \textit{amd1} and \textit{aah1} knockout strains. Nor were any obvious interactions between \textit{amd1} or \textit{aah1} and any of the other genes conferring HAP resistance seen. However, the HAP resistance conferred by \textit{ADE4} overexpression was abolished in the \textit{ade1} and \textit{ade2} strains (Fig 6). This is consistent with the known function of \textit{ADE4} which encodes phosphoribosylpyrophosphatase amidotransferase, the first enzyme in the purine biosynthetic pathway, and supports the notion that \textit{ADE4} overexpression reduces the toxicity of HAP by diluting it with freshly synthesized purines. There was also a small effect of the \textit{ade1} knockout on the HAP resistance conferred by \textit{HMS1} overexpression (Fig 6). The HAP resistance conferred by the \textit{LOG1},
HAM1, DUT1, APT2 and ATR1 genes were all unaffected by the ade1 and ade2 knockouts, and thus apparently independent of de novo purine biosynthesis. As expected, the amd1, aah1, ade1 and ade2 knockouts had no effects on the resistance to 5-FU in any of the strains (Fig 7).

Effect of resistance genes on the sensitivity to other purine and pyrimidine analogues

In order to test the generality of our findings with HAP and 5-FU, we also tested our cloned resistance genes with several other purine and pyrimidine analogues. A problem with such experiments is that not all drugs are toxic in yeast, due to poor uptake or failure of conversion to the active metabolite. In total, we tested six purine analogues (2-chloroadenine, 6-thioguanine, 6-mercaptopurine, 6-N-hydroxylaminopurine, 2-amino-6-hydroxylaminopurine, and 8-azaguainine) and three pyrimidine analogues (6-azauracil, 5-azacytosine and 5-fluorocytosine). Six of the drugs (2-chloroadenine, 6-thioguanine, 6-mercaptopurine, 6-N-hydroxylaminopurine, 2-amino-6-hydroxylaminopurine, and 5-azacytosine) were not toxic at the highest concentrations that could be tested without precipitation of the drug. The purine analogue 8-azaguainine (8-AzaG) and the pyrimidine analogues 6-azauracil (6-AzaU) and 5-fluorocytosine (5-FC) did show toxicity, and we proceeded to test the effects of the resistance genes on sensitivity to these three drugs. The results are shown in Fig 9, with HAP and 5-FU included as controls.

For the purine analogue 8-AzaG, the pattern was similar to that seen with HAP in that overexpression of ADE4, ATR1, LOG1 and HAM1 all caused resistance to the drug. We presume that the mechanisms involved are similar to those for HAP resistance. However, overexpression of DUT1, APT2 or HMS1 did not cause any significant resistance to 8-AzaG (Fig 9). For the pyrimidine analogue 6-AzaU, we saw that overexpression of CPA1 and CPA2 caused resistance, similar to the case with 5-FU. However, the other plasmids did not have much of an effect. The other pyrimidine analogue, 5-FC, was more similar to 5-FU in its pattern of sensitivity. However, a striking difference is that overexpression of ADE4 confers resistance to 5-FC but not to 5-FU (Fig 9). This was an unexpected finding since ADE4 confers resistance to HAP and other purine analogues by boosting the de novo synthesis of purines. (Fig 9).

Discussion

We have performed a screen for genes whose overexpression confer resistance to HAP. To facilitate detection of weak effects, the screen was carried out both in a wild type yeast strain and in a ham1 knockout strain that has increased sensitivity to HAP [17]. In addition to HAM1, we found four genes conferring HAP resistance: ADE4, ATR1, DUT1 and APT2 (Figs 1 and 2). To find out more about the mechanisms by which the genes cause resistance to HAP, we also tested knockout mutants for sensitivity to HAP. Furthermore, we carried out a cross-dependency test in which all plasmids were transformed into strains with knockouts of each one of the other genes, and tested for the abilities to confer HAP resistance (Fig 6). Also included in this experiment were the five genes that we previously found to confer resistance to 5-FU when overexpressed: CPA1, CPA2, HMS1, LOG1 and HAM1 [16], and four genes involved in purine de novo synthesis and salvage: ADE1, ADE2, AMD1, AAH1. All strains and plasmids were also tested for resistance to 5-FU in order to identify common and unique mechanisms of drug resistance. The observed effects and genetic interactions are summarized in Fig 8. We found that four of the genes confer resistance to both HAP and 5-FU: HMS1, LOG1, HAM1, and ATR1. APT2 and ADE4 only confer HAP resistance, whereas CPA1 and CPA2 only confer resistance to 5-FU. DUT1 mainly confers HAP resistance, though a barely detectable effect was also seen on resistance to 5-FU.
### Fig 9. Resistance to 5-FC, 6-azauracil and 8-azaguanine conferred by overexpression of different genes.

The genes isolated in our HAP resistance screen were tested for resistance to 5-FC, 6-AzaU and 8-AzaG. No drug controls are also shown. Also included were the genes isolated in our previous screen for 5-FU resistance [16]. Cells transformed with the empty vector pHR81 [30] were included as a negative control. Transformants were grown in liquid medium to late exponential phase, serially diluted, and then spotted onto uracil- and adenine-less SC galactose (Gal-UA) plates with or without HAP, 5-FU, 5-FC, 6-AzaU or 8-AzaG at the indicated concentrations. An empty row (Empty) was left below the pADE4 and pCPA1 transformants in order to prevent effects on adjacent strains due to the release of adenine and uracil into the media.

![Image of yeast colonies](https://doi.org/10.1371/journal.pone.0196840.g009)
The cloned genes represent four different mechanisms by which a cell may acquire resistance to nucleotide analogues. The first resistance mechanism is to promote efflux of the drug, and is exemplified by ATR1, ATR2 and HMS1. The ATR1 gene encodes a multidrug efflux pump, and a likely reason for the strong resistance to both HAP and 5-FU conferred by overexpression of ATR1 is that Atr1p pumps out the non-canonical nucleotides, (d)HAPMP and 5-F(d)UMP, or the corresponding free bases. HMS1 encodes a myc-related transcription factor that was found to activate ATR1 expression in a microarray experiment [45]. This provides a likely explanation for our findings that overexpression of HMS1 has no effect on the atr1 knockout strain, indicating that Atr1p functions downstream of Hms1p in conferring resistance to boric acid.

https://doi.org/10.1371/journal.pone.0196840.g010

The cloned genes represent four different mechanisms by which a cell may acquire resistance to nucleotide analogues. The first resistance mechanism is to promote efflux of the drug, and is exemplified by ATR1, ATR2 and HMS1. The ATR1 gene encodes a multidrug efflux pump, and a likely reason for the strong resistance to both HAP and 5-FU conferred by overexpression of ATR1 is that Atr1p pumps out the non-canonical nucleotides, (d)HAPMP and 5-F(d)UMP, or the corresponding free bases. HMS1 encodes a myc-related transcription factor that was found to activate ATR1 expression in a microarray experiment [45]. This provides a likely explanation for our findings that overexpression of HMS1 has no effect on the atr1 knockout strain, indicating that Atr1p functions downstream of Hms1p in conferring resistance to boric acid.

https://doi.org/10.1371/journal.pone.0196840.g010

The cloned genes represent four different mechanisms by which a cell may acquire resistance to nucleotide analogues. The first resistance mechanism is to promote efflux of the drug, and is exemplified by ATR1, ATR2 and HMS1. The ATR1 gene encodes a multidrug efflux pump, and a likely reason for the strong resistance to both HAP and 5-FU conferred by overexpression of ATR1 is that Atr1p pumps out the non-canonical nucleotides, (d)HAPMP and 5-F(d)UMP, or the corresponding free bases. HMS1 encodes a myc-related transcription factor that was found to activate ATR1 expression in a microarray experiment [45]. This provides a likely explanation for our findings that overexpression of HMS1 has no effect on the atr1 knockout strain, indicating that Atr1p functions downstream of Hms1p in conferring resistance to boric acid.

https://doi.org/10.1371/journal.pone.0196840.g010

The cloned genes represent four different mechanisms by which a cell may acquire resistance to nucleotide analogues. The first resistance mechanism is to promote efflux of the drug, and is exemplified by ATR1, ATR2 and HMS1. The ATR1 gene encodes a multidrug efflux pump, and a likely reason for the strong resistance to both HAP and 5-FU conferred by overexpression of ATR1 is that Atr1p pumps out the non-canonical nucleotides, (d)HAPMP and 5-F(d)UMP, or the corresponding free bases. HMS1 encodes a myc-related transcription factor that was found to activate ATR1 expression in a microarray experiment [45]. This provides a likely explanation for our findings that overexpression of HMS1 has no effect on the atr1 knockout strain, indicating that Atr1p functions downstream of Hms1p in conferring resistance to boric acid.

https://doi.org/10.1371/journal.pone.0196840.g010
and Log1p. We interpret these interactions as synergisms between drug efflux (Atr1p and Hms1p) and other detoxification mechanisms (Log1p and Ham1p). Finally, our finding that the ATR1-related gene ATR2 (YMR279C) also confers resistance to HAP and 5-FU (Fig 4) suggests that its gene product acts as a multidrug efflux pump similar to Atr1p.

The second resistance mechanism is to dilute the drug or its activated metabolites by boosting the de novo synthesis of nucleotides. This mechanism is exemplified by CPA1, CPA2 and ADE4. CPA1 and CPA2 overexpression boosts de novo pyrimidine synthesis, thus preventing 5-FU from exerting its toxic action [16]. Overexpression of ADE4, which encodes the first enzyme in purine biosynthesis, similarly boosts de novo purine synthesis, which dilutes HAP and prevents it from exerting its toxic action. As expected, we found that these resistance genes are highly specific for the type of nucleotide: CPA1 and CPA2 do not confer resistance to HAP (Fig 6), and ADE4 does not confer resistance to 5-FU (Fig 7). However, CPA1 and CPA2 do confer resistance to other pyrimidine analogues such as 5-fluorocytosine and 6-azauracil (Fig 9).

A third resistance mechanism is to interfere with activation of the drug, and it is possible that APT2 could act in this way. Our finding that APT2 overexpression confers resistance to HAP is surprising since previous studies did not detect a knockout phenotype or enzymatic activity associated with APT2, which was therefore proposed to be a pseudogene [37]. The homology to APT1 which encodes APRT suggests that APT2, if active, should encode an enzyme with similar activity. APRT is needed for activation of HAP into its toxic metabolite HAPMP [24], so overexpression of a protein with APRT activity would be expected to make cells more rather than less sensitive to HAP. Consistent with this, we found that overexpression of APT1 did make the cells slightly more sensitive to HAP (Fig 3). One possible explanation for our finding that overexpression of APT2 confers resistance to HAP could be that it interferes with the expression of Apt1p and thus with its ability to activate HAP. Such interference could occur by promoter competition for an activator of both genes, since APT2 is known to be transcribed [37]. Alternatively, since APRT is a dimeric enzyme [37], overexpression of APT2 might lead to the formation of inactive Apt1p-Apt2p heterodimers. However, a second possible explanation for our finding could be that Apt2p has a previously undetected activity that helps to detoxify HAP. APRT catalyzes a reversible reaction [47], so one possibility is that Apt2p is an APRT that favours the reverse reaction, converting HAPMP to HAP. Yeast isozymes can have opposite favoured directions; one example of this is the alcohol dehydrogenases Adh1p and Adh2p [48]. Our finding that the apt2 knockout is moderately sensitive to HAP (Fig 5A) is consistent with the second explanation, since it suggests that APT2 is not just a pseudogene. Our finding that APT1 overexpression confers resistance to 5-FU (Fig 3) could be due to interference with activation of 5-FU to 5-FUMP (Fig 11), since the 5-FU activating enzyme Fur1p and the adenine activating enzyme Apt1p both use the same substrate, PRPP. Purines have been shown to relieve 5-FU toxicity in a cell line, and it was suggested that this is due to PRPP being depleted during activation by nucleobase phosphoribosyltransferases [49].

The fourth resistance mechanism is to detoxify the drug by degrading it or its activated metabolites. All organisms need to keep their nucleotide pools free from non-canonical nucleotides that might cause damage if incorporated into DNA or RNA. Some non-canonical nucleotides are generated continuously from the metabolism, such as dUTDP produced as an intermediate in TMP synthesis, or IMP and XMP which are intermediates in AMP and GMP synthesis. Other non-canonical nucleotides are generated by oxidation or deamination of canonical nucleotides. Several enzymes have evolved to deal with the threat posed by non-canonical nucleotides [50], and overexpression of such enzymes is expected to confer resistance to nucleotide analogues. This resistance mechanism is exemplified by the DUT1 and HAM1 genes and, as argued below, we also think that LOG1 belongs to this class of genes.
**DUT1** encodes dUTP pyrophosphatase, which degrades the genotoxic dUTP generated from the dUDP produced during TMP synthesis [51]. It is an essential enzyme in many organisms including yeast [52]. Dut1p was long thought to be specific for dUTP, but it was more recently found to have significant activity also against the non-canonical purine nucleotide dITP [52]. Our finding that overexpression of **DUT1** confers resistance to HAP suggest that Dut1p may have an even broader specificity, including purine analogue triphosphates such as dHAPTP. In this context, our finding that overexpression of **DUT1** has little or no effect on the resistance to 5-FU is surprising since 5-FdUTP is more similar to dUTP, and since the *dut1/DUT1* heterozygote, as expected, was sensitive to 5-FU but not to HAP (Fig 5). However, dephosphorylation of 5-FdUTP generates 5-FdUMP which is also highly toxic due to its inhibition of thymidylate synthase [4]. It is conceivable that increased production of 5-FdUMP could explain why overexpression of **DUT1** has little or no beneficial effect on 5-FU toxicity. Since dHAPMP is not toxic, a similar situation would not exist for HAP toxicity, which could explain why **DUT1** overexpression has an effect in that case.

Interestingly, we observed a significantly higher sensitivity to 5-FU in the diploids, both in the wild type and in the *dut1/DUT1* heterozygote (Fig 5B). A likely reason for this is that the response to genotoxic stress differs between haploids and diploids. Thus, Li and Tye [53] found that the replication stress induced by a defective *mcm4* allele caused a diploid-specific severe genetic instability and reduced viability. It was suggested that this is due to different

---

https://doi.org/10.1371/journal.pone.0196840.g011
repair pathways being favoured in haploid and diploid cells. In haploids, replication stress mainly induces Rad6-dependent pathways that resume stalled forks, whereas diploids use the Rad52- and MRX-dependent pathways that repair double strand breaks. Presumably, the latter type of repair events are lethal when massively induced, which may explain both the reduced viability of mcm4 diploids [53] and our finding that diploids are more sensitive to 5-FU than haploids (Fig 5B).

The HAM1 gene was discovered in a screen for yeast genes that cause increased sensitivity to HAP when mutated [17]. Ham1p and its orthologues in other species are purine nucleoside triphosphate phosphatases with specificity for (d)ITP and (d)XTP [18,54–55]. This suggested that Ham1p has evolved to deal with the threat posed by these naturally occurring non-canonical purine nucleotides. However, it was subsequently shown that overexpression of HAM1 also confers resistance to the pyrimidine analogues 5-bromodeoxyuridine [56] and 5-FU [16]. This indicates that the Ham1p enzyme has a broader specificity, being active also against non-canonical pyrimidine nucleotides. Our finding that overexpression of DUT1 is fully able to compensate for the increased HAP sensitivity of the ham1 strain (Fig 6) is interesting, as Dut1p is thought to be specific for deoxyribonucleotides, and would thus presumably target only dHAPTP but not HAPTP. It suggests that the genotoxic effects of dHAPTP are more important for HAP toxicity than the effects of HAPTP incorporation into RNA.

Our finding that LOG1 confers resistance to HAP when overexpressed is consistent with the previous finding that a log1 knockout is sensitive to HAP [23]. The function of the yeast Log1 protein remains to be determined. However, its homology to the plant LOG proteins, which produce free cytokinins by cleaving off the N6-modified adenine from cytokinin nucleoside monophosphates [26,43], is intriguing since it is the same N6-position of adenine that is modified in HAP. It is therefore likely that Log1p confers resistance to HAP by cleaving off the HAP nucleobase from the activated (deoxy)ribonucleoside monophosphate, (d)HAPMP, thus preventing its conversion into (d)HAPTP that can be incorporated into RNA or DNA. This would be consistent with the partial dependence of LOG1 on HAM1 for its ability to confer HAP resistance when overexpressed (Fig 6), since Log1p would then function downstream of Ham1p in the degradation of non-canonical purine nucleotides (Fig 11). The fact that LOG1 confers resistance to 5-FU when overexpressed further suggests that Log1p is active also against non-canonical pyrimidine nucleoside monophosphates such as 5-FUMP. The partial dependence of HAM1 on LOG1 for the ability to confer 5-FU resistance (Fig 7) is consistent with this notion.

In order to assess how general our findings were, we also tested the effects of the cloned resistance genes on the sensitivity to nine additional purine and pyrimidine drugs. Six of the drugs failed to produce any toxicity in yeast, but results were obtained with the pyrimidine analogues 5-FC and 6-AzaU and the purine analogue 8-AzaG, which are shown in Fig 9. The resistance gene profile of 8-AzaG was similar to that of HAP, except for the fact that overexpression of DUT1, APT2 and HMS1 did not confer any significant resistance to 8-AzaG. The absence of an effect of DUT1 is likely due to 8-AzaG toxicity being mainly caused by its incorporation into RNA, which inhibits protein synthesis [57]. Hence, a reduction in any 8-aza-dGTP formed is not expected to relieve toxicity. The absence of an effect of APT2 is likely due to the fact that activation of guanine and 8-AzaG to ribonucleotides is catalyzed by hypoxanthine-guanine phosphoribosyltransferase, encoded by the yeast HPT1 gene [58], in contrast to HAP, which is mainly activated by APT1 [24]. The lack of a detectable effect of HMS1 on 8-AzaG toxicity could simply be due to the fact that HMS1 was the weakest resistance gene recovered in our screen (Fig 2A).

The resistance gene profile of 6-AzaU was more narrow than that of 5-FU. Thus, we saw a significant effect only with the CPA1 and CPA2 genes (Fig 9). A likely explanation for this is
that unlike 5-FU, 6-AzaU does not get incorporated into RNA or DNA, but exerts its toxic
effect by inhibiting the pyrimidine biosynthetic enzyme OMP-decarboxylase [57]. It is there-
fore not surprising that overexpression of CPA1 and CPA2 which boosts pyrimidine biosyn-
thesis and thus provides more substrate for OMP-decarboxylase can relieve the 6-AzaU
toxicity.

The resistance gene profiles of 5-FC and 5-FU were similar, but interestingly, overexpres-
sion of ADE4, which boosts de novo synthesis of purines, confers resistance to 5-FC but not
5-FU (Fig 9). A likely explanation is that the uptake of 5-FC and 5-FU in yeast is mediated by
different transporters. Uracil and 5-FU are taken up by the uracil permease Fur4p, which is
feedback-inhibited by intracellular pyrimidines [59–61]. 5-FC is instead taken up by the purine
and cytosine permease Fcy2p, which is not inhibited or repressed by cytosine [59], but possibly
by an adenine metabolite [62]. Furthermore, ADE4 overexpression has been shown to cause
excretion of inosine and hypoxanthine [63], and hypoxanthine acts as a competitive inhibitor
of Fcy2p mediated cytosine uptake that can also relieve 5-FC toxicity [60,62]. We conclude
that the resistance to 5-FC conferred by ADE4 overexpression most likely is due to its effect on
Fcy2p-mediated uptake of 5-FC.

In conclusion, it seems that Ham1p, Log1p and Dut1p all have broader specificities than
initially thought, affecting both purines and pyrimidines (Fig 11). Together, they serve as gate-
keepers that prevent non-canonical bases from being incorporated into nucleic acids, by
dephosphorylating nucleoside triphosphates (Ham1p and Dut1p) and by cleaving the resulting
nucleoside monophosphates into free bases and ribose-1-phosphate (Log1p). Ham1p targets
both ribo- and deoxyribonucleoside triphosphates, thereby preventing the incorporation of
non-canonical bases into RNA and DNA. Dut1p only dephosphorylates deoxyribonucleoside
triposphates. It may have evolved to deal with the special threat posed by the genotoxic dUTP
generated during biosynthesis of TTP, but also targets non-canonical purine deoxyribonucleo-
tides such as dITP and dHAPTP. Log1p, finally, acts downstream of Ham1p by cleaving its
products, which should facilitate the Ham1p reaction by keeping the concentrations of these
products low, and prevent reactivation by phosphorylation. Based on the wide phylogenetic
distribution of the LOG (LONELY GUY) family of enzymes, it seems likely that keeping the
nucleotide pool free from non-canonical nucleotides is their original function, and that their
role in cytokinin production in plants and some microorganisms [26–28] is a more recent
development in these organisms.

Acknowledgments

This work was supported by grants to HR from the Swedish Cancer Society (CAN 2014/910)
and the Knut and Alice Wallenberg Foundation (KAW 2015.0056).

Author Contributions

Conceptualization: Mattias Carlsson, Hans Ronne.
Funding acquisition: Hans Ronne.
Investigation: Mattias Carlsson, Guo-Zhen Hu.
Visualization: Mattias Carlsson, Guo-Zhen Hu, Hans Ronne.
Writing – original draft: Mattias Carlsson, Hans Ronne.
Writing – review & editing: Mattias Carlsson, Guo-Zhen Hu, Hans Ronne.
References

1. Heidelberger C, Chaudhuri NK, Danneberg P, Moore D, Griesbach L, Duschinsky R, Schnitzer RJ, Pleven E, Scheiner J. Fluorinated pyrimidines, a new class of tumour-inhibitory compounds. Nature 1957; 178: 663–666. https://doi.org/10.1038/179663a0 PMID: 13418758

2. Fukushima M, Nomura H, Murakami Y, Shirasaka T, Aiba K. Estimation of Pathways of 5-Fluorouracil Anabolism in Human Cancer Cells in Vitro and in Vivo. Gan to Kagaku Ryoho. Cancer & Chemotherapy. 1996; 23(6): 721–31.

3. Longley DB, Harkin DP, Johnston PG. 5-Fluorouracil: Mechanisms of Action and Clinical Strategies. Nat Rev Cancer. 2003; 3(5): 330–38. https://doi.org/10.1038/nrc1074 PMID: 12724731

4. Hoskins J, Butler JS. RNA-Based 5-Fluorouracil Toxicity Requires the Pseudouridylation Activity of Cbf5p. Genetics. 2008; 179(1): 323–30. 179/1/323. https://doi.org/10.1534 genetics.107.082727 PMID: 18493057

5. Bunz F, Hwang PM, Torrance C, Waldman T, Zhang Y, Dillehay L, Williams J, Lengauer C, Kinzler KW, Vogelstein B. Disruption of p53 in Human Cancer Cells Alters the Responses to Therapeutic Agents. J Clin Investig. 1999; 104(3): 263–269. https://doi.org/10.1172/JCI6863 PMID: 10439067

6. Ladner RD. The Role of dUTPase and Uracil-DNA Repair in Cancer Chemotherapy. Curr Protein Peptide Sci. 2001; 2(4): 361–70. https://doi.org/10.2174/13892030345004991

7. Yoshioka A, Tanaka S, Hiraoka O, Koyama Y, Hirota Y, Ayusawa D, Seno T, Garrett C, Wataya Y. Deoxyribozyme and Nucleoside Triphosphate Imbalance. 5-Flurouracil-induced DNA Double Strand Breaks in Mouse FM3A Cells and the Mechanism of Cell Death. J Biol Chem. 1987; 262(17): 8235–41. https://doi.org/10.1016/0006-291X(87)90719-4 PMID: 2954951

8. Santi DV, McHenry CS, Sommer H. Mechanism of Interaction of Thymidylate Synthetase with 5-Fluorodeoxyuridine. Biochemistry. 1974; 13(3): 471–81. https://doi.org/10.1021/bi00700a012 PMID: 4203910

9. Giaever, Flaherty P, Kumm J, Proctor M, Nislow C, Jaramillo DF, et al. Chemogenomic profiling: identifying the functional interactions of small molecules in yeast. Proc Natl Acad Sci USA. 2004; 101: 793–798. https://doi.org/10.1073/pnas.0307490101 PMID: 14718668

10. Lum PY, Armour CD, Stepantsians SB, Cavet G, Wolf MK, Butler JS, et al. Discovering modes of action for therapeutic compounds using a genome-wide screen of yeast heterozygotes. Cell. 2004; 116:121–137. https://doi.org/10.1016/S0092-8674(03)01035-3 PMID: 14718172

11. Hoskins J, Butler JS. Evidence for Distinct DNA- and RNA-Based Mechanisms of 5-Fluorouracil Cytoxicity in Saccharomyces cerevisiae. Yeast. 2007; 24(10): 861–70. https://doi.org/10.1002/yea.1516 PMID: 17640085

12. Gustavsson M, Ronne H. Evidence That tRNA Modifying Enzymes Are Important in Vivo Targets for 5-Fluorouracil in Yeast. RNA. 2008; 14(4): 666–74. https://doi.org/10.1261/ma.966208 PMID: 18314501

13. Spedaliero CJ, Mueller EG. Not All Pseudouridine Synthases Are Potently Inhibited by RNA Containing 5-Fluorouridine. RNA. 2004; 10(2): 192–9. https://doi.org/10.1083/jmbi.2003104 PMID: 14730018

14. Fang F, Hoskins J, Butler JS. 5-Flourouracil Enhances Exosome-Dependent Accumulation of Polyadenylated RNAs. Mol Cell Biol. 2004; 24(24): 10766–76. https://doi.org/10.1128/MCB.24.24.10766-10776.2004 PMID: 15572680

15. Zhao X, Yu Y-T. Incorporation of 5-Fluorouracil into U2 snRNA Blocks Pseudouridylation and PremRNA Splicing in Vivo. Nucl Acids Res. 2007; 35(2): 550–58. https://doi.org/10.1093/nar/gkl084 PMID: 17169984

16. Carlsson M, Gustavsson M, Hu G-Z, Murén E, Ronne H. A Ham1p-Dependent Mechanism and Modulation of the Pyrimidine Biosynthetic Pathway Can Both Confer Resistance to 5-Fluorouracil in Yeast. PloS One. 2013; 8(10): e52094. https://doi.org/10.1371/journal.pone.0052094 PMID: 24124444

17. Noskov VN, Staak K, Shcherbakova PV, Kozmin SG, Negishi K, Ono B-C, et al. HAM1, the Gene Controlling 6-N-Hydroxylaminopurine Sensitivity and Mutagenesis in the Yeast Saccharomyces cerevisiae. Yeast. 1996; 12(1): 17–29. PMID: 8789257

18. Davies O, Mendes P, Smallbone K, Malys N. Characterisation of Multiple Substrate-Specific (d)ITP/(d) XTPase and Modelling of Deaminated Purine Nucleotide Metabolism. BMB Rep. 2012; 45(4): 259–64. https://doi.org/10.5483/BMBRep.2012.45.4.259 PMID: 22531138

19. von Ahnen N, Armstrong VW, Behrens C, von Tirpitz C, Stallmach A, Herfarth H, et al. Association of Inosine Triphosphatase 94C-A and Thiopurine S-Methyltransferase Deficiency with Adverse Events and Study Drop-Outs under Azathioprine Therapy in a Prospective Crohn Disease Study. Clin Chem. 2005; 51(12): 2282–88. https://doi.org/10.1373/clinchem.2005.057158 PMID: 16214825

20. Stocco G, Cheok MH, Crews KR, Dervieux T, French D, Pei D, et al. Genetic Polymorphism of Inosine Triphosphatase Pyrophosphatase Is a Determinant of Mercaptopurine Metabolism and Toxicity during

21. Pleven E, Scheiner J. Fluorinated pyrimidines, a new class of tumour-inhibitory compounds. Nature 1957; 178: 663–666. https://doi.org/10.1038/179663a0 PMID: 13418758
Roles of yeast LOG1, HAM1 and DUT1 genes in detoxification of nucleotide analogues

Byrne KP, Wolfe KH. The Yeast Gene Order Browser: Combining curated homology and syntic content reveals gene fate in polyploid species. Genome Res. 2005; 15(10): 1456–61. https://doi.org/10.1101/gr.3672305 PMID: 16169922

Alfonzo JD, Crother TR, Guetsova ML, Daignan-Fornier B, Taylor MW. APT1, but Not APT2, Codes for a Functional Adenine Phosphoribosyltransferase in Saccharomyces Cerevisiae. J Bacteriol. 1999; 181 (1): 347–52. PMID: 9864350

Kanazawa S, Driscoll M, Struhl K. ATR1, a Saccharomyces Cerevisiae Gene Encoding a Transmembrane Protein Required for Aminotriazole Resistance. Mol Cell Biol. 1988;(2): 664–73. https://doi.org/10.1128/ MCB.8.2.664 PMID: 3280970

Tkach JM, Yimit A, Lee AY, Riffle M, Costanzo M, Jaschob D, et al. Dissecting DNA Damage Response Pathways by Analysing Protein Localization and Abundance Changes during DNA Replication Stress. Nature Cell Biol. 2012; 14(9): 966–76. https://doi.org/10.1038/ncb2549 PMID: 22842922

Bozdag GO, Ulusikiz I, Gulculer GS, Karakaya HC, Koc A. Roles of ATR1 Paralogs YMR279c and YOR378w in Boron Stress Tolerance. Biochem Biophys Res Commun. 2011; 409(4): 748–51. https://doi.org/10.1016/j.bbrc.2011.05.080 PMID: 21621519
41. Brauer MJ, Saldanha AJ, Dolinski K, Botstein D. Homeostatic adjustment and metabolic remodeling in glucose-limited yeast cultures. Mol. Biol. Cell 2005; 16: 2503–17. https://doi.org/10.1091/mbc.E04-11-0968 PMID: 1575808

42. Lopez R, Silventoinen V, Robinson S, Kibria A, Gish W. WU-Blast2 Server at the European Bioinformatics Institute. Nucl Acids Res. 2003; 31(13): 3795–98. https://doi.org/10.1093/nar/gkg573 PMID: 12824421

43. Kuroha T, Tokunaga H, Kojima M, Ueda N, Ishida T, Nagawa S, et al. Functional Analyses of LONELY GUY Cytokin-in-Activating Enzymes Reveal the Importance of the Direct Activation Pathway in Arabidopsis. Plant Cell. 2009; 21(10): 3152–69. https://doi.org/10.1105/tpc.109.068676 PMID: 19837870

44. Merker DJ, Wall AS, Taylor J, Schramm VL. AMP Deaminase from Yeast. Role in AMP Degradation, Large Scale Purification, and Properties of the Native and Proteolyzed Enzyme. J Biol Chem. 1989; 264(35): 21422–30. PMID: 2687280

45. Chua G, Morris QD, Sopko R, Robinson MD, Ryan O, Chan ET, et al. Identifying Transcription Factor Functions and Targets by Phenotypic Activation. Proc Natl Acad Sci U S A. 2006; 103(32): 12045–50. https://doi.org/10.1073/pnas.0601540103 PMID: 16880382

46. Galperin MY, Moroz OV, Wilson KS, Murzin AG. House cleaning, a part of good housekeeping. Mol Microbiol. 2005; 59: 5–19. https://doi.org/10.1111/j.1365-2958.2005 .04950.x PMID: 16359314

47. Vertessy BG, Toth J. Keeping uracil out of DNA: physiological role, structure and catalytic mechanism of dUTPases. Acc Chem Res. 2009; 42: 97–106. https://doi.org/10.1021/ar800114w PMID: 18837522

52. Tchigvintsiev A, Singer AU, Flick R, Petit P, Brown G, Evdokimova E, et al. Structure and Activity of the Saccharomyces Cerevisiae dUTP Pyrophosphatase DUT1, an Essential Housekeeping Enzyme. Biochim J. 2011; 437(2): 243–53. https://doi.org/10.1042/BJ20110304 PMID: 21548881

53. Li XC, Tye BK. Ploidy dictates repair pathway choice under DNA replication stress. Genetics 2011; 187:1031–1040. https://doi.org/10.1534/genetics.110.125450 PMID: 21242538

55. Simone PD, Pavlov YI, Borgstahl GEO. ITPA (inosine triphosphate pyrophosphatase): From surveillance of nucleotide pools to human disease and pharmacogenetics. Mut. Res. 2013; 735: 131–46. https://doi.org/10.1016/j.mrrev.2013.08.001 PMID: 23969025

57. Takayama S, Fujiw Kurosa A, Adachi N, Ayusawa D. Overexpression of HAM1 gene detoxifies 5-bromodeoxyuridine in the yeast Saccharomyces cerevisiae. Curr. Genet. 2007; 52:203–11. https://doi.org/10.1007/s00294-007-0152-z PMID: 17899088

59. Heidelberger C. Cancer Chemotherapy with Purine and Pyrimidine Analogues. Ann Rev Pharmacol 1967; 7(1): 101–24. https://doi.org/10.1146/annurev.pa.07.040167.000533 PMID: 5337075

60. Jund R, Lacroute F. Genetic and Physiological Aspects of Resistance to 5-Fluoropyrimidines in Saccharomyces Cerevisiae. J. Bacteriol. 1970; 102 (3): 607–615. PMID: 5429721

61. Polak A, Grenson M. Evidence for a Common Transport System for Cytosine, Adenine and Hypoxanthine in Saccharomyces Cerevisiae and Candida Albicans. Eur. J. Biochem. 1973; 32 (2): 276–82. https://doi.org/10.1111/j.1432-1033.1973.tb02608.x PMID: 4569075
62. Chevallier MR, Jund R, Lacroute F. Characterization of Cytosine Permeation in Saccharomyces Cerevisiae. J. Bacteriol. 1975; 122 (2): 629–41. PMID: 47858

63. Rebora K, Desmoucelles C, Borne F, Pinson B, Daignan-Fornier B. Yeast AMP Pathway Genes Respond to Adenine through Regulated Synthesis of a Metabolic Intermediate. Mol. Cell. Biol. 2001; 21 (23): 7901–12. https://doi.org/10.1128/MCB.21.23.7901-7912.2001 PMID: 11689683