Metabolic Regulation and Anticancer Drug Resistance in the Yeast *Saccharomyces cerevisiae*

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Abstract

Cancer is a malignant neoplastic tissue. The pyrimidine analogue 5-fluorouracil is an anticancer drug used against several types of cancer. It owes its specificity against neoplasms due to the higher rate of nutrient uptake, RNA and DNA synthesis, and in particular the higher rate of nucleobase scavenging and salvage of neoplastic cells. However, tumours can develop resistance to 5-fluorouracil and other anticancer drugs, rendering the therapy useless. Due to genetic variation, humans can also be inherently more or less sensitive to 5-fluorouracil and other anticancer drugs such as the purine analogue mercaptopurine.

In this thesis I have investigated the mechanisms of resistance to 5-fluorouracil and the model purine analogue 6-N-hydroxylaminopurine in the budding yeast *Saccharomyces cerevisiae*. The mechanisms found to confer resistance in *S. cerevisiae* to 5-fluorouracil and 6-N-hydroxylaminopurine included both deregulation of the *de novo* synthesis of nucleotides, and detoxification of non-canonical nucleotides. I proceeded to study genetic interactions between genes involved in resistance to both 5-fluorouracil and 6-N-hydroxylaminopurine by using plasmid overexpression in yeast strains knocked out for different resistance genes and other genes involved in nucleotide metabolism.

HAM1 is a yeast gene coding for a nucleotide pyrophosphatase, the overexpression of which we found to confer resistance to both 5-fluorouracil and 6-N-hydroxylaminopurine. *HAM1* has a human homologue called *ITPA*. We proceeded to test if a known genetic polymorphism in *ITPA* could help predict toxicity in patients receiving a chemotherapeutic regimen including 5-fluorouracil. Our results suggest that loss of *ITPA* function may lead to increased sensitivity to 5-fluorouracil.

We have also investigated the mechanism of transcriptional regulation by the yeast Rph1p protein, a zinc finger transcription factor which also contains a jmjC domain with histone demethylase activity. Rph1p functions downstream of the RAS-cAMP-PKA pathway and several other nutrient sensing pathways. We found, surprisingly, that the histone demethylase activity of Rph1p is largely dispensable for its role in transcriptional regulation.

Keywords: *Saccharomyces cerevisiae*, 5-Fluorouracil, 6-N-hydroxylaminopurine, histone demethylase, nucleotide analogues, toxicity, drug resistance, cancer.

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Dedication

Whom shall research whither hence.

Contents

List of Publications

This thesis is based on the work contained in the following papers, referred to by Roman numerals in the text:

I Carlsson, M., Gustavsson, M., Hu, G-Z., Murén, E., Ronne, H. (2013). A Ham1p-dependent mechanism and modulation of the pyrimidine biosynthetic pathway can both confer resistance to 5-fluorouracil in yeast. PloS One 8: e52094. doi:10.1371/journal.pone.0052094.

II Nordberg, N., Olsson, I., Carlsson, M., Hu, G-Z., Orzechowski Westholm, J., Ronne, H., (2014). The histone demethylase activity of Rph1 is not essential for its role in the transcriptional response to nutrient signaling. PloS One 9(7): e95078. doi: 10.1371/journal.pone.0095078.

III Mattias Carlsson, Mia Wadelius, Hugo Kohnke, Bengt Glimelius, Hans Ronne, 2014. Genetic variant of inosine triphosphate pyrophosphatase (ITPA) predicts toxicity of 5-fluorouracil and irinotecan in patients with metastatic colorectal cancer. (manuscript).

IV Mattias Carlsson, Guo-Zhen Hu, Hans Ronne, 2014. Cloning of genes that confer resistance to 6-N-hydroxylaminopurine when overexpressed in yeast and their interaction with genes that confer resistance to 5-fluorouracil. (manuscript).

Publications I-II are reproduced with the permission of the publishers.

The contribution of Mattias Carlsson to the papers included in this thesis was as follows:

I Took part in project planning. Major contribution to the laboratory work including screening, cloning, resistance phenotyping, and radioactive labelling experiments. Minor contribution to manuscript writing.

II Took part in the laboratory work with cDNA and qPCR. Made analysis of qPCR data and took part in the analysis when comparing sets of genes. Minor contribution to manuscript writing.

III Took part in project planning. Took part in the laboratory work of DNA-preparation and genotyping. Major contribution to data analysis and manuscript writing.

IV Took part in project planning. Major contribution to the laboratory work including screening, cloning, resistance phenotyping and genetic interactions. Major contribution to analysis of results and manuscript writing.

Abbreviations

5-FU	5-Fluorouracil
5-FOA	5-Fluoroorotic acid
6-MP	6-Mercaptopurine
HAP	6-N-Hydroxylaminopurine
AMPK	5' AMP-activated protein kinase
AP-site	Apurinic/Apyrimidinic site
ABC	ATP-binding cassette transporters
BER	Base excision repair
cAMP	Cyclic AMP
CAD	Multifunctional protein including CPSase
CDK	Cyclin-dependent kinase
Clb, Cln	Late and early cyclins
CP	Carbamoyl phosphate
CPSase	Carbamoyl-phosphate synthetase
DDR	DNA damage response
DSB	DNA double stranded break
dsDNA	Double stranded DNA
G0, G1, G2	Cell cycle phases
GAP	GTPase-activating protein
GC-RMA	Gene ChipRobust Multiarray Averaging
GEF	Guanine nucleotide exchange factor
GGR	Global genomic repair
GTPase	Signalling GTP/GDP binding protein
H3K36	Lysine 36 of histone protein 3
HAP	6-N-hydroxylaminopurine
HIP	Haploinsufficiency profiling
HOP	Homozygous deletion profiling
HRR	Homologous recombination repair
IMP	Inosine monophosphate

ITPA	Inosine triphosphate pyrophosphatase
kanMX	Kanamycin resistance cassette
Ku	Yku70p-Yku80p DNA encircling complex
Log	Logarithmic growth phase
LOG	Lonely Guy cytokinin-activating gene/protein
LP-BER	Long-patch base excision repair
M	Cell cycle phase of mitosis and cytokinesis
MEN	Mitotic exit network
MMEJ	Microhomology-mediated end joining
MMS	Methyl methanesulfonate, DNA alkylating agent
MODS	Multiple organ dysfunction syndrome
MRX	Heterotrimeric complex Mre11p-Rad50p-Xrs2p.
MTHF	5,10-Methylenetetrahydrofolate
NAG	N-acetylglutamate
NER	Nucleotide excision repair
NHEJ	Non-homologous end joining
ORC	Origin recognition complex of DNA replication
ORF	Open reading frame
PDS	Post diauxic shift growth phase
PKA	Protein Kinase A
PRPP	Phosphoribosyl pyrophosphate
Rad17p-Clamp	Heterotrimeric complex Rad17p-Mec3p-Ddc1p
RAS	A small GTPase
RNR	Ribonucleotide Reductase
RPA	Replication protein A
S	Cell cycle phase of DNA synthesis
SBF	SCB binding (transcription) factor, complex of Swi4p-Swi6p
SN-BER	Single-nucleotide base excision repair
SSB	DNA single-stranded break
ssDNA	Single stranded DNA
STRE	Stress response element (DNA sequence motif)
TCR	Transcription coupled repair
TLS	Translesion synthesis
TORC1	Target of rapamycin complex 1
U2-snRNA	U2 spliceosomal small nuclear RNA
YAC	Yeast artificial chromosome

1 Yeast

The budding yeast *Saccharomyces cerevisiae*, a eukaryotic microorganism, got its genus and species names from the Latin words for 'sugar fungus' and beer. It is also named 'budding yeast' in the scientific literature due to the fact that it has an asymmetric cell division, where a small daughter cell buds off the larger mother cell. It is however most widely known as just 'yeast', since it is the yeast used in human culture to make bread, wine and beer since ancient times. Even more ancient is the time point when the ancestors of humans and *S. cerevisiae* diverged. It is estimated to have taken place more than 1.2 billion years ago (Wu et al., 1986).

The laboratory strains of budding yeast are often single cells when grown in liquid culture, and form round smooth colonies when grown on solid media. However, at least some strains of *S. cerevisiae* also have the capacity to form biofilms or invasive pseudohyphae on solid media and can form multicellular aggregates (flocculate) in liquid culture (Reynolds and Fink, 2001; Smukalla et al., 2008).

S. cerevisiae can be easily propagated both in its haploid and in its diploid form. A diploid strain is generated by mating of two haploid strains with opposite mating types. A haploid strain is regenerated when the diploid sporulates (*i.e.* goes through meiosis). This sexual life cycle facilitates the construction of yeast strains with new genotypes since new combinations of mutations or gene knockouts can be generated by mating of single mutant haploids and sporulation, after which double mutant haploids can be recovered by tetrad dissection. The efficient homologous recombination of *S. cerevisiae* makes it relatively easy to both knock in and knock out chromosomal genes. New genes and multiple copies of existing genes can also be introduced by transformation of yeast cells with shuttle plasmids or yeast artificial chromosomes (YACs).

The genome of *S. cerevisiae* has been fully sequenced and contains about 6000 genes, whereof about 20% are essential and 3% show haploinsufficiency (a growth deficiency of heterozygous wild type/knockout mutant diploids) on standard rich media (Deutschbauer et al., 2005; Goffeau et al., 1996). The Saccharomyces Genome Deletion Project made start to stop codon deletions of all non-essential yeast genes, in which the open reading frame was replaced by a uniquely tagged *kanMX* module, which confers kanamycin resistance. The project generated both homo- and heterozygous knockout strain libraries, which have greatly facilitated the discovery of gene functions and phenotypes (Kelly et al., 2001).

1.1 Chemical genetics and drug discovery in yeast

Around 2700 of the 6000 predicted *S. cerevisiae* proteins have human homologues with e-values less than 10^{-10} when blasted against the corresponding yeast protein. Several hundred of these predicted human orthologues of yeast genes have been implicated in human diseases. Significantly, drugs targeting human proteins may also inhibit the corresponding yeast orthologue, *e.g.* lovastatin which inhibits sterol biosynthesis both in human cells and in yeast. Yeast can therefore be used as a tool to discover new drugs for use in humans, at least when the targeted processes or enzymes are conserved between the two organisms.

The cellular processes that are most highly conserved between humans and yeast are the primary metabolism, protein synthesis, cell division, intracellular transport, and DNA synthesis and repair. This implies that yeast can be useful as a tool for studying cancer and cytostatic drugs, to the extent that cancer is viewed as a cell division disorder (Hughes, 2002).

One purpose of chemical genetics is to find macromolecular targets of small molecules. By modulating the functions of such macromolecules, using small molecules, one can bring about cellular changes in signal transduction, enzyme activities and cellular organization. The interactions between small molecules and macromolecules can be studied from two different perspectives. The focus could thus either be on a small molecule and which kind of effect it causes to the cell, or on a macromolecule, whose function could potentially be altered by some small molecule. The strategies to find an interaction are different depending on which perspective one has. If the mechanism behind the growth inhibition caused by a small molecule is sought, one could use HIP, HOP or resistance screens, as described below. In contrast, if one wants to find a small molecule that inhibits the function of a specific macromolecule, HIP could be used to screen a chemical library for potential inhibitors.

1.1.1 Haploinsufficiency profiling

Haploinsufficiency profiling (HIP) is a technique used to identify proteins whose functions are affected by a specific condition. This is done by looking for growth defects of yeast strains that are heterozygous for different gene deletions under a specific condition, *e.g.* the exposure to a small molecule inhibitor. This kind of growth defect due to a 50% reduced gene dosage (as compared to the homozygous wild type strain) is called haploinsufficiency. The technique can also be used the other way around, to screen for a specific condition that inhibits the growth of a yeast strain that is heterozygous for a given gene knockout.

HIP can be massively parallelized by pooling genetically marked ("bar coded") yeast strains during growth and testing, followed by a microarray analysis to determine which strains survived in the presence of the drug and which strains did not (Giaever et al., 2004).

The HIP technique only works if one can find conditions where the strain that is heterozygous for the deleted gene has a growth defect, as compared to the wild type strain. For example, to find an inhibitor of the Ura2p enzyme, one could compare the growth of the heterozygous *URA2/ura2* strain and the wild type *URA2/URA2* strain in media devoid of uracil (which requires Ura2p function), with and without a potential inhibitor of Ura2p.

1.1.2 Homozygous deletion profiling

The homozygous deletion profiling (HOP) technique is similar to HIP in set-up, but different in interpretation. In the HOP assay one compares the growth of a haploid wild type strain to that of a haploid strain where the single copy of a specific gene has been knocked out. If the knockout strain has a growth defect under a specific condition, it cannot be due to the condition inhibiting the knocked out protein, since the protein is not there.

It is, however, still possible to find clues to the target of the condition, since the knocked out protein whose absence causes an increased sensitivity to the condition may act in redundancy or in synergy with an unknown target that is directly affected by the condition.

For example one could hypothetically find that arginine inhibits the growth of a strain defective in the carbamoyl phosphate synthetase subfunction of Ura2p, since arginine represses the functionally redundant carbamoyl phosphate synthetase function of the Cpa1p/Cpa2p complex (Lacroute et al., 1965).

1.1.3 Plasmid overexpression screens for genetic suppression and resistance

Under a condition where wild type or mutant cells have a growth inhibited phenotype, one can also find the protein affected by the condition, or other proteins which can rescue the growth defect when overexpressed, by transforming the cells with a yeast genomic library made in a high copy number plasmid.

In the scenario where the condition is the exposure to an enzyme inhibitor, either the overexpression of the inhibited enzyme, a compensatory enzyme, or a detoxifying enzyme would be expected to restore growth. In the screen in publication I for 5-fluorouracil (5-FU) resistance, we did not isolate strains that overexpressed Cdc21p, thymidylate synthetase, which is inhibited by 5-FU. However, we isolated strains overexpressing genes, *HAM1* and *YJL055w*, known and expected respectively to be involved in detoxification of non-canonical nucleotides.

Overexpressor screens can also be employed to find suppressor genes, *i.e.* genes whose overexpression compensates a reduced viability resulting from the loss or mutation of a given protein. With this approach it is for example possible to find proteins acting downstream in the signalling pathway of the targeted protein, or subunits in the protein complex which the protein is part of.

2 The cell cycle and nutrient signalling

The progression of the cell cycle can be divided into four phases designated G1, S, G2 and M. The G1, S and G2 phases are sometimes collectively called the interphase, while the M-phase is composed of mitosis and cytokinesis. *S. cerevisiae* has an asymmetrical cytokinesis *i.e.* a cell division where the larger mother cell buds off a smaller daughter cell; hence the colloquial name of *S. cerevisiae* is budding yeast.

Each phase of the cell cycle is associated with specific cyclins, *e.g.* Cln cyclins during G1 and Clb cyclins during the S, G2 and M phases. Cdc28p/Cdk1p, which is the major cyclin dependent protein kinase (CDK) in yeast, performs phase-specific signalling during the cell cycle by associating with the different cyclins. Another CDK is Pho85p, which has its own set of cyclins, and is involved in the response to different external phosphate levels. The ordered progression of the cell cycle is subject to several checkpoints when the cell cycle can be halted to ensure coordination of different processes (Pringle et al., 1997).

2.1 START of the cell cycle and TORC1 and PKA signalling

The metabolic regulation of the cell cycle takes place in the G1 phase. During G1 *S. cerevisiae* decides if conditions are favourable enough to commit the cell to progress through the next cell cycle. If conditions are favourable, the association of the early cyclin Cln3p with Cdc28p, and the resulting activation of the CDK, induces the START condition in which over 200 genes are upregulated, including the genes coding for the next set of cyclins, Cln1p and Cln2p. Cln3p is regulated at the transcriptional level by glucose and acetyl-CoA, and the translation of Cln3p is regulated by the TOR and RAS-cAMP-PKA nutrient sensing pathways (Shi and Tu, 2013).

S. cerevisiae has two RAS proteins, Ras1p and Ras2p. The N-terminal of the RAS-GEF Cdc25p is involved in glucose sensing through mechanisms that are still poorly understood; Ras1p and Ras2p have been shown to be activated by intracellular acidification independent of glucose and Cdc25p. Cdc25p or intracellular acidification or both propagates a signal through the Cdc25p-RAS-cAMP-PKA pathway. Moreover, Cdc25p is thought to be regulated by a negative feedback involving protein kinase A (PKA) phosphorylation, which could prevent overproduction of cAMP during growth on high glucose (Colombo et al., 1998; Gross et al., 1999).

Hypothetically, since cAMP is produced from ATP, the energy status of the cell (reflected by the ATP concentration) could synergistically interact with both intracellular acidification and glucose to determine the subsequent activity of PKA.

Furthermore, the G-protein Gpa2p together with the G-protein coupled receptor Gpr1p can produce cAMP and activate PKA via a separate glucose induced signalling pathway (Colombo et al., 1998; Kraakman et al., 1999; Wang et al., 2004).

TORC1 is a signalling complex containing either of the redundant Tor1p or Tor2p protein kinases. TORC1 senses the availability and quality of nutrients including carbon and nitrogen in the form of amino acids and ammonium. TORC1 phosphorylates and activates the protein kinase B homologue Sch9p if conditions are favourable for proliferation. There is also another complex TORC2, which only contains the Tor2p kinase, and differs from TORC1 in its upstream effectors and downstream targets. There are indications that TORC2 is activated by ribosomal association, and inhibited by environmental stress (Loewith and Hall, 2011; Wanke et al., 2008). TORC2 is involved in regulating actin organization, maintaining cell wall integrity, and regulating ceramide biosynthesis via phosphorylation of the downstream targets Slm1p, Slm2p, and Ypk2p; Ypk2p being the major target (Liao and Chen, 2012). TORC2 has furthermore been shown to positively regulate the activity of the pentose phosphate pathway, which generates the nucleotide precursor ribose-5-phosphate (Kliegman et al., 2013).

2.2 The G0 phase and the diauxic shift

S. cerevisiae cells can also exit from G1 into a stationary G0 phase. This eventually happens when yeast cells run out of nutrients, but stationary phase is preceded by the diauxic shift, during which *S. cerevisiae* switches from fermentation to respiration. The AMPK homologue Snf1p is important for the regulation of transcription during entry into the non-fermentative post diauxic

shift growth phase. When the energy status of the cell goes down, the concentration of ADP rises. Snf1p is activated due to this rise in ADP concentration. Activated Snf1p phosphorylates and activates several transcription factors, but deactivates both the Sch9p protein kinase mentioned above, and the transcriptional repressor Mig1p. Deactivation of Mig1p by Snf1p-dependent phosphorylation allows the expression of genes responsible for the utilization of non-fermentable and other non-preferred carbon sources, such as galactose (Celenza and Carlson, 1984; Lu et al., 2011; Mayer et al., 2011; Nehlin and Ronne, 1990).

In a parallel pathway the protein kinase Rim15p integrates signals from several nutrient sensing pathways and controls entry into stationary phase. Either loss of nitrogen- or carbon-dependent TORC1-Sch9p signalling or loss of phosphate-dependent Pho80p-Pho85p signalling causes dephosphorylation and nuclear import of Rim15p. A simultaneous loss RAS-cAMP-PKA signalling due to lack of fermentable carbon sources releases the inhibition of Rim15p protein kinase activity and promotes entry into G0 (Pedruzzi et al., 2003; Wanke et al., 2008, 2005).

2.2.1 The Gis1p and Rph1p transcriptions factors

Genome wide transcription profiling indicates that the effect of Rim15p on gene expression during the diauxic shift is almost entirely mediated by the transcription factors Msn2p, Msn4p, Gis1p and Rph1. Msn2p and Msn4p are STRE-element binding transcription factors, which control transcription in response to a variety of stress conditions. Gis1p is a Post Diauxic Shift, PDS-element binding transcription factor, which also binds to the STRE element (Cameroni et al., 2004; Swinnen et al., 2006). However, it has been reported that Rim15p does not directly phosphorylate Gis1p *in vitro*, but only Msn2p, which suggest that a unknown protein kinase might convey the signal from Rim15p to Gis1p (Lee et al., 2013).

Gis1p also has a homologue called Rph1p. Both are C_2H_2 zinc finger proteins, and are involved in growth phase dependent gene regulation. In the log phase Gis1p and Rph1p mainly act as redundant repressors of gene expression. In contrast to the log phase, Rph1p and Gis1p act as both activators and repressors, and target different sets of genes in the PDS (Orzechowski Westholm et al., 2012). Similar to Gis1p, Rph1p also gets phosphorylated after treatment with the TORC1 inhibitor rapamycin (Huber et al., 2009). Taken together, this suggests that also Rph1p is a downstream effector of Rim15p.

Sch9p inactivation dependent activation of Gis1p is also DNA-protective by causing downregulation of Rev1p, which is a component of the error prone translesion DNA polymerase Zeta complex, and by causing upregulation of

Sod2p, which prevents superoxide dependent DNA oxidation (Fabrizio et al., 2003; Madia et al., 2009).

2.2.2 Rph1p and H3K36 histone methylation

Rph1p and Gis1p both have JmjN and JmjC domains (Balciunas and Ronne, 2000). The JmjC domain, which is found in many eukaryotic proteins, has histone demethylase activity. This activity is present in Rph1p, but the JmjC domain of Gis1p seems to lack histone demethylase activity, since it is mutated in a key amino acid residue involved in co-factor coordination. Rph1p demethylates the tri- and di-methylated form of the lysine found at position 36 in histone H3, called H3K36 (Klose et al., 2007, 2006).

The H3K36 residue is methylated by the H3K36 methyltransferase Set2p during transcription. This in turn recruits the histone deacetylase complex Rpd3S, which represses spurious ORF-internal transcription by chromatin deacetylation (Carrozza et al., 2005; Drouin et al., 2010; Krogan et al., 2003). Given the fact that the methylation state of H3K36 indirectly modifies the accessibility of the chromatin for transcription, one might expect transcription regulation by Rph1p to be dependent on its H3K36 demethylase activity. However, we found in publication II that the histone demethylase activity of Rph1p is largely dispensable for transcriptional regulation by Rph1p.

In a human cell line and in the fission yeast *Schizosaccharomyces pombe*, the Set2p dependent methylation of H3K36, which is removed by Rph1p, is facilitating DNA-repair by the non-homologous end joining pathway (NHEJ) (Fnu et al., 2010; Pai et al., 2014). NHEJ is an error prone repair mechanism, and hence mutagenic, for double strand breaks in DNA. NHEJ is described in more detail in chapter 3.2.4 in this thesis.

3 Cell cycle arrest and DNA damage

3.1 DNA damage and DNA breaks

Common single strand lesions in DNA during normal growth are caused by the processing of DNA bases that have been damage by oxidation. Oxidative damage turns guanosine into 8-oxoguanosine, and thymidine into thymidine glycol. Another major DNA lesion is the spontaneous deamination of deoxycytidine resulting in a deoxyuridine. The removal and repair of damaged bases results in intermediate apyrimidinic or apurinic sites (AP-sites), and single strand breaks (SSB) via the base excision repair (BER) pathway described below. If two SSBs happen in close proximity, or the replication fork stalls at a SSB then the DNA molecule can break in two parts, with either 3' or 5' overhangs at the ends. This type of lesion is called a double strand break (DSB). Virtually all DSBs happen during the S phase. From the estimated rate of production of single strand lesions under normal conditions, 1% of the SSBs are estimated to be converted into DSBs. The estimated rate of DSB formation is around one DSB per 10^8 base pairs per cell cycle in a range of organisms. This is in line with the finding that 22% of all *S. cerevisiae* cells in S phase has Rad52p foci indicative of active DSB repair (Collura et al., 2012; Impellizzeri et al., 1991; Lisby et al., 2001; Vilenchik and Knudson, 2003).

DSBs in *S. cerevisiae* can be repaired more or less accurately by three mechanisms. DSBs with short overhangs of up to 10 bases tend to be religated with or without errors by non-homologous end joining (NHEJ). In spite of its name NHEJ can efficiently religate compatible overhangs without loss of bases, but bases may also be lost in the process. DSBs with longer overhangs tend to be repaired precisely and error free by a homologous recombination repair mechanism (HRR). Longer non-compatible overhangs, which could be generated by exonucleases, are repaired by a mechanism independent of NHEJ and HRR, the microhomology mediated end joining (MMEJ) which is error

prone and causes deletions (Daley and Wilson, 2005). The frequency of SSBs and DBSs can be increased by growth in the presence of 5-fluorouracil, see chapter 5.6.2 in this thesis.

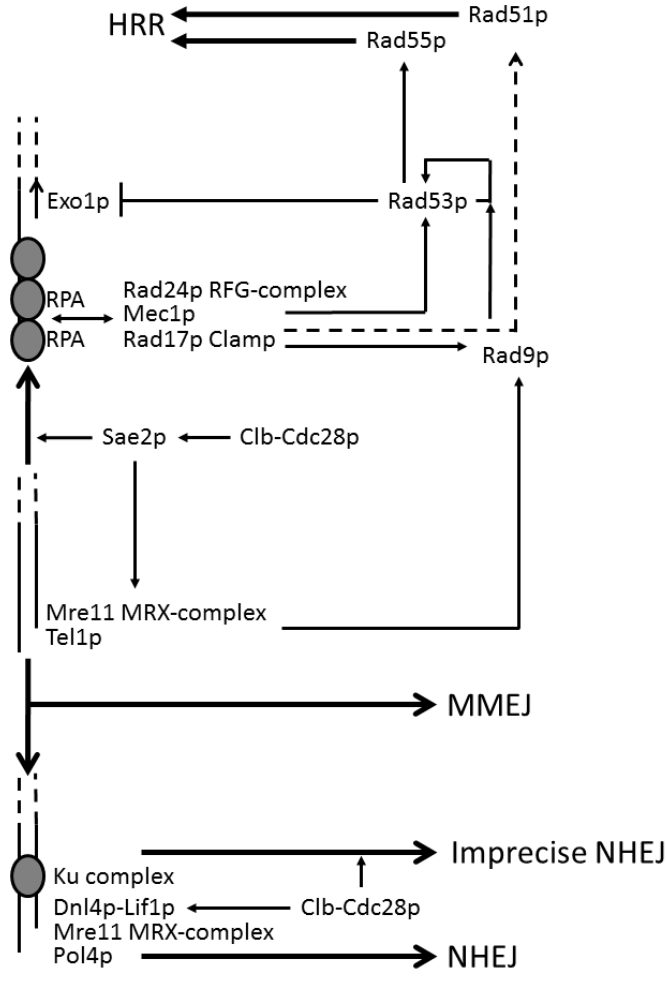

Figure 1. Simplified outline of the major DNA repair pathways in yeast, from DSB to DDR signalling and cell cycle regulated DNA repair. Damaged DNA molecules with DSBs at the lower ends, and with different bound repair complexes are shown to the left in the figure.

3.2 DNA repair mechanisms and DNA damage signalling

The DNA damage response (DDR) has a central signal protein Rad53p, which becomes phosphorylated and activated after DNA damage. The Chk1p protein

is another mediator of the DDR. DNA damage activation of Chk1p is mediated by Rad9p, and is enhanced by phosphorylation of Rad9p by Cdc28p in association with B-type cyclins during the S and G2 phases. This makes it possible to modulate DDR in coordination with the cell cycle (Abreu et al., 2013). Rad53p is also activated at stalled replication forks in a Mec1p and Mrc1p dependent manner. Stalled replication can indicate DNA-lesions, or lack of replication factors (Osborn and Elledge, 2003). Rad53p phosphorylates the Dun1p kinase, which subsequently phosphorylates the ribonucleotide reductase (RNR) inhibitor Sml1p. The phosphorylation of Sml1p promotes its degradation and activation of RNR. RNR is the rate limiting step in production of dNTPs from NTP. The increased synthesis of dNTPs by activated RNR can help recover from DNA damage and stalled replication (Chen et al., 2007; Zhao and Rothstein, 2002).

The main initiator of Rad53p DDR signalling is DSBs, but nucleotide excision repair (NER) can also activate the DDR signalling. Initially Tel1p is recruited to the ends of the DSB by the Mre11p MRX-complex. Tel1p then activates Rad53p DDR signalling via activation of Rad9p (Clerici et al., 2014; Corda et al., 2005; Schwartz et al., 2002).

As it is involved in all major DSB repair pathways, the Mre11p MRX-complex has several important features: dsDNA 3'-5' exonuclease activity, 3' overhang endonuclease activity, and 5'-3' stepwise dsDNA exonuclease activity (Trujillo and Sung, 2001; Zhu et al., 2008). See Figure 1 for a simplified outline of the major DNA repair pathways in yeast, from DNA damage to DDR signalling and cell cycle regulated DNA repair.

3.2.1 Base excision repair

Non-canonical bases in DNA can be recognized and removed by enzymes called DNA glycosylases; this generates an apurinic or apyrimidinic site (an AP-site), *i.e*. a DNA-bound deoxyribose-5'-phosphate. Examples of DNA-glycosylases in *S. cerevisiae* are Ung1p, which recognizes uracil, Ogg1p, which recognizes 8-oxyguanine, Ntg1p and Ntg2p, which recognize oxidized pyrimidines such as thymine glycol, and Mag1p, which recognizes 3-methyladenine, *e.g.* adenine alkylated with methylmethanesulfonate (MMS). After the removal of the damaged nucleobase, the repair can proceed either through single nucleotide base excision repair (SN-BER), or through long patch base excision repair (LP-BER).

In SN-BER, the Ogg1p, Ntg1p or Ntg2p proteins, which also have AP-site lyase activity, cleave the 3' phosphodiester bond of the AP-site. The AP-site endonuclease Apn1p then cleaves the 5' phosphodiester bond leaving a single nucleotide gap. The gap is filled by a DNA-polymerase, possibly Pol4p (since

Pol4p has been shown to be of importance for MMS tolerance). The resulting DNA-strand nick is then sealed by the DNA-ligase Cdc9p or Dnl4p. Alternatively, SN-BER starts with cleavage of the AP-site 5' phosphodiester bond by the AP-site endonuclease Apn1p. The lyase activity of Trf4p then cleaves the AP-site 3' phosphodiester bond leaving a single nucleotide gap, which is repaired by Pol4p and Cdc9p or Dnl4p, as described above (Chen et al., 1989; Gellon et al., 2008; Meadows et al., 2003; Sterling and Sweasy, 2006).

LP-BER starts with cleavage of the AP-site 5' phosphodiester bond by the AP-site endonuclease Apn1p. DNA-polymerase epsilon or delta then repairs the lesion by strand displacement. This process is especially useful if the deoxyribose is damaged, which would inhibit the subsequent Trf4p lyase activity in SN-BER. The 5´ flap endonuclease Rad27p then cleaves the displaced strand containing the AP-site in its 5' end. The resulting DNA-strand nick can then be sealed by the DNA ligase Cdc9p or Dnl4p (Blank et al., 1994; Wang et al., 1993; Wu and Wang, 1999)

The inactivation of the AP-site endonucleases Apn1p and Apn2p by knocking out of the corresponding genes results in constant DDR signalling via phosphorylation of Rad53p, and causes a delay in the progression of the cell cycle at the G2/M transition. This indicates that a malfunctional BER results in genetic instability since Rad53p DDR signalling is activated by the major DNA lesions: DSB, stalled replication, or resected nucleotide excision repair (NER) repair (see below). Phenotyping of strains mutated in other DNA repair pathways in combination with a disabled BER suggest that NER, HRR, and mutagenic translesion bypass synthesis (TLS) are all involved in compensating for loss of BER (Collura et al., 2012; Swanson et al., 1999).

3.2.2 Nucleotide excision repair

The NER pathway repairs a wide range of DNA lesions: intrastrand crosslinks, bulky nucleobase adducts, and other helix distorting lesions. NER is also involved in repair of interstrand crosslinks. NER relies on two mechanisms to localize the lesion. One mechanism is coupled to transcription and is initiated when RNA polymerase II encounters a lesion on the transcribed strand; this pathway is called transcription coupled repair (TCR). The other pathway relies on continuous surveillance of the helix structure; this pathway is called global genomic repair (GGR). When a lesion has been found, the NER complex excises a stretch of around 25 nucleotides centred at the lesion. The gap is refilled by DNA polymerase delta or epsilon, and the last nick in the DNA is sealed by Cdc9p (Enoiu et al., 2012; Tatum and Li, 2011).

NER repair can in turn induce Rad53p-dependent DDR signalling. Either lack of replication factors, or the presence of another lesion on the template strand may cause the DNA polymerase to stall during the refilling of the gap. This leaves an exposed 5' end that is substrate for the 5'-3' exonuclease Exo1p. Exo1p then generates a long stretch of SSDNA that induces the Rad53p DDR signal as described below under homologous recombination repair (HRR) (Giannattasio et al., 2010).

3.2.3 Homologous recombination repair

HRR is a repair mechanism with high fidelity that relies on a homologous DNA template, either a sister chromatid or a homologous chromosome in diploid cells. The 3' DNA strands of the two broken ends invade the homologous DNA by strand exchange and are then elongated using the homologous DNA as a template. Depending on how the repair process resolves it may also result in recombination between the two DNA helices.

The initial processing of a DSB is influenced by the cell cycle. The Sae2p protein is phosphorylated and activated by Cdc28p in the S and G2 phases. Activated Sae2p in association with the Mre11p-complex processes the DSB end to generate a short 3' overhang. This primes the processed DSB end for further 5'-3' exonuclease processing by either Exo1p, or the Sgs1p helicase complex and the Dna2p exonuclease; this 5'-3' exonuclease processing extends 3' ssDNA. Moreover, the initial processing of the DSB reduces its affinity for the Ku-complex, which is essential for the non-homologous end joining repair, NHEJ, thus blocking the latter. Furthermore, the Dna2p is also activated by Cdc28p during the S and G2 phases (Chen et al., 2011; Foster et al., 2011; Huertas et al., 2008; Mimitou and Symington, 2008; Zhu et al., 2008).

A model for DDR activation ssDNA has been proposed. Replication protein A (RPA) first binds to the protruding ssDNA. The Mec1p/Dcd2p heterodimer in association with the Rad24p-RFG-Clamploader then binds to the RPA molecules and loads the Rad17p-Clamp. The association between Mec1p/Dcd2p, RPA, and the Rad17p-Clamp activates the kinase activity of Mec1p. Mec1p then phosphorylates and activates Rad9p and the DDR signalling protein Rad53p. Rad9p further induces the autophosphorylation and activation of Rad53p, which in turn activates the Rad55p/Rad57p complex (see below). Rad9p and Rad53p also provide a negative feedback by inhibiting Exo1p, which prevents further generation of SSDNA (Clerici et al., 2014; Gilbert et al., 2001; Jia et al., 2004; Majka et al., 2006; Schwartz et al., 2002; Vialard et al., 1998).

The RecA homologue Rad51p is an ssDNA binding protein that facilitates homology search by DNA strand exchange between homologous DNA helices,

something which is strictly required for HRR. Rad52p and the Rad55p/Rad57p complex facilitate the loading of Rad51p onto ssDNA while displacing RPA. Rad53p, the DDR signalling protein, activates the Rad55p/Rad57p complex, and Mec1p bound to RPA sitting on ssDNA is thought to activate Rad51p (Flott et al., 2011; Herzberg et al., 2006; Miyazaki et al., 2004; Sugiyama and Kowalczykowski, 2002).

3.2.4 Non-homologous end joining

The efficiency of the NHEJ pathway is dependent on it being error-prone. NHEJ either trims or fills in the DSB DNA overhangs and then religates them, which often result in point mutations and loss of bases. Several protein complexes are engaged in NHEJ. The Ku complex preferentially binds, encircles, and stabilizes DSBs with short overhangs or blunt ends (Foster et al., 2011). The Ku complex then recruits the Mre11p-MRX nuclease complex, the Dnl4p/Lif1p/Nej1p DNA ligase complex, and the mismatch tolerant Pol4p DNA polymerase (Pardo et al., 2006; Zhang et al., 2007).

In the S and G2 phases Cdc28p phosphorylates Lif1p, which makes the NHEJ more efficient in performing mutagenic religations. This mode of repair is called imprecise NHEJ (Matsuzaki et al., 2012).

In a human cell line and in the fission yeast *S. pombe* Set2p dependent methylation of H3K36 has been shown to facilitate DNA repair by NHEJ pathway (Fnu et al., 2010; Pai et al., 2014). The H3K36 methylation is removed by the histone demethylase Rph1p in *S. cerevisiae*. The role of the histone demethylase enzymatic activity of Rph1p in gene regulation was investigated in publication II in this thesis.

3.2.5 Microhomology mediated end joining, MMEJ

The MMEJ pathway is independent of the Ku complex of the NHEJ pathway and the Rad52p protein of the HRR pathway, and is therefore considered a separate mode of DSB repair. MMEJ is dependent on the Mre11-MRX nuclease complex, the Rad1p/Rad10p 3’ flap nuclease complex, and the Dnl4p DNA ligase. MMEJ can ligate non-compatible ends by processing them until an imperfect microhomology of about 8-10 bases is found. Dnl4p then ligates the DSB with the result of a deletion spanning the region between the two microhomologies (Ma et al., 2003).

3.3 DNA and RNA damage signalling and cell cycle arrest

Activation of the DDR pathway causes a temporary cell cycle arrest in *S. cerevisiae* in order to give the cell time to repair the lesion. However, even if

DSBs persist the cell eventually adapts after about 8-15 hours and attempts to progress though the cell cycle regardless of the unrepaired DSBs. The adaptation correlates with a reduction in activity and phosphorylation of Chk1p and Rad53p, *i.e.* with deactivation of both branches of the DDR signalling (Vidanes et al., 2010). Shown in Figure 2 is a simplified outline of the DDR signalling pathways leading to cell cycle arrest.

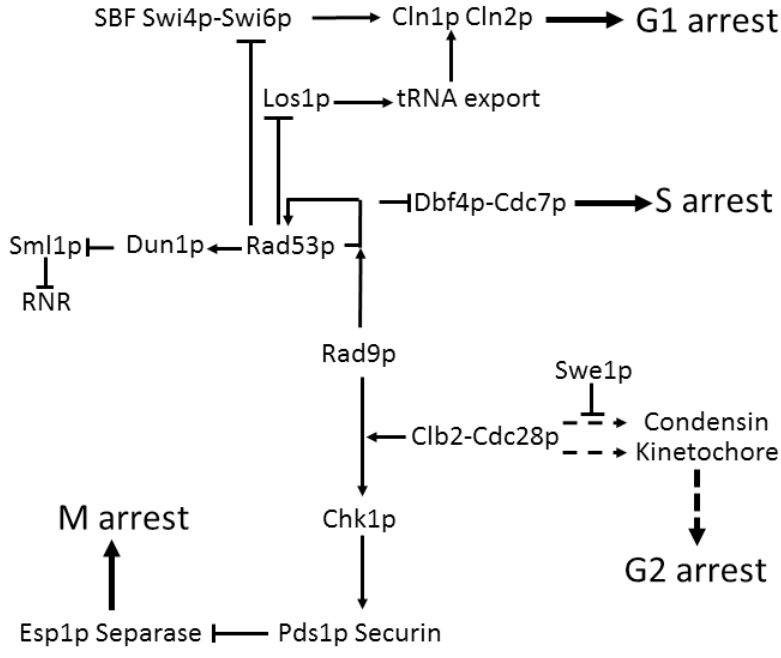

Figure 2. Simplified outline of the DDR signalling pathways leading to cell cycle arrest. Dashed arrows indicate proposed interactions that are still poorly characterized.

3.3.1 G1 arrest

G1 arrest from DDR signalling

DNA damage by MMS during the G1 phase causes an immediate drop in the late G1 cyclins Cln1p and Cln2p, which prevents progression of the cell cycle; this is an uncharacterized pathway which appears to be independent of DDR signalling via Rad53p. However, during DDR signalling, activated Rad53p phosphorylates Swi6p and thereby inhibits the Swi4p/Swi6p transcriptional activator complex SBF. This also extends the G1 phase since SBF positively

regulates the same late G1 cyclins Cln1p and Cln2p, which are necessary for progression through START into the S phase of the cell cycle (Sidorova and Breeden, 1997).

G1 arrest from intranuclear accumulation of immature tRNA

tRNA biogenesis is a complex process. The tRNA genes are transcribed by RNA polymerase III. About 20% (59 of 286) of the tRNA genes have introns, and *S. cerevisiae* tRNAs undergo 25 different modifications of nucleobases and ribose, but not all modifications are found in each tRNA. The modifying enzymes act both inside and outside of the nucleus. Before nuclear export the 3' and 5' ends of the pre-tRNA are processed. Both spliced and unspliced tRNAs are exported via nuclear pores in association with the Ran1p GTPase and exportins. Intron-containing tRNAs are spliced in the cytoplasm. tRNAs are then reimported into the nucleus constitutively and at least one spliced tRNA, tRNA(Phe), is then further modified. Los1p is the main exportin for intron-containing tRNAs, and Msn5p is the main exportin for spliced and intron-less tRNAs. However, since the double knockout *los1 msn5* is viable, there must also exist other means to export tRNA out of the nucleus. It is thought that intranuclear aminoacylation and nuclear-cytoplasmic tRNA cycling are parts of a tRNA surveillance system that prevents aberrant tRNAs from accumulating in the cytoplasm and interfere with translation (Chan and Lowe, 2009; Hopper, 2013; Kramer and Hopper, 2013; Murthi et al., 2010; Ohira and Suzuki, 2011). Accordingly, nuclear export of tRNAs is dependent on a mature structure of the tRNA backbone including post-transcriptional modifications *e.g.* pseudouridylation by Pus1p (Großhans et al., 2001; Hopper, 2013).

Retention of immature tRNAs in the nucleus induces the expression of the Gcn4p transcription factor independently of Gcn2p, which induces transcription of Gcn4p in response to amino acid starvation. The retention of immature tRNA in the nucleus can also be an active process. DDR signalling via Rad53p induces relocalization of the tRNA exportin Los1p to the cytoplasm. Unspliced tRNAs then accumulate in the nucleus and cause activation of Gcn4p. Gcn4p in turn reduces the accumulation of the late G1 cyclin Cln2p and thereby delays the progression of the cell cycle into S phase (Ghavidel et al., 2007; Qiu et al., 2000; Vazquez de Aldana et al., 1994, p. 4).

3.3.2 S-phase arrest

The Cdc7p protein kinase associates with its regulatory subunit Dbf4p, which accumulates at the G1 to S transition. The Dbf4p-Cdc7p is required for both early and late S-phase replication firing (*i.e.* start of DNA replication). Dbf4p-

Cdc7p associates with origin of replication complexes (ORCs) and phosphorylates the proteins Mcm2p and Mcm4p. The DDR signalling protein Rad53p inhibits this process by phosphorylating Dbf4p. This prevents association of the Dbf4p-Cdc7p with ORCs, which results in an inhibition of late origin firing, and induction of an S-phase arrest (Duncker et al., 2002; Lei et al., 1997; Sheu and Stillman, 2006).

Finally, it should be noted that replication fork progression also slows down in response to DNA damage, but this response is independent of Rad53p (Tercero and Diffley, 2001).

3.3.3 G2/M arrest

Mitotic entry is driven by Cdc28p activation, and prevented by Swe1p-dependent phosphorylation of Cdc28p. The Swe1p-dependent phosphorylation inhibits the Clb2p-Cdc28p kinase, thereby preventing phosphorylation of targets critical for mitotic entry (Hu and Aparicio, 2005; Rossio and Yoshida, 2011).

Prophase chromosome condensation is mediated by the Smc2p-Smc4p condensin complex. Cdc28p homologues in vertebrates, *S. pombe* and *Xenopus* induce chromosome condensation, in *S. pombe* by phosphorylation of the condensin subunit Smc4p, but it is still unknown if Clb2-Cdc28p activates condensin in *S. cerevisiae*. Finally, Cdc28p signalling is also of importance for the attachment of microtubules to kinetochores, which is needed for chromatid separation (Enserink and Kolodner, 2010; Li and Elledge, 2003).

Inhibition of anaphase

The late B cyclin Clb2p in association with Cdc28p phosphorylates the DDR signalling protein kinase Rad9p. This primes Rad9p for phosphorylation and activation of the downstream effector Chk1p (Abreu et al., 2013). Chk1p in turn phosphorylates the securin Pds1p, which prevents its degradation; degradation of Pds1p would otherwise have been be induced by the anaphase promoting complex. Phosphorylated Pds1p continue to inhibit the separase Esp1p, which is responsible for loss of cohesion between sister chromatids during progression from metaphase to anaphase, thereby preventing progression into the anaphase (Ciosk et al., 1998; Wang et al., 2001).

Inhibition of mitotic exit

Rad53p also inhibits the polo kinase Cdc5p, which results in hypophosphorylation and activation of the GAP Bfa1p-Bub2p. Bfa1p-Bub2p inhibits the GTPase Tem1p, which would otherwise promote the initiation of mitotic exit by the mitotic exit network (MEN) which is responsible for the

completion of mitosis. However, this checkpoint seems to respond only to DNA damage at the telomeres, and not to DSBs in general (Valerio-Santiago et al., 2013). Inhibition of the polo kinase Cdc5p by Rad53p also indirectly prevents spindle elongation and chromosome segregation during anaphase B (Zhang et al., 2009).

4 Cancer

The survival of a multicellular organism requires that all cells, both germ line and somatic, stay in line and act according to the plan for the common good. Individualistic endeavour is strongly discouraged; upon tendencies to deflect, the cell is simply ordered to commit suicide, which it does without hesitation. However, the plan is not written in stone, but with deoxynucleotides, and perhaps the plan was not perfectly waterproof to begin with. Over time and cell divisions the inherent imperfectnesses of DNA replication and DNA repair starts to obscure the plan, until a day comes when the cell discovers that it has a brand new plan: I am.

In reality, cells in multicellular organisms need several transformations to gradually develop into a malignant neoplasm, *i.e.* a cancer. Malignancy means that the tumour spreads by dislodging tumour cells into blood vessels, cells which act as seeds for new tumours throughout the body. These new tumours are called metastases.

When the cancer has metastasized in its host, what eventually causes death can be direct organ disruption from tumour growth, but is often (around 30% of deaths) a metabolic state called cachexia. Cachexia is a catabolic state in which body tissue is continuously wasted in spite of adequate nutrient intake. Cachexia is caused by persistent release of cytokines and other factors not only from the tumour but mainly from the host (Onesti and Guttridge, 2014). Death can also be caused by abnormal inflammation that induces the multiple organ dysfunction syndrome, MODS (M E McFadden, 1992).

Cancer-causative mutations typically involve tumour suppressor genes or oncogenes. Both alleles of a tumour suppressor gene generally need to be deactivated for loss of an anticancer phenotype whereas a gain of function mutation in one allele of an oncogene is enough for a pro cancer phenotype. The review Hallmarks of Cancer (Hanahan and Weinberg, 2011) stresses that there is an underlying phenotype of genetic instability and increased mutation

rate that enables cells to acquire the other hallmarks of cancer; hallmarks are characteristics which are needed for transformation to malignancy. In contrast, others emphasize the role of selection and clonal expansion as being more important. In these models, a normal mutation rate is considered high enough to cause cancer. However, all models agree that genetic instability is important in a subset of cancers, and that an increased mutation rate generally would transform healthy tissue into a cancer faster. They further point to the fact that there are solid tumours with no apparent form of genomic instability and that most haematological malignancies do not show clear signs of genomic instability (Abdel-Rahman, 2008; Tomlinson et al., 2002).

Hanahan and Weinberg (2011) also emphasized the importance of tumour induced inflammation as a malignancy enabling characteristic. The inflammation, which is caused mainly by innate immune system cells inside the tumour, provides essential factors fostering tumourgenesis. They also emphasize the important role of the stroma for sustaining tumourgenesis. The stroma is composed of recruited and subverted healthy non-immune system cells inside the tumour, and provides signalling feedback and structural and vascular support.

To transform into a malignant neoplasm cells need to acquire several hallmarks according to Hanahan and Weinberg (2011):

1. The cells need to sustain proliferative signalling, either by becoming truly self-sustaining for growth signals, by over-responding to growth signals from its environment, or by making surrounding cells provide growth signals via feed-back regulation.
2. The cells need to overcome both intracellular and extracellular inhibitory signals and resist induced cell death, *i.e.* apoptosis. The evasion from destruction by the immune system seems to be important, but Hanahan and Weinberg argue that it remains to be shown that anti-tumour immunity is a significant barrier to tumour formation.
3. The uninhibited and constantly proliferating cell will eventually have to develop replicative immortality, in order to avoid senescence.
4. The growing tumour needs angiogenesis, the creation of new blood vessels, for supporting its expanding cell mass with nutrients.
5. In order to metastasize, tumour cells also need to develop traits to remodel and degrade their surrounding extracellular matrix, loose there attachment to neighbour cells, and become more migratory.
6. Reprogramming of the energy metabolism is very common in cancers. The Warburg effect, in which cells ferment glucose to lactate even in the presence of oxygen, has been suggested as a mean to generate NADPH and to divert glycolytic and citric acid cycle intermediates into

precursors for biosynthetic pathways (Vander Heiden et al., 2009). In agreement with this, a broad spectrum of tumours have been found to not only upregulate glycolytic enzymes, but also enzymes in the *de novo* synthesis pathways of nucleotides (Weber, 2001). It should be noted, however, that increased fermentation in tumour tissue can also result from lack of oxygen due to poor vascularization.

4.1 *Saccharomyces cerevisiae* as a model in cancer research

The yeast *S. cerevisiae* cannot be considered multicellular, though it has the capacity to form pseudohyphae, and a unicellular organism cannot by definition develop cancer. However, to the extent that the underlying mechanisms are conserved between yeast and human cells, research in *S. cerevisiae* can contribute to the understanding of several important aspects of human cancers *e.g.* basic metabolism, DNA-damage and repair, and detoxification and resistance to cytotoxic drugs.

Even though the same signalling pathways frequently are found in yeast and human cells, there can be noteworthy differences in how they function. One example involves the notorious oncogene RAS: RAS driven PKA signalling is pro-proliferative in *S. cerevisiae*, whereas in human cancers trimeric G-protein driven PKA signalling may instead dampen proliferative signals. PKA signalling in these human cancers acts by inhibiting the aberrant constitutive binding between human RAS carrying oncogenic mutations and its downstream effectors B-Raf and C-Raf. This aberrant binding is responsible for persistent growth signalling in some cancers (Li et al., 2013). See also chapter 2.1 in this thesis for RAS signalling in *S. cerevisiae*.

5 Nucleotide metabolism

Nucleobases are nitrogen rich heterocyclic compounds which can be components of nucleotides. The canonical nucleobases are uracil, cytosine, thymine, adenine and guanine. Nucleobases bound to the 1' position of a deoxyribose or ribose sugar form compounds called deoxyribonucleosides and ribonucleosides respectively. The prefix/infix ribo is often left out. Nucleotides are composed of nucleosides with a mono, di, or tri phosphate linked to the 5' position of the ribose, see Table 1 for nucleobases, nucleosides, and nucleotides of importance for this thesis. Ribonucleotide triphosphates are polymerized into RNA molecules and deoxyribonucleotide triphosphates into DNA molecules. The polymerization is coupled to the removal of a pyrophosphate from each nucleotide triphosphate.

Table 1. *Selected nucleobases, nucleosides and nucleotides of importance for this thesis.*

Nucleobase	Nucleoside	Nucleotide (triphosphate)
Uracil, U	Uridine,Urd	UTP and dUTP
5-Fluorouracil, 5-FU	5-Fluorouridine, 5-FUrd	5-FUTP
Cytosine, C	Cytidine, Cyd	CTP
Thymine, T	Thymidine, dThd	dTTP
6-N-Hydroxylaminopurine, HAP		HAPTP
Adenine, A	Adenosine, Ado	ATP
Hypoxanthine, I	Inosine, Ino	ITP
Guanine, G	Guanosine, Guo	GTP

Nucleotides can be biosynthesized *de novo* from simpler precursors, or salvaged from nucleobases, which is called recycling or scavenging. Deoxyribonucleotides are synthesized in one step from the corresponding ribonucleotides. This enzymatic reaction, where the ribose of ribonucleotide-

diphosphates is reduced, is catalyzed by the ribonucleotide reductase complex (RNR).

The description of nucleotide metabolism in this thesis is mainly focused on the salvage and turn-over pathways, since the non-canonical nucleobases used in paper I and IV 5-fluorouracil (5-FU) and 6-N-hydroxylaminopurine (HAP) owe their toxicity to activation via the salvage pathways. The pyrimidine metabolism is summarized in Figure 3 and the purine metabolism in Figure 8.

5.1 Nucleotides as energy carriers

A nucleotide is more energy rich the more phosphates it has bound to the ribose at the 5' position, the upper limit being a triphosphate. Cells synthesize nucleotide triphosphates in two steps. First, ATP is synthesized from ADP and inorganic phosphate through either fermentation or oxidative phosphorylation. Subsequently, GTP, CTP and UTP are produced from GDP, CDP, and UDP by transfer of one phosphate from ATP; the same process is also used to make deoxyribonucleotide triphosphates. This equilibration is catalyzed by the nucleoside-diphosphate kinase enzyme, which is called Ynk1p in *S. cerevisiae* (Jong and Ma, 1991).

By coupling an energetically unfavorable reaction, such as polymerization of DNA, RNA, and polypeptides to the removal of phosphates from nucleotides the total reaction can become energetically favorable. Moreover, the coupling of nucleotide dephosphorylation makes possible otherwise energetically unfavorable biosynthetic reactions, *e.g.* carbamoyl-phosphate biosynthesis which is discussed in chapter 5.5.

5.2 The role of nucleotides in signalling pathways

Apart from their roles as energy carriers and precursors of RNA and DNA, nucleotides have additional roles in signalling pathways: ATP is commonly used for protein phosphorylation by protein kinases, such as PKA; GTP is used as a molecular switch by GTPases, such as RAS; and cAMP and ADP are used as second messenger signalling molecules. See chapter 2.1 and 2.2 in this thesis for more details.

5.3 Nucleotide pool balance and regulation of *de novo* synthesis

The balance between individual nucleotides within both the ribonucleotide and deoxyribonucleotide pool is important. Not only would depletion of one or more deoxyribonucleotides result in S-phase cell cycle arrest, but

deoxyribonucleotide imbalance alone, even without one or more limiting deoxyribonucleotides, results in mutagenesis due to incorporation of incorrect nucleotides during DNA synthesis (Kumar et al., 2010).

5.3.1 Ribonucleotide pool balance and *de novo* synthesis

S. cerevisiae uses several mechanisms to regulate the balance in the *de novo* synthesis of ribonucleotides. One notable strategy for balancing the ribonucleotide pool is to use one nucleotide as a co-reactant for the biosynthesis of another nucleotide. This increases the rate of synthesis of the former nucleotide when the latter nucleotide is abundant.

The first enzyme in the *de novo* synthesis of pyrimidines, Ura2p, thus uses ATP, an end product of purine synthesis, as a co-reactant. Ura2p is also negatively feedback regulated by UTP, an end product in pyrimidine synthesis (Antonelli et al., 1998).

On the contrary, the purine *de novo* synthesis is not thought to be regulated by the availability of pyrimidines; however, the first enzyme in this pathway, Ade4p, is feedback inhibited by the end product ATP (Rebora et al., 2001) .

Inosine monophosphate, IMP, is a branch point intermediate in purine biosynthesis. One branch leads to the synthesis of ATP, the other branch to GTP. The first enzyme in the branch leading to ATP synthesis, Ade12p, uses GTP as a co-reactant (Lipps and Krauss, 1999). In contrast, the enzymes Imd2p, Imd3p and Imd4p, which all catalyze the first step in the branch leading to GTP synthesis, are transcriptionally feedback inhibited by GDP (Escobar-Henriques and Daignan-Fornier, 2001). The aminase reaction, catalyzed by Gua1p, that transforms XMP to GMP furthermore uses ATP as a co-activator (Saint-Marc and Daignan-Fornier, 2004).

The ATP:GTP ratio is also balanced by the AMP-deaminase, Amd1p, which degrades AMP back to the branch-point intermediate IMP. Amd1p is allosterically activated by dATP and ATP, but inhibited by GTP. However, Amdp1 balancing is essential only when adenine is used as a source for purine biosynthesis, and *de novo* synthesis of purines is downregulated (Merkler and Schramm, 1990; Saint-Marc et al., 2009).

CTP is synthesized from UTP by an amination reaction coupled to ATP dephosphorylation, which is catalyzed by the enzymes Ura7p and Ura8p. Both enzymes are negatively feedback regulated by CTP, but activated by GTP, which contributes to the CTP:GTP pool balance (Nadkarni et al., 1995; Yang et al., 1994).

The *de novo* synthesis pathways of pyrimidines and purines are both feed-forward activated by pathway intermediates. The purine intermediate SAICAR thus activates purine biosynthesis (Rebora et al., 2001), whereas the pyrimidine

intermediates dihydroorotic acid and orotic acid activate pyrimidine biosynthesis (Flynn and Reece, 1999).

5.3.2 Deoxyribonucleotide pool balance

All deoxyribonucleotides diphosphates except dTDP are directly synthesized from the corresponding ribonucleotide diphosphate by the ribonucleotide reductase complex (RNR). dTDP is instead made from dUDP which is synthesized from UDP by the RNR. dUDP is first phosphorylated to dUTP by Ynk1p, after which dUTP is dephosphorylated to dUMP by the dUTPase Dut1p. Thymidylate synthetase Cdc21p then catalyzes the methylation of dUMP to dTMP. dTMP is subsequently phosphorylated to dTDP by thymidylate kinase Cdc8p. The *CDC8, CDC21* and *RNR1* genes are upregulated during the G1 to S phase transition (Puig et al., 2013; White et al., 1987).

RNR catalyzes the conversion of ribonucleotides into deoxyribonucleotides, and thereby balances the deoxyribonucleotide pool. Balance means *in vivo S. cerevisiae* that the concentration of dTTP is 2-fold higher than that of dATP and dCTP, and 4-fold higher than that of dGTP. ATP allosterically activates RNR, and dATP allosterically inactivates RNR; consequently, RNR measures and balances the ratio between deoxyribonucleotides and ribonucleotides. RNR also balances the individual deoxyribonucleotides. dATP thus increases the specificity of RNR for CDP and UDP, dTTP increases the specificity of RNR for GDP, and dGTP increases the specificity of RNR for ADP (Kumar et al., 2010).

5.4 Pyrimidine salvage and turnover

S. cerevisiae can utilize extracellular nucleotides through the Npp1, Npp2, and Pho5p mediated periplasmic degradation of nucleotides to phosphates and nucleosides (Kennedy et al., 2005).

Cytidine is then taken up by the cytidine permease Fcy2p and uridine is taken up by the uridine permease Fui1p. However, nucleobases are preferred over nucleosides for nucleotide scavenging in *S. cerevisiae*, and pyrimidine nucleosides are also converted to uracil, in addition to direct nucleoside phosphorylation. When used as sources for pyrimidines, uridine, cytidine and deoxycytidine are first degraded to uracil and cytosine respectively, a reaction which is catalyzed by the uridine hydrolase Urh1p. Cytosine is then further deaminated to uracil by Fcy1p. Cytidine can also be deaminated to uridine by the Cdd1p deaminase, but the reaction is inefficient *in vivo.* In addition, the nucleosides cytidine, uridine and deoxycytidine can be phosphorylated by the

Urk1p kinase to CMP, UMP and dCMP respectively. Deoxyuridine cannot, however, be utilized by yeast (Kurtz et al., 1999, 2002; Wagner et al., 1998).

It should be noted that Urh1p does not hydrolyze thymidine to thymine (Mitterbauer et al., 2002). Moreover, thymidine kinase is absent from yeast, and neither thymine nor thymidine can therefore be used as a substrate for synthesis of dTMP. dTMP can however be used directly to sustain DNA synthesis in some strains, but only with limited efficiency (Brendel and Haynes, 1973; Grivell and Jackson, 1968).

Cytosine is taken up by the purine-cytosine transporter Fcy2p, and as mentioned above intracellular cytosine is always deaminated to uracil by the Fcy1p deaminase prior to conversion into pyrimidine nucleotides (Ferreira et al., 1997; Jund and Lacroute, 1970). Uracil is taken up by the uracil specific permease Fur4p (Jund et al., 1977). Uracil is then directly activated to UMP through a reaction with phosphoribosyl pyrophosphate (PRPP) which is catalyzed by Fur1p. *FUR1* is moreover transcriptionally activated by its own substrate uracil (Kern et al., 1991). The *FUR4* uracil permease is in contrast downregulated by uracil (Seron et al., 1999).

Pyrimidine scavenging and salvage thus converges on UMP, which is converted to UDP by the UMP specific kinase Ura6p (Ma et al., 1990). No CMP or dCMP kinases have so far been found in *S. cerevisiae*. CMP could possibly be recycled via the Sdt1p nucleotidase, which can dephosphorylate CMP, UMP, 5-FUMP, and 5-FCMP, but not deoxyribonucleotides such as dCMP (Nakanishi and Sekimizu, 2002). dCMP can, however, be converted into dUMP by the Dcd1p deaminase. This dUMP is then further converted into dTMP by Cdc21p catalyzed methylation. Hence, dCMP would not be recycled, but converted into dTMP, through this pathway (McIntosh and Haynes, 1984).

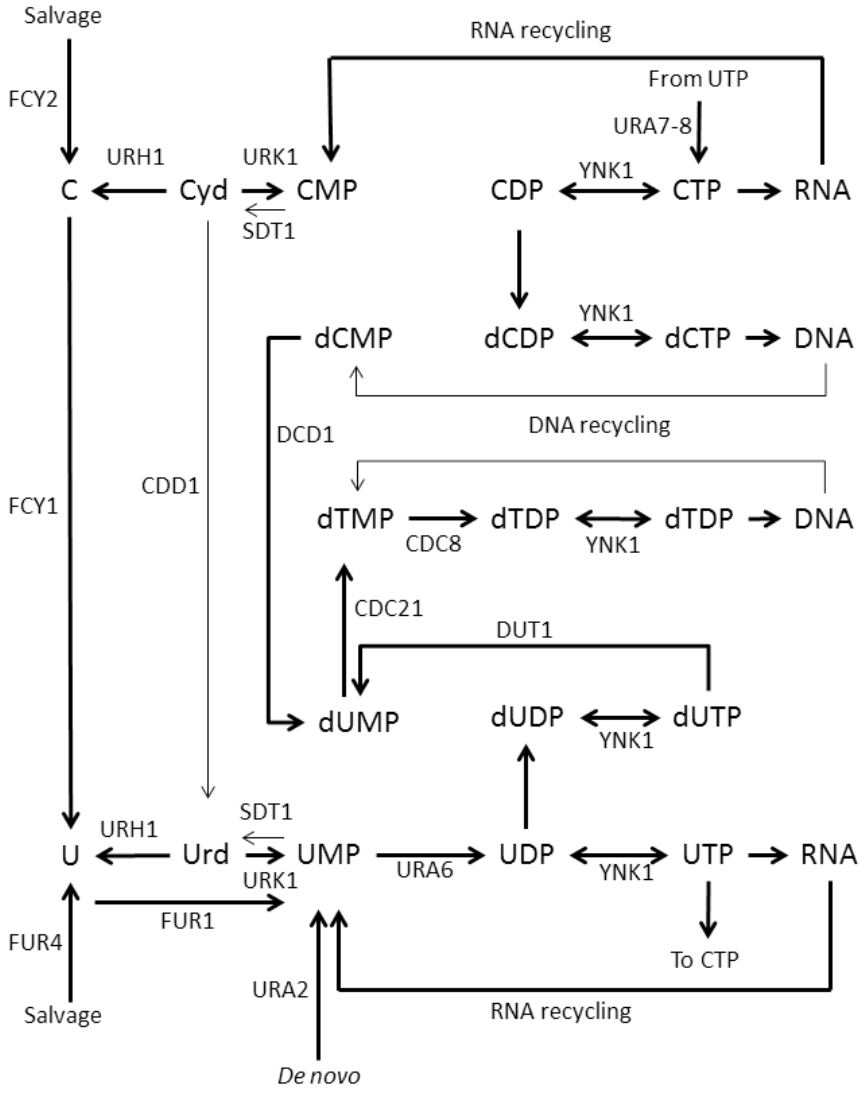

Figure 3. Pyrimidine metabolism in *S. cerevisiae* with a focus on salvage and turnover. Thinner arrows mean that the reactions are known or expected to be of relatively minor importance. Most enzymatic reactions in the *de novo* synthesis of pyrimidines are not shown.

5.5 Pyrimidine and arginine *de novo* synthesis

The uridylate, cytidylate, and thymidylate nucleotides are all synthesized from a UMP intermediate. Interestingly, this *de novo* synthesis of UMP shares its first intermediate with the *de novo* synthesis of the amino acid arginine. The

first enzymatic step in the synthesis of either arginine or UMP is thus the condensation of carbonate, ammonium from glutamine, and phosphate from ATP, into carbamoyl phosphate (CP). The enzyme carrying out this reaction is called carbamoyl phosphate synthetase, often abbreviated CPSase, but sometimes CPase in yeast, and CPS in animals. Both yeast and animals have two different carbamoyl phosphate synthetases, one used in arginine biosynthesis and another one used in UMP biosynthesis.

5.5.1 Carbamoyl phosphate synthetases of the pyrimidine biosynthetic pathway

The most complex protein containing a carbamoyl phosphate synthetase domain is the multi-enzyme CAD, which is part of the UMP synthesis pathway in man. It contains two domains which together form carbamoyl phosphate synthetase 2 CPS2, but it also harbours domains that carry out the two subsequent steps in UMP synthesis: aspartate carbamoyl transferase (ATCase) and dihydroorotase (DHOase), producing carbamoyl aspartate (CAspate) and dihydroorotate (DHOate) respectively , see Figure 4, (Evans and Guy, 2004).

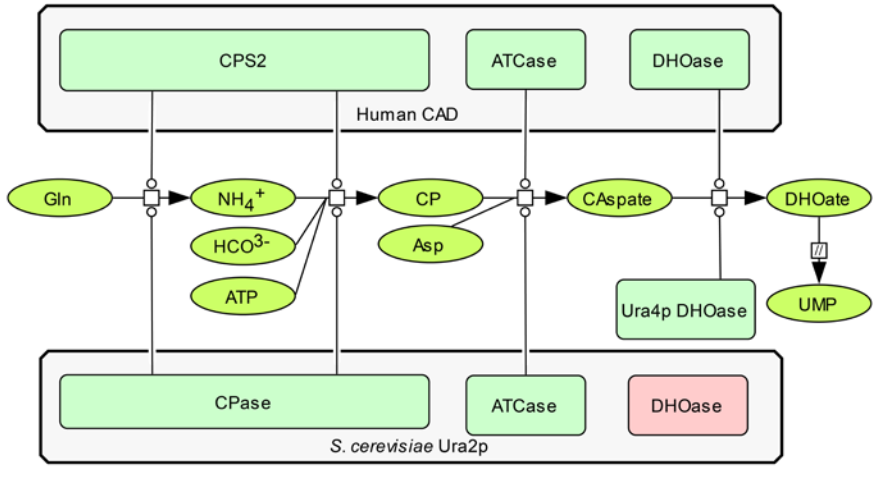

Figure 4. Comparison of the first reactions in the *de novo* synthesis of UMP in human cells (top) and *S. cerevisiae* (bottom). Several reaction steps from DHOate to UMP are left out.

Human CAD is found in the cytosol associated with mitochondria, but about a third of the proteins translocates to the nucleus during S-phase (Evans and Guy, 2004; Sigoillot et al., 2005). Its yeast orthologue Ura2p is also cytosolic

(Benoist et al., 2000) contradicting an earlier finding that it was associated with the nucleus (Nagy et al., 1989, 1982).

A striking difference between human and yeast enzymes is the fact that the human CAD has an active dihydroorotase domain, whereas the yeast orthologue Ura2p only has a homologous but enzymatically dead domain (Souciet et al., 1989). As depicted in Figure 4, this synthesis step is instead mediated by the protein Ura4p in yeast (Lacroute, 1968).

5.5.2 Carbamoyl phosphate synthetases of the arginine biosynthesis and the urea cycle

In this pathway the differences between animals and fungi are more pronounced, both regarding localizations and functions of the enzymes, see Figure 5.

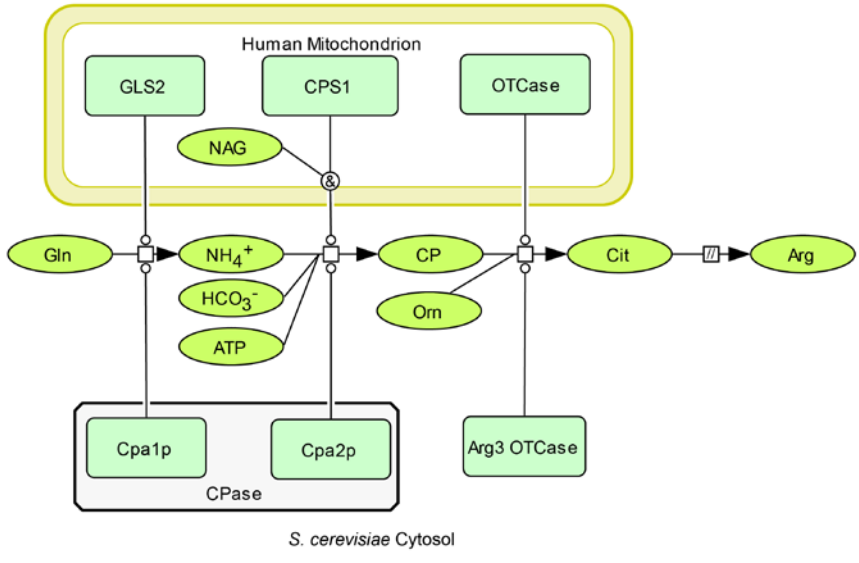

Figure 5. Comparison of the first few reactions in the *de novo* synthesis of arginine, in human cells (top) and yeast (bottom).

In yeast, the two unlinked genes *CPA1* and *CPA2* code for the two domains of this CPSase. Cpa1p is the smaller subunit, and contains the activity which removes ammonium from glutamine. The larger subunit Cpa2p catalyzes the condensation of carbonate, ammonium and phosphate into carbamoyl phosphate.

In the human orthologue of Cpa1/2p, CPS1, the two domains are present within one protein. However, the domain of CPS1 which is homologous to Cpa1p is no longer involved in deamination of glutamine, and CPS1 instead utilizes free ammonium cleaved from glutamine by the GLS2 glutaminase. It should be noted that the Cpa2p subunit of the yeast CPSase also can use free ammonium, but it is not as efficient a substrate as glutamine. The CPS1 enzyme is allosterically activated by N-acetylglutamate (NAG) unlike the yeast CPSase. The yeast CPSase complex is instead allosterically activated by its natural substrate glutamine (Aledo et al., 2000; Funghini et al., 2003; Lacroute et al., 1965; Pierard and Schroter, 1978).

A further difference between the two species is the fact that the yeast CPSase is located in the cytosol (Urrestarazu et al., 1977) whereas the human CPS1 protein is located in the mitochondrial matrix of hepatocytes, where it functions in the urea cycle (Funghini et al., 2003).

5.5.3 Feedback inhibition of CPSase and cross-utilization of carbamoyl phosphate between arginine and UMP synthesis

Interestingly, in isolated human hepatocytes, 66-90% of the *de novo* synthesis of pyrimidines uses CPS1 derived carbamoyl phosphate that is leaking from the mitochondria rather than CPS2 derived carbamoyl phosphate from the CAD multi-enzyme. This CPS1 dependent *de novo* synthesis of pyrimidines is not feedback inhibited by an expanded pyrimidine nucleotide pool, in contrast to the *de novo* synthesis that is dependent on CPS2. This cross-utilization of carbamoyl phosphate and lack of feedback regulation is most likely tissue specific since CPS1 is expressed only in the hepatocytes where it is involved in the urea cycle, and in the epithelial cells of the intestinal mucosa (Monks et al., 1985; Wendler et al., 1983).

In yeast, the carbamoyl phosphate produced by either Cpa1p/Cpa2p or Ura2p is freely available for arginine or pyrimidine synthesis, as both enzymes are cytosolic and a single pool of cytosolic carbamoyl phosphate therefore exists (Lacroute et al., 1965).

UTP, which is a terminal product of pyrimidine synthesis, inhibits both the transcription of the *URA2* gene as well as the CPSase and ATCase activities of the Ura2p enzyme. In human cells, it is only the CPSase activity of the orthologue CAD which is inhibited by UTP (Antonelli et al., 1998).

The yeast CPSase subunit Cpa1p is similarly feedback inhibited by its end product arginine, at the translational level (Messenguy et al., 1983). In contrast, the human CPS1 protein is allosterically regulated by NAG, and the production of NAG by the enzyme N-acetylglutamate synthase is activated by glutamate

and arginine (Morizono et al., 2004). This makes sense since glutamate is the main carrier of the amine groups that are disposed of through the urea cycle whereas arginine is an intermediate in the urea cycle, and thus needed for its proper function.

5.6 5-Fluorouracil

5-FU was the first drug designed to specifically target cancer cells, and it has been used in the treatment of solid tumours for more than fifty years. It was rationally designed based upon the finding that rat hepatomas metabolized uracil faster than normal tissues. Heidelberger and colleagues therefore synthesized 5-FU in 1957 (Heidelberger et al., 1957), in an attempt to make a non-metabolizable uracil analogue. Its structure differs from uracil in that it has a fluorine atom substituting a hydrogen at the fifth position in the heterocyclic pyrimidine ring, the same position where thymine carries a methyl group, see Figure 6. This modification does not hinder base pairing with adenine and 5-FU metabolites are therefore incorporated instead of uracil during DNA and RNA synthesis (Grem, 2000).

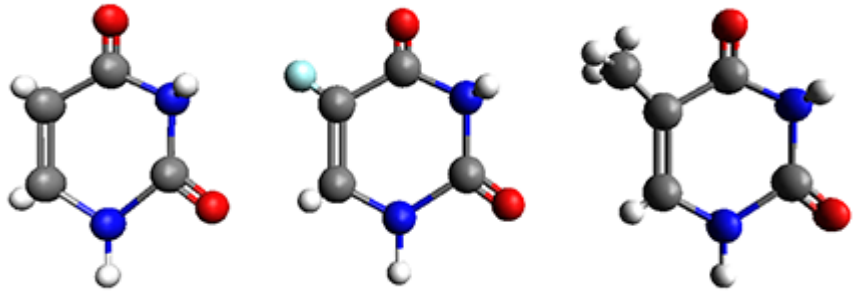

Figure 6. Comparison of the structures of uracil, 5-fluorouracil and thymine.

5.6.1 Metabolism of 5-FU

5-FU and uracil are taken up into mammalian cells by the same facilitated transport system (Wohlhueter et al., 1980). Similarly, in *S. cerevisiae*, uracil and 5-FU are both taken up by the proton symporter Fur4p (Jund and Lacroute, 1970). Inside the cell, 5-FU can then either be activated or degraded, see Figure 7. In *S. cerevisiae*, 5-FU is activated to 5-FUMP by the uracil phosphoribosyltransferase Fur1p (Kern et al., 1991). In mammalian cells, the same activation reaction is carried out by orotate phosphoribosyltransferase (OPRTase) but also through two other pathways (Fukushima et al., 1996).

In one of these pathways, uridine phosphorylase (UP) and uridine kinase (UK) produces 5-FUMP. In the other pathway, thymidine phosphorylase (TP) and thymidine kinase (TK) produces 5-FdUMP. This deoxyfluoropyrimidine can however also be generated by the conversion of 5-FUDP to 5-FdUDP, a reaction catalyzed by ribonucleotide reductase (RNR) in both *S. cerevisiae* and human cells (Longley et al., 2003). The activated metabolites of 5-FU: FdUMP, FdUTP, and FUTP have several toxic effects on the nucleotide metabolism.

Unactivated 5-FU is degraded and detoxified by dihydropyrimidine dehydrogenase (DPD) which degrades pyrimidines such as 5-FU and similar compounds in a wide range of organisms from certain yeasts to humans. However, this pathway is absent in *S. cerevisiae* (Andersen et al., 2006).

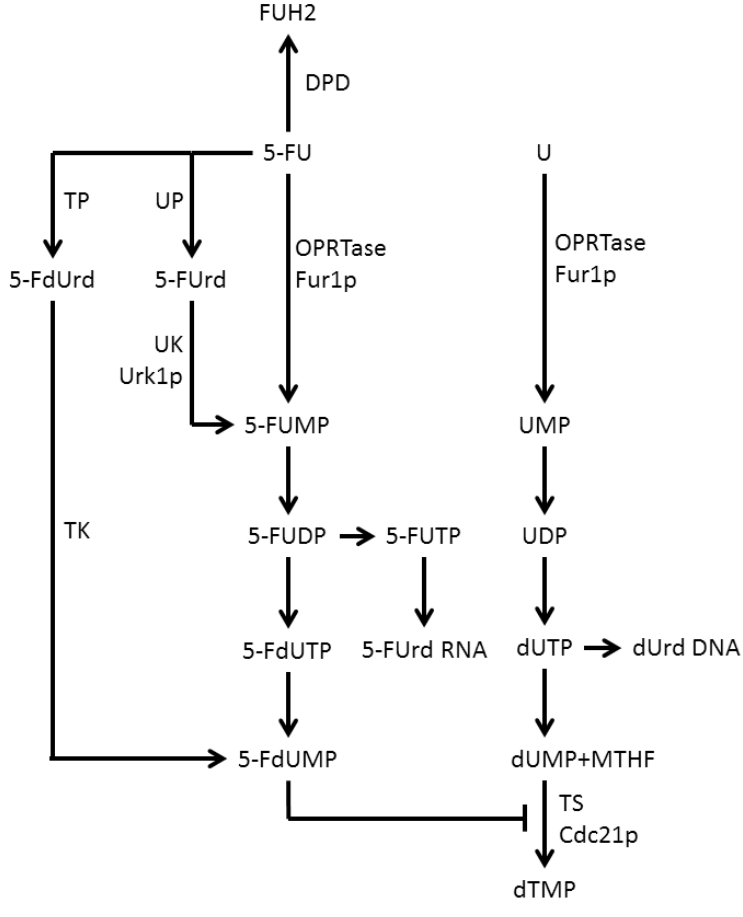

Figure 7. Metabolic activation of 5-FU and uracil, with focus on enzymes which differ between *S. cerevisiae* and man. *S. cerevisiae* lacks DPD, UP, TP and TK.

5.6.2 Effects of 5-FU on DNA metabolism

The 5-FU metabolite FdUMP forms a stable ternary complex with thymidylate synthase (TS or Cdc21p in *S. cerevisiae*) and the cofactor 5,10-methylenetetrahydrofolate (MTHF) resulting in the inhibition of the synthesis of dTMP from dUMP and MTHF, see Figure 7. This inhibition results in an accumulation of dUMP and subsequently also dUTP. Under these conditions, dUTP and FdUTP both become used as substrates in DNA replication, see Figure 7. Uracil DNA glycosylase removes the uracil and 5-FU nucleobases that are misincorporated into DNA, but the lack of dTTP due to inhibition of TS sends the repair machinery into a futile cycle of mal-repairing the lesion with uracil and then removing the uracil again (Ladner, 2001). This eventually leads to loss of genome integrity, through the formation of double-strand DNA breaks, and cell death (Yoshioka et al., 1987).

S. cerevisiae ung1 strains lacking uracil DNA glycosylase are therefore significantly protected from the toxic effects of 5-FU. The deletion of *UNG1* prevents the generation of abasic sites and causes the strain to accumulate massive amounts of T to U substitutions in its DNA. These substitutions are not detrimental in the short term. The next enzyme after Ung1p in the base excision repair process, Apn1p, nicks the DNA at the abasic position. The deletion of *APN1* in combination with 5-FU is highly toxic, but its deletion does not cause an accumulation of abasic unrepaired sites, which implies that another repair process takes over and causes loss of viability (Seiple et al., 2006). See chapter 3.2.1 about BER in this thesis.

It should further be noted that 5-FU induced cell death in some human cell lines is dependent on p53 mediated apoptosis, which can be prevented by externally added uridine but not by externally added thymidine. This in turn implies that RNA mediated effects may be the primary cause of 5-FU toxicity, at least in some cells (Bunz et al., 1999).

5.6.3 Effects of 5-FU on RNA metabolism

5-FU has detrimental effects on most if not all parts of the RNA metabolism, from ribosome biogenesis to mRNA biosynthesis and processing. The reduced growth rate of 5-FU treated *S. cerevisiae* is in general more easily suppressed by the addition of UMP than of dTMP to the growth medium. This implies that RNA damage mediates most of the toxicity of 5-FU in *S. cerevisiae* (Hoskins and Butler, 2007).

S. cerevisiae cells treated with 5-FU accumulate polyadenylated 27s rRNAs, and this is even more pronounced in the exosome exonuclease mutant *rrp6*, implying that 5-FU interferes with RNA processing in the exosome, of which Rrp6p is a component (Fang et al., 2004).

Interestingly, the 5-FU hypersensitivity of the *rrp6* mutant can be suppressed by knocking out the pseudouridine synthase gene *CBF5*. Cbf5p and other pseudouridine synthases, which convert uridine to pseudouridine in small RNAs, can become covalently attached to 5-FU moieties that are present in RNA. It is likely that RNA molecules containing these probably toxic adducts are recognized as malformed and targeted for degradation by polyadenylation, but hyperaccumulate in the absence of Rrp6p (Hoskins and Butler, 2008; Spedaliere and Mueller, 2004)

5-FU also becomes incorporated into U2-snRNA, where it blocks U2-snRNA pseudouridylation. The hypopseudouridylated U2-snRNA makes the spliceosome defective in pre-mRNA splicing, which disturbs regulation of protein expression at the mRNA level (Zhao and Yu, 2007).

Furthermore, 5-FU inhibits tRNA uridine methyltransferases and tRNA pseudouridine synthases, and thereby interfere with tRNA maturation (Frendewey et al., 1982; Gu et al., 1999; Santi and Hardy, 1987). 5-FU also acts synergistically to worsen the effects of tRNA modifying enzyme knockouts and elevated temperatures, both of which are known to destabilize tRNA (Alexandrov et al., 2006). This in turn implies that one toxic effect of 5-FU treatment may be destabilization of tRNA (Gustavsson and Ronne, 2008).

5.6.4 5-FU tolerance and sensitivity

Human cancer cells and *S. cerevisiae* cells both have the ability to acquire resistance to 5-FU. Loss of function mutations in either of the uracil salvation proteins, the uracil permease Fur4p or the uracil phosphoribosyltransferase Fur1p thus renders yeast cells highly resistant to 5-FU, but at the same time makes them dependent on alternative pathways such as a functional *de novo* synthesis of UMP, or cytosine-to-UMP scavenging. Other known resistant genotypes in yeast are mutations that cause a loss of feedback inhibition of the carbamoyl phosphate synthetase protein Ura2p, overexpression of the uncharacterised gene *YJL055w*, and overexpression of the nucleotidase *SDT1* (Jund and Lacroute, 1970; Ko et al., 2008; Nakanishi and Sekimizu, 2002).

Work in *Aspergillus nidulans* further suggested that loss of feedback inhibition of the arginine specific carbamoyl phosphate synthetase, and loss of function of the ornithine carbamoyl transferase, both can cause resistance to 5-FU by increasing the *do novo* synthesis of UMP, through redirecting carbamoyl phosphate away from arginine synthesis toward UMP synthesis (Palmer et al., 1975).

A gene expression analysis on colon cancer cell lines which were in the process of developing a 5-FU resistant phenotype, revealed a quite complex pattern of change in gene expression during the acquisition of an early weakly

resistant phenotype. After the transition to a strongly resistant phenotype, changes in the regulation of genes involved in pyrimidine metabolism could be observed, such as over-expression of the CAD and thymidylate synthase genes (Schmidt et al., 2004). However, it should be noted that changes in the pyrimidine metabolism, *i.e.* down regulation of pyrimidine catabolism and up regulation of *de novo* synthesis and scavenging, are generally present in cancers even in the absence of 5-FU selection, and probably reflect the faster proliferation rate of cancers cells (Weber, 2001).

High expression of either thymidylate synthase, dihydropyrimidine dehydrogenase or thymidine phosphorylase has also been associated with resistance to 5-FU in tumours (Salonga et al., 2000). In contrast, a higher expression of orotate phosphoribosyltransferase has been associated with increased sensitivity to 5-FU in cancer cell lines (Taomoto et al., 2006). However, overexpression of thymidine phosphorylase can also make some cell lines more sensitive to 5-FU (Schwartz et al., 1995). This apparent contradiction can possibly be explained by the fact that thymidine phosphorylase catalyses the first step of activation of 5-FU to 5-FdUMP, *i.e.* the conversion of 5-FU to 5-FdUrd. The latter is the growth inhibiting metabolite of 5-FU in some but not all cells (Codacci-Pisanelli et al., 2008). In other cells, where RNA toxicity mediated by 5-FUMP is more important, a redirection of 5-FU into 5-FdUrd might instead release the growth inhibition.

5.7 Purine salvage and turnover

S. cerevisiae appears to lack capacity to salvage extracellular purine nucleosides (Vickers et al., 2000). However, the purine nucleobases, adenine, guanine, and hypoxanthine are scavenged from the extracellular environment via the Fcy2p transporter (Ferreira et al., 1997). The nucleobases are then converted to nucleotides in a reaction with PRPP which is catalyzed by the phosphoribosyltransferases: Hpt1p for hypoxanthine and guanine, Apt1p for adenine, and Xpt1p for guanine and xanthine (Alfonzo et al., 1999; Ali and Sloan, 1982; Guetsova et al., 1999).

The interconversion between the purine salvage pathways is asymmetric. Adenine can be used as a source for guanylates either via deamination of AMP by Amd1p, or direct deamination and phosphoribosylation by Aah1p and Hpt1p respectively, into the purine biosynthesis branch point intermediate IMP. However, guanine cannot be used as a source for adenylates. Hypoxanthine is salvaged directly to the branch point intermediate IMP and is therefore a source for both adenylates and guanylates (Saint-Marc and Daignan-Fornier, 2004; Saint-Marc et al., 2009).

AMP is converted into ADP by Adk1p, which is the major ATP-dependent adenylate (AMP) kinase. However, Adk1p only has minor dAMP kinase activity (Gu et al., 2005; Su and Russell, 1967). GMP is converted into GDP by Guk1p, which is the ATP-dependent guanylate (GMP) kinase. Guk1p also has dGMP kinase activity to a lesser degree (Konrad, 1992).

Deamination of purines happens spontaneously. This generates xanthosine triphosphate, (d)XTP from (d)GTP, and hypoxanthosine triphosphate (d)ITP from (d)ATP. (d)XTP and (d)ITP are degraded by the Ham1p pyrophosphatase, which prevents their incorporation into DNA and RNA (Davies et al., 2012). In publication I, we found that overexpression of Ham1p also confers resistance to 5-FU, which suggests that Ham1p may also prevent the incorporation of 5-FU into RNA and DNA. In this context it should be noted that the UTPase Dut1p, which degrades dUTP to dUMP, also has activity against dITP (Tchigvintsev et al., 2011).

There are also several other enzymes involved in purine nucleoside turnover, which could be of importance for detoxification of purine analogues: the Isn1p IMP-nucleotidase, the Pnp1p guanine and inosine ribohydrolase, the Ado1p adenosine kinase, the Gud1p guanine deaminase, and the Aah1p adenine deaminase (Saint-Marc and Daignan-Fornier, 2004). See Figure 8 for a summary of purine salvage and turnover.

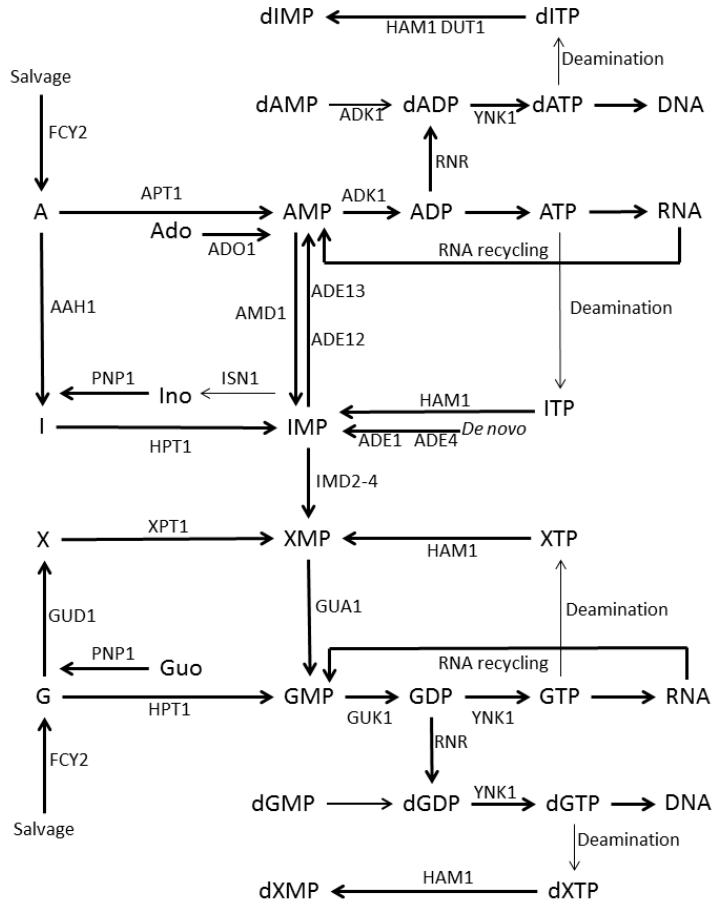

Figure 8. Purine metabolism in *S. cerevisiae* with a focus on salvage and turnover. Thinner arrows mean that the reactions are known or expected to be of relatively minor importance. Most enzymatic reactions in the *de novo* synthesis of purines are not shown.

5.8 6-N-Hydroxylaminopurine

6-N-Hydroxylaminopurine, HAP, is converted into HAPMP by the Apt1p adenine phosphoribosyl transferase, and is further phosphorylated to HAPDP. HAPDP is assumed to be transformed to dHAPTP, via RNR and Ynk1p. dHAPTP is capable of ambiguous base pairing, and causes mutations by

replacing both dGTP and dATP during DNA replication. The toxicity of HAP is considered to be due to its role as a mutagen. However, it is conceivable that HAP could also affect RNA metabolism (Abdul-Masih and Bessman, 1986).

Even though HAP is a mutagen, it can be utilized as a source of exogenous purines, and support growth of *S. cerevisiae.* This is dependent on HAP being converted into the purine biosynthesis branch-point intermediate IMP. This reaction is assumed to be catalysed by either of the deaminases Amd1p or Aah1p, since adenine aminohydrolases are known to have broad specificity against 6-N substituted adenines. Aah1p might play the more important role since its deletion confers sensitivity against HAP. Two other genes, among several known to be of importance for HAP tolerance, is the *HAM1* nucleotide pyrophosphatase and the *YJL055w* gene, whose function is unknown (Noskov et al., 1996; Stepchenkova et al., 2005). In publication I, we found that overexpression of both *HAM1* and *YLJ055w* also reduce the incorporation of 5-fluorauracil into RNA.

HAP is not used as an anticancer drug although it has shown therapeutic activity against transplanted animal tumours. The reason for this is that HAP is haemolytic in humans at doses below those that might produce anticancer effects (Dollinger and Krakoff, 1975). However, other purine analogues are used as drugs *e.g.* 6-mercaptopurine (6-MP) which is used for treatment of leukaemia (Adam de Beaumais et al., 2011). The 6-MP metabolite methylthioinosine monophosphate (meTIMP) is a potent inhibitor of purine *de novo* synthesis, which may contribute to the cytotoxicity of 6-MP. However, a knock-down of the thiopurine methyltransferase gene, which catalyzes one step in the conversion of 6-MP to meTIMP, did not significantly affect the toxicity of 6-MP in a human cell line (Karim et al., 2013).

It is conceivable that 6-MP could also contribute to cytotoxicity by being incorporated into DNA or RNA. This is implied from the fact that reduced activity of inosine triphosphate pyrophosphatase (ITPA) correlates with more frequent hepatotoxicity during 6-MP treatment; ITPA degrades non-canonical nucleotide triphosphates, *e.g.* (d)ITP and (d)XTP and thereby prevents their incorporation into RNA and DNA (Davies et al., 2012; Tanaka et al., 2012). In manuscript III we found that a polymorphism of the ITPA gene, which reduces the enzymatic activity with 78% in heterozygous carries (Simone et al., 2013), also correlates with toxicity during combined treatment with 5-FU and irinotecan.

6 Genetic association studies

The correlation, and sometimes also the causation between phenotypes and the underlying genotypes can be studied in different ways. In a forward genetic genome-wide association study, many genetic polymorphisms in each genome are simultaneously analysed in an attempt to find which of them associate with, *i.e.* correlate with, a given phenotype. However, genetic association studies do not necessarily have to be genome-wide. In manuscript III we used genetic information about polymorphisms at five specific loci to build a model for correlation with 5-FU toxicity in cancer patients.

In contrast, a reverse genetics microarray study can determine which phenotypes, *i.e.* changes in mRNA expressions levels, are caused by a given genetic modification. In publication II we investigated the effect of a mutation in the Rph1p transcription factor that abolishes its histone demethylase activity on genome-wide gene regulation.

6.1 Microarray analysis and gene expression

A commonly used method to measure gene expression is the Affymetrix Genechip microarray platform, which was used in publication II. To measure the abundance of an mRNA transcript, a Genechip array has a set of probes that hybridize to different parts of each mRNA transcript. It has furthermore both perfect match and mismatch probes, which facilitates the transformation of microarray signal values to mRNA transcript levels.

In order to estimate the expression level of each RNA transcript, different normalization methods can be used. In publication II we used the GC-RMA normalization method (Wu and Irizarry, 2004). The normalization of microarrays is an area of development, and an alternative normalization method is the IRON algorithm (Welsh et al., 2013).

6.1.1 Limma

Microarray mRNA expression can be analysed using the package Linear Models for Microarray Data, limma, which is available in R, a programming environment for statistical analysis. Limma accepts log2 transformed expression data, which is the type of output data generated by the GC-RMA normalization. Limma can be used to fit a generalized linear model between the mRNA expression data for each gene, and the explanatory variables *e.g.* experimental conditions such as the genetic modifications studied in publication II. However, if limma is used with the default settings, it will simply model an ordinary linear regression between the mRNA expression levels and the explanatory variables using the least squares method.

Limma facilitates the finding of sets of significantly differently expressed genes in comparisons between different experimental conditions by using false-detection-rate correction of P-values, smoothening of sample variance and moderation of the t-test for differential expression (Smyth, 2004).

An interaction formula in the Wilkinson-Rogers notation is frequently used to describe a model between a dependent variable and explanatory variables (Wilkinson and Rogers, 1973). An interaction formula of this kind can be supplied to both the limma package used in publication II, and to the glm function used for logistic regression in manuscript III. The interaction formula provides the modelling program, *e.g.* limma or glm, with information on whether to include interactions between explanatory variables, and whether or not the fitted model should pass through origo, *i.e.* the dependent variable should be zero if all explanatory variables are zero (Wilkinson and Rogers, 1973).

6.2 Generalized linear models

Depending on the nature of the explanatory and dependent variables, different regression methods can be used to construct a model, and test the significance of the contribution from each explanatory variable. A common regression method is linear regression, which tests the dependence of a continuous variable on one or more variables. To build models with a dependent variable that is not continuous, the regression method can be generalized by a transformation of the dependent variable. In contrast to a continuous variable, the dependent variable in manuscript III is a dichotomous variable with toxicity or no toxicity as possible outcomes. The regression used to build a model with a dependent dichotomous variable is called logistic regression. Logistic regression builds a model between the explanatory variables, the regression coefficients of which become determined, and the transformed dependent

variable, called the logit. The logit is the logarithm of the odds that correspond to the probability of the outcome (Petrie and Sabin, 2005).

An iterative algorithm performs a maximum likelihood estimation in logistic regression and other types of generalized linear modelling. This procedure estimates the regression coefficients for each explanatory variable of the model in such a way that the data of the explanatory variables has the highest possible probability of generating the data of the dependent variable. In logistic regression, the regression coefficients correspond to the natural logarithm of the odds ratio for an increase in the corresponding explanatory variable. To assess the significance of explanatory variables in the model, or to assess the adequacy of fit between the model and the data, the likelihood ratio statistic is used. This statistic is often referred to as deviance (Petrie and Sabin, 2005).

In manuscript III we used the c-statistic for evaluating the performance of the model in correctly classifying patients into either the 5-FU sensitive, or 5-FU insensitive group. The c-statistic has a possible range between 0.5 and 1. A value of 0.5 indicates that the model lacks classification performance, and a model with value 1 means that it classifies perfectly (Petrie and Sabin, 2005). A hypothetic model with a c-statistic of 1 could thus be considered to explain all variation in the dependent variable, and could consequently be considered to include all explanatory variables.

7 Aims

The general aim of this thesis has been to use the budding yeast *S. cerevisiae* as a model organism to study mechanisms involved in cancer, with the aim of using the knowledge gained in yeast to improve anticancer therapies.

Specifically, I have investigated the mechanisms of resistance to the nucleobase analogues 5-fluorouracil (5-FU; publication I) and 6-N-hydroxylaminopurine (HAP; manuscript IV). 5-FU is a cytotoxic drug used in the treatment of cancer, whereas 6-N-hydroxylaminopurine is a purine analogue used primarily to study the metabolism of purine analogues. The details of the metabolism of purine analogues are important for the understanding of the fate of other purine analogues such as 6-mercaptopurine, which are used for the treatment of leukaemia. In manuscript III, the effect on 5-FU sensitivity of a mutation in the human orthologue of one of the genes identified in publication I was investigated.

I have also participated investigating the mechanism(s) of transcriptional regulation of the yeast Rph1p transcription factor, which functions downstream of several nutrient sensing pathways (publication II). These nutrient signalling pathways are conserved in eukaryotic evolution, and are a part of the stimulus a cell needs for the decision whether to proliferate or to arrest. Much research has been devoted to gaining a better understanding of the mechanisms behind the sustained proliferative signalling in cancer. A better understanding of the downstream molecular consequences of nutrient signalling could therefore also help to elucidate the mechanisms behind cancer.

7.1 Publication I

Through mutations in single tumour cells and subsequent clonal expansion, tumours often develop resistance to anticancer drugs. Possible resistance mechanisms include mutations in the molecular target(s) of the drug that make

these targets less sensitive, as well as improved degradation and exclusion of the drug. Furthermore, some drugs need to be converted from a pro-drug to an active drug, a step that can also be affected by mutations. In publication I, we used a gene overexpression screen in *S. cerevisiae* to find proteins that may be involved in resistance to the cytotoxic anticancer drug 5-FU. We also characterized the mechanisms of the resistance conferred by overexpression of some of the genes identified in our screen.

7.2 Publication II

Transcription factors lie at the end of many signal transduction pathways, and their activation changes the proteome to adapt the cell to the cause of the signal. Stimuli which induce signal transduction can for example be nutrients, stressors or cell to cell signalling.

The Rph1p transcription factor lies downstream of the nutrient sensing pathways of TORC1 and PKA. In the exponential growth phase, Rph1p together with the closely related protein Gis1p repress many genes redundantly, whereas other genes instead are activated independently by Rph1p and Gis1p in the post diauxic shift growth phase. Genes that are regulated by Rph1p and/or Gis1p have STRE and PDS elements in their promoters, to which the two proteins bind. Rph1p, unlike Gis1p, has two known roles; it is a transcription factor, but it also has histone demethylase activity. Modification of histone acetylation is one of the major mechanism by which transcription factors influence gene expression, which suggested that the histone demethylase activity of Rph1p might be important for its role as a transcription factor. We carried out transcriptome profiling of both an *rph1* deletion mutant and an *rph1 demethylase-deficient* point mutant using microarray technology in order to elucidate the connection between the histone demethylase activity of Rph1p and its transcription factor activity.

7.3 Manuscript III

Genetic variation can render humans hypersensitive to drugs. For example, polymorphisms causing reduced activity of the 5-FU degrading enzyme dihydropyrimidine dehydrogenase are associated with severe toxicity in patients receiving 5-FU treatment. Similarly, a polymorphism in the inosine triphosphate pyrophosphatase gene *ITPA*, which causes reduced enzyme activity, has been shown to influence toxicity from 6-mercaptopurine.

The human *ITPA* gene is the orthologue of the *S. cerevisiae* gene *HAM1*, which we found in publication I to confer resistance to 5-FU when

overexpressed. By reanalysing toxicity data from a clinical trial on patients treated with a 5-FU based regimen and genotyping tissue samples from the same patients for the *ITPA* polymorphism, we aimed to elucidate if the *ITPA* polymorphism correlates with 5-FU toxicity.

7.4 Manuscript IV

To gain more knowledge concerning tolerance against nucleobase analogues, we did another gene overexpression screen in *S. cerevisiae* for resistance to the mutagenic purine analogue 6-N-hydroxylaminopurine (HAP). A gene involved in resistance to nucleobase analogues can be more or less specific. By finding genes involved in resistance to the purine analogue HAP, and test the effect of those genes on resistance to 5-FU resistance, we aimed to find more genes which could influence 5-FU resistance. We also aimed to get a deeper understanding of genes known to confer 5-FU resistance by testing the effect of those genes regarding HAP resistance. Furthermore, we investigated if genetic interactions between 5-FU and HAP resistance genes could reveal possible molecular functions of the resistance genes.

8 Results and discussion

8.1 Publication I

In publication I, we sought to find genes which confer 5-FU resistance when overexpressed in *S. cerevisiae*. We found six genes:

- The subunits of the CPSase, *CPA1* and *CPA2,* which catalyze the first step in the *de novo* synthesis of arginine.
- The CPSase, *URA2*, which catalyze the first step in the *de novo* synthesis of UMP.
- The *HMS1* basic helix-loop-helix transcription factor which is known to positively regulate pseudohyphal growth.
- The *YJL55w* gene encoding a protein of unknown function, but of which overexpression is known to confer resistance to 5-FU, and deletion is known to confer sensitivity to purine analogues, *e.g.* HAP.
- The *HAM1* gene, which encodes a (d)ITP and (d)XTP pyrophosphatase. The overexpression of *HAM1* is known to confer resistance to 5-bromodeoxyuridine, and the deletion of *HAM1* is known to confer sensitivity to purine analogues, *e.g.* HAP.

8.1.1 Pyrimidine *de novo* synthesis

The Ura2p enzyme and the Cpa1p/Cpa2p enzymatic complex catalyse the synthesis of the same compound, carbamoyl phosphate, which is a precursor in both UMP and arginine biosynthesis. This suggests that the Cpa1p/Cpa2p enzymatic complex could confer 5-FU resistance by increasing the synthesis of UMP, which would dilute the toxic 5-FU metabolite 5-FUMP. To this end we investigated if a functional UMP synthesis pathway is required to confer the 5-FU resistance. We found that a functional UMP synthesis pathway is required for 5-FU resistance mediated by these genes. We further investigated if alterations of the arginine biosynthesis pathway could influence 5-FU

resistance. Based on a model where available carbamoyl phosphate can be channelled into either arginine or UMP synthesis, we argued that blocks in arginine synthesis could divert more carbamoyl phosphate into the synthesis of UMP, and thereby contribute to 5-FU resistance. In accordance, we found that blocks in arginine biosynthesis conferred a moderately increased 5-FU resistance. We also investigated if arginine could block the resistance conferred by Cpa2p overexpression. The logic behind this was that the enzymatic activity of Cpa2p is largely dependent on Cpa1p, and *CPA1* is downregulated by arginine. We found that arginine could indeed block 5-FU resistance conferred by *CPA2* overexpression. However, this required a high arginine concentration and growth on ammonium-less media.

8.1.2 Mechanisms of resistance

To broadly characterize the mechanisms of resistance by each gene, we tested if the overexpression of each gene affected either uracil or 5-FU on the level of cellular uptake or expulsion, and incorporation into RNA. We assayed this by using ^{14}C radiolabeled nucleobases *i.e.* uracil and 5-FU.

The overexpression of the transcription factor *HMS1* displayed the same pattern as overexpression of the *CPA1* and *CPA2* genes in that it blocked both uptake and RNA incorporation of both 5-FU and uracil. Since we also found that 5-FU resistance resulting from *HMS1* overexpression was dependent on a functional pyrimidine *de novo* synthesis, this suggested that *HMS1* acts by upregulating the pyrimidine *de novo* synthesis. In addition to the diluting effect of *de novo* synthesized UMP on the incorporation of externally supplied 5-FU or uracil into RNA, the upregulated *de novo* synthesis resulting from either *HMS1*, *CPA1* or *CPA2* overexpression could also feedback inhibit the uptake of both uracil and 5-FU through the Fur4p uracil permease; *de novo* synthesized UMP has been shown to be degraded to uracil, and uracil has been shown to downregulate *FUR4* (Grenson, 1969; Seron et al., 1999).

In contrast, the overexpression of *YJL055w* and *HAM1* did not reduce the uptake of either uracil or 5-FU, but instead specifically reduced the incorporation of 5-FU into RNA while having no effect on incorporation of uracil. This suggested that *HAM1* and *YLJ055w* act by detoxifying 5-FU or a 5-FU metabolite *e.g.* 5-FUTP. In accordance with this, Ham1p pyrophosphatase has been found to dephosphorylate the purine nucleotides (d)XTP and (d)ITP to (d)XMP and (d)IMP, thereby preventing the incorporation of these non-canonical nucleotides into RNA and DNA. Overexpression of *HAM1* has moreover been shown to confer resistance to 5-bromodeoxyuridine, which suggests that Ham1p also might detoxify non-canonical pyrimidine analogues

(Davies et al., 2012; Takayama et al., 2007). We conclude that this is a likely mechanism for the effect of *HAM1* overexpression on 5-FU resistance.

8.1.3 Resistance to 5-FU-related compounds

We furthermore tested if overexpression of the resistance genes also confer resistance to related compounds. We found that the resistance genes also confer resistance to 5-fluorocytosine and 6-azauracil, but differed in their ability to confer resistance to methotrexate and 5-fluoroorotic acid (5-FOA). The peculiarity of lack of resistance to the 5-FU precursor 5-FOA by overexpression of any of the CPSases, is presumably because CPSase overexpression, and coupled overexpression of the plasmid marker *URA3* would also contribute to the activation of 5-FOA into 5-FU; pyrimidine *de novo* synthesis has a feed-forward mechanism. Overexpression of Cpa1p and Cpa2p conferred resistance to the antifolate methotrexate. In contrast Ura2p did not confer resistance. However, the lack of resistance from overexpression of Ura2p could hypothetically be due to a more increased arginine synthesis in the Cpa1/Cpa2p overexpressors compared to the Ura2p overexpressor. Arginine could then influence the resistance to methotrexate through some unknown mechanism *e.g.* increased synthesis of nitrogen oxide.

8.2 Publication II

In the log phase Gis1p and Rph1p mainly act as redundant repressors of gene expression. This is inferred from the fact that genes are mainly upregulated *i.e.* derepressed in the double knockout *gis1 rph1* strain in the log phase. In contrast to the log phase, Rph1p and Gis1p act as both activators and repressors, and target different sets of genes in the PDS phase (Orzechowski Westholm et al., 2012).

A H235A mutation in Rph1p removes its histone demethylase activity. By comparing mRNA expression patterns in different mutants, we sought to identify the contribution of the Rph1p enzymatic activity to regulation of gene expression. We proceeded to compare gene expression in wild type and mutant yeast strains. The included mutants were: *gis1Δ, rph1Δ, rph1-H235A, gis1Δ rph1Δ,* and *gis1Δ rph1-H235A*. Analyses of the transcriptomes were done in both the log and PDS growth phases. Surprisingly, we found that the changes in gene expression in the *rph1-H235A* mutants were relatively small; the transcriptome of the *rph1-H235A* mutant was thus much more similar to that of the wild type than to that of the *rph1Δ* strain, and the transcriptome of the *gis1 rph1-H235A* double mutant was more similar to that of the *gis1Δ* mutant than to that of the *gis1Δ rph1Δ* double mutant.

8.2.1 The effect of the *rph1-H235A* mutation on gene expression

The *rph1-H235A* demethylase mutation affected gene expression more in PDS phase, where 25 genes become upregulated due to the mutation, than in log phase. However, this is still a low number of genes compared to those affected in the *rph1Δ* strain. We further found that although relatively few genes were differently expressed above the 1.5-fold threshold that we used in the *rph1-H235A* mutant, genes that were up or downregulated in the *rph1Δ* mutant were also generally affected in the same direction in the *rph1-H235A* mutant, but to a smaller degree. This suggests that the transcriptional activity of Rph1 is generally impaired in the *rph1-H235A* mutant, but the effect is big enough to be significant only for a small number of genes. The result of this comparison between sets of genes was also significant when the small number of genes that were significantly regulated at least 1.5-fold by the *rph1-H235A* mutant were excluded. We concluded that while most of the transcriptional regulation mediated by *Rph1p* seems to be largely independent of its demethylase activity, this regulation is to some extent enhanced by the same demethylase activity.

A total of 45 genes were significantly affected in one of the two growth phases (log or PDS) by the *rph1-H235A* mutation in either of the wild type or *gis1* backgrounds. Several of these genes are involved in sporulation, and several are also located close to (less than 30 kb) telomeres. Interestingly, two genes, *SNZ1* and *SNO1*, which are downregulated in both the *rph1Δ* and *rph1-H235A* strains, have been found to be derepressed in *set2* and *rpd3* mutants. This result indicates that at least these two genes, but most probably more of the 45 genes, are regulated by Rph1p-dependent demethylation of H3K36.

There are other groups of genes whose expression seems to be affected by the *rph1-H235A* mutation, but the effect of the mutation on the expression of each individual gene was too small to be significant. However, as noted above, when these genes are analysed as a group, the effect of the *rph1-H235A* mutation becomes significant. One example is the 290 genes that have been found to be affected by a *set2* deletion. In accordance with the opposing effect of Set2p and Rph1p on H3K36 methylation, the *rph1-H235A* mutation affects the transcription of these genes, as a group, in the opposite direction of *set2*. In the PDS phase, the *rph1-H235A* mutation furthermore increased the expression of genes within 20 kb of the telomeres, when considered as a group. Moreover, the demethylase activity of Rph1p seems to be important for the repression of genes involved in meiosis and sporulation. This is inferred from the fact that genes whose expression is increased or decreased more than 5-fold during sporulation are affected by the *rph1-H235A* mutation, when considered as a group. The effect is particularly pronounced for genes that are repressed in the

log phase, and the effect of the *rph1-H235A* mutation is, surprisingly, more pronounced in this case than the effect of either the *gis1* or *rph1* deletions.

8.3 Manuscript III

The human orthologue of the *S. cerevisiae gene HAM1*, the overexpression of which we found to confer resistance to 5-FU in publication I, is *ITPA*. Reduced activity of ITPA due to the 94 C>A *ITPA* polymorphism, correlates with more frequent toxicity during treatment with the purine analogue 6-MP (Tanaka et al., 2012). We genotyped blood and tissue samples previously obtained from colorectal adenocarcinoma patients for the *ITPA* 94 C>A polymorphism. The patients were genotyped for the *UGT1A1* *28, *MTHFR* 677 C>T, *ABCB1* 3435 C>T, and *TYMS* *2 or *3 polymorphisms in a previous study (Glimelius et al., 2011) and were also examined for clinically relevant early toxicity resulting from the treatment by a combinatorial regimen with irinotecan and 5-FU. It was possible to genotype the *ITPA* 94C>A polymorphism for 126 out of 140 saved blood or tissue samples from these patients. 14 of the patients were heterozygous for the *ITPA* polymorphism, and none were A/A homozygous, which does not deviate significantly from a Hardy-Weinberg equilibrium.

To determine the contribution from the *ITPA* polymorphism to clinically relevant early toxicity a multivariate logistic regression model was built. To build this model the above mentioned polymorphisms were included since they had previously been found to correlate with clinically relevant early toxicity. First, a univariate analysis was done for each polymorphism to determine if the effect of the polymorphic alleles on toxicity were better explained by an additive or dominant allelic model. Two alleles were found to significantly ($P<0.05$) influence toxicity when considered in isolation; the *MTHFR* 677 T allele correlated with a decreased toxicity, and the *ABCB1 3435* T allele correlated with a significantly increased toxicity. Subsequently, when the multivariate logistic regression was done, the *UGT1A1* locus, but neither the *ITPA* nor the *TYMS* locus, could be directly fitted into the model. To fit a polymorphism into the model all regression coefficients had to be significant at $P<0.05$, and the improvement of the goodness-of-fit of the model had to be significant $P<0.05$ in a likelihood ratio test.

The model was then extended to test for contributions from genetic interactions. For example, one could reason that *TYMS*, thymidylate synthetase and *MTHFR*, methylenetetrahydrofolate reductase, should show some genetic interaction since they both presumably influence the same 5-FU related DNA toxicity. However, TYMS could not be fitted into the model by any genetic interaction. *ITPA*, on the other hand, could be fitted into the model when a

genetic interaction between the *ITPA* and *ABCB1* loci was included into the model. The regression coefficient of this interaction was negative, which means that the simultaneous presence of the *ITPA* 94 A, and the *ABCB1* 3435 T alleles has a less than additive effect on clinically relevant early toxicity. A possible molecular explanation for this interaction could be that ITPA dephosphorylates a 5-FU metabolite into a fluorinated nucleotide monophosphate which is then pumped out from the cell by the ABCB1 efflux pump. If the ABCB1 substrate concentration is substantially reduced by the *ITPA* defect, then the presence or absence of ABCB1 might be of less importance for toxicity.

To get a measure of the goodness-of-fit of the model the c-statistic was calculated. A c-statistic of 0.5 would have indicated that the model had no discriminatory power, and a c-statistic of 1.0 would have indicated that the model would have been perfectly discriminatory between toxicity and no toxicity. The c-statistic of the full model including *ITPA* was calculated to 0.73, which indicates that the model is reasonable good, but does not explain all variation in toxicity between patients. Other yet to be identified polymorphisms could presumably be included to strengthen the discriminatory power of the model.

8.4 Manuscript IV

To get a better understanding of detoxification and resistance to toxic nucleobase analogues, we screened a shuttle vector based genomic library for genes which confer resistance to the mutagenic purine analogue HAP, when overexpressed in *S. cerevisiae*. The genes which were found to confer HAP resistance, and the genes which conferred resistance to 5-FU in publication I were all tested for genetic interactions affecting both 5-FU and HAP resistance. The new genes we found to confer resistance to HAP were:

- The *DUT1* gene encoding deoxyuridine triphosphate pyrophosphatase (dUTPase), an enzyme required for synthesis of thymidylate and DNA. dUTPase has also been found to be essential for genomic stability. Dut1p, which primarily dephosphorylates the pyrimidine dUTP, has furthermore been shown to dephosphorylate the purine deoxyribonucleotide dITP. However, *DUT1* overexpression, surprisingly, only conferred resistance to HAP, but not 5-FU; while the *DUT1/dut1* heterozygous strain was only sensitive to 5-FU, but not HAP.
- The *APT2* gene, a paralogue of the *APT1* gene, which encodes adenine phosphoribosyltransferase. However, the *APT2* encoded protein has not been shown to possess adenine phosphoribosyltransferase activity. We

found that while *APT2* overexpression conferred weak resistance to HAP, the *apt2* deletion strain was not HAP sensitive.

- The *ATR1* gene encoding a membrane bound efflux pump belonging to the major facilitator superfamily, which has been shown to confer resistance to the nitrogen-containing heterocyclic compound aminotriazole, and also borate. *ATR1* overexpression conferred strong resistance to both 5-FU and HAP, but the *atr1* deletion strain was only sensitive to 5-FU, and not to HAP.
- The *ADE4* gene which encodes the enzyme 5-phosphoribosyl-1-pyrophosphate amidotransferase, Ade4p. Ade4p catalyses the first step in the *de novo* synthesis of purine nucleotides. *ADE4* overexpression conferred resistance to HAP, and also weak resistance to 5-FU.

Two genes, *YJL055w* and *HMS1*, which conferred resistance to 5-FU in publication I, also conferred resistance to HAP in the genetic interaction assay. However, these two genes were not recovered in the genomic library screen for HAP resistance. The gene that was used as a positive control for HAP resistance, *HAM1*, was however recovered five times in the screen. This suggests that the screen was quite exhaustive, but still not complete.

8.4.1 Genetic interactions and models for HAP and 5-FU resistance

We included the deletion strains *amd1*, *aah1*, and *ade1* in our studies of genetic interactions in order to further elucidate the possible molecular functions of the cloned resistance genes. From the genetic interactions found, possible molecular mechanisms of resistance to HAP or 5-FU were proposed.

The strong genetic interaction between the *HAM1* overexpression, and the *amd1* deletion regarding 5-FU resistance could be due to an effect of the purine nucleotide balance on 5-FU metabolism. However, a more likely possible explanation of this effect is that Amd1p, which is known to deaminate AMP, also deaminates the 5-FU metabolite 5-FCMP.

Interestingly, although they are not members of the same gene family as *ATR1*, some of the genes in the ATP-binding cassette (ABC) family of drug efflux transporters have been found to confer resistance to 5-FU, 6-thioguanine and 6-mercaptopurine in human cell lines. Thus, 5-FU resistance was shown to be conferred by the ABC efflux pump MRP5 through ATP-dependent transport of the monophosphorylated nucleotides 5-FUMP and 5-FdUMP (Chen et al., 2001; Pratt et al., 2005). This is analogous to the suggested role of Atr1p in our models for HAP and 5-FU resistance, where Atr1p is proposed to pump out the monophosphorylated nucleotides dHAPMP and 5-FCMP.

DNA toxicity due to increased mutagenesis is the primary mode of action of HAP that has been proposed by others (Abdul-Masih and Bessman, 1986). Our

results are consistent with this hypothesis. In contrast, 5-FU is also toxic to RNA metabolism. In addition to its known inhibition of tRNA pseudouridine synthases *e.g.* Pus1p and tRNA uridine methylases, 5-FU may also affect RNA metabolism by being converted into 5-FCTP and incorporated into RNA. Analogously to the inhibition of tRNA uridine methylases, 5-FCTP incorporation into tRNA could inhibit the tRNA methylases such as Trm4p. The inhibition of multiple tRNA modifying enzymes *e.g.* Pus1p and Trm4p could in turn cause tRNA instability (Gustavsson and Ronne, 2008) and possibly also erroneous tRNA mediated gene regulation.

9 Conclusions and future perspectives

9.1 Pharmacogenomics of 5-fluorouracil

The (d)ITP pyrophosphatase gene *HAM1* in *S. cerevisiae*, the overexpression of which we found to confer 5-FU resistance in publication I, and HAP resistance in manuscript IV, is the orthologue of the human gene *ITPA*. In manuscript III we found that the 94C>A polymorphism in the *ITPA* gene correlated with an increased incidence of toxicity from combined 5-FU and irinotecan treatment in cancer patients. However, this result, which was based on logistic regression modelling, was only significant when we included a genetic interaction between the *ITPA* 94C>A polymorphism, and the 3435C>T polymorphism in the *ABCB1* ATP-binding cassette gene. This genetic interaction was negative, which means that the co-occurrence of both polymorphisms has a less than additive effect on the incidence of toxicity.

With data from more 5-FU treated patients it could be possible to more stringently determine the correlation between 5-FU sensitivity and the *ITPA* polymorphism, *i.e.* score the correlation without taking genetic interactions into account. The correlation could presumably be established by a regular Fisher's exact test from a larger patient group. Moreover, by studying the effects of the *ITPA* polymorphism on other combination treatments or on treatment with 5-FU alone, any conceivable effects of irinotecan could be ruled out.

9.2 Resistance to nucleobase analogues in *S. cerevisiae*

As discussed in manuscript IV, 5-FU seems to target mainly RNA metabolism in yeast, but also to a lesser extent DNA metabolism. It would benefit the elucidation of 5-FU metabolism if it was possible to specifically target either DNA or RNA metabolism with 5-FU.

Deoxyuridine is claimed to not be utilizable by *S. cerevisiae* (Kurtz et al., 2002), see also chapter 5.4 this thesis. This suggests that it should not be possible to specifically target DNA metabolism with 5-fluorodeoxyuridine, which could otherwise have been converted to 5-FdUMP, which presumably would inhibit thymidylate synthetase, but not interfere with RNA metabolism. However, *HAM1* overexpression has been shown to confer resistance to 5-bromodeoxyuridine (Takayama et al., 2007). This suggests the possibility that DNA metabolism might still be specifically targeted by modified pyrimidine deoxyribosides such as 5-fluorodeoxyuridine, contrary to the earlier results. 5-Fluorodeoxyuridine, if it could be used to specifically target the DNA metabolism, would according to our model in manuscript IV result in different resistance phenotypes due to overexpression than 5-FU. *HAM1* overexpression would thus be expected to confer lower resistance, and *DUT1* overexpression to confer relatively higher resistance to the drug. It would furthermore be possible to study drug interactions between the DNA toxic 5-fluorodeoxyuridine, and other cell cycle inhibitors.

A yeast immobilization technique makes it possible to study *S. cerevisiae* that are in cell cycle arrest, with downregulated *CDC28* and *CLN1*, but still retaining a high fermentative capacity (Nagarajan et al., 2014). Since DNA synthesis is absent under these conditions, this technique could make it possible to more specifically study the RNA toxicity of both 5-FU, and HAP. HAP is considered to be mutagenic, and toxic for DNA metabolism, but could hypothetically also target RNA metabolism.

9.2.1 5-FU and tRNA

A model for 5-FU toxicity is proposed in manuscript IV. In this model 5-FU also targets Trm4p in addition to its known targets, see chapter 5.6.3 in this thesis. Trm4p methylates the 5’ position of cytidines in several tRNAs. This is of importance for gene regulation in response to oxidative stress (Chan et al., 2012). The methylation status of cytidines in tRNA can be determined by bisulfite treatment of RNA, which transforms cytidine, but not 5’-methylcytidine to uridine, followed by sequencing of cDNA generated from treated RNA (Schaefer et al., 2009). Consequently, it would be possible to assay the impact of 5-FU on Trm4p-mediated tRNA cytidine methylation with this method.

Another tRNA methylase, Trm9p, methylates the 5’ position of uridines in some tRNAs, and is thereby involved in the translational regulation of DNA damage response (DDR) genes such as the ribonucleotide reductase subunit genes *RNR1* and *RNR3* (Begley et al., 2007). It would be interesting to investigate whether 5-FU mediated RNA toxicity causes a translational

downregulation of DDR genes such as *RNR1* and *RNR3*, and thereby increases the DNA toxicity *per se*. However, since the dysregulation would happen at the translational level, RT-PCR or microarrays could not be used to assay this. Conceivably, one could instead use protein electrophoresis or possibly quantitative protein mass spectrometry.

In addition to the lethal effects of 5-FU on RNA metabolism, 5-FU might cause an arrest in the G1 phase of the cell cycle, which could be mediated by tRNA destabilization (Gustavsson and Ronne, 2008). Initially, this could be investigated in *S. cerevisiae* treated with growth inhibiting, but not lethal, concentrations of 5-FU; a non-lethal concentration would presumably be required to investigate an ordered regulated response, such as cell cycle arrest.

tRNA destabilization prevents tRNA maturation. *GCN4* has moreover been shown to be upregulated by the presence of immature tRNA in the nucleus, and prevent the progression of the cell cycle from G1 to S by delaying the accumulation of late G1 cyclins, see chapters 3.3.1 and 5.6.3 in this thesis. Thus, 5-FU mediated tRNA toxicity could possibly affect the transcriptional status of *GCN4* and the late G1 phase cyclins.

9.2.2 5-FU and HAP metabolism and degradation

Possible models for 5-FU and HAP metabolism and toxicity are discussed in manuscript IV, but several of the proposed molecular mechanisms remain to be biochemically validated. For example, it is important to determine if the adenine deaminase Amd1p can deaminate 5-FCMP, CMP or both; in particular the ability to deaminate 5-FCMP would be important for its role in 5-FU detoxification. The suggested 5-FCMP deaminase activity of Amd1p would have to be tested *in vitro*, but a possible CMP deaminase activity of Amd1p, which if found would imply 5-FCMP deaminase activity, could be tested by genetics. Thus, a comparison of an *urh1 cdd1 fcy1 ura3* strain to an *urh1 ccd1 fcy1 ura3 amd1* strain, for the ability to utilize externally provided cytidine as a source of pyrimidines, might reveal if Amd1p has CMP deaminase activity. If the former, but not the latter strain is able to grow in the presence of cytidine, it would strongly imply that Amd1p also has CMP deaminase activity in addition to its known AMP deaminase activity. Since Amd1p has been found to be allosterically regulated by ATP, this should be tested on both fermentable and non-fermentable carbon sources.

In manuscript IV, we also discussed the possibility that Ham1p causes 5-FU resistance by being a pyrophosphatase acting on 5-FCTP. It is possible to assay this activity by combining the purified Ham1p enzyme and inorganic pyrophosphatase, and detecting the generation of phosphate after the addition

of 5-FCTP as a substrate. However, the proposed Ham1p substrate 5-FCTP would first have to be synthesized.

9.3 Nutrient signalling and metabolic regulation

In publication II we found that the histone lysine demethylase (H3K36) activity of Rph1p was largely dispensable for its role in transcriptional regulation. This raises the question what the primary role of the Rph1p-dependent histone demethylation is. One possible clue are results obtained in other organisms, see chapter 2.2.2 in this thesis, that have shown that H3K36 methylation preferentially enhances the non-homologous end joining (NHEJ) DNA repair pathway (Fnu et al., 2010); thus H3K36 methylation could be important for the choice between DNA repair pathways. This could be of importance for the generation of mutations, since NHEJ is error prone compared to the other major repair pathway homologous recombination repair (HRR). See chapter 3.2.3 and 3.2.4 in this thesis.

Transcribed DNA has been found to be more vulnerable to mutagenesis (Kim et al., 2007) and Rph1p positively regulates gene expression mainly in the PDS phase (Orzechowski Westholm et al., 2012) when mitochondria are active, and, consequently, the burden of DNA damaging reactive oxygen species (ROS) is comparably high. This implies that genes regulated by Rph1p, and also Gis1p, in the PDS phase could be more vulnerable to DNA damage due to ROS exposure. Hypothetically, the Rph1p histone demethylase activity, which counteracts H3K36 methylations by Set2p inside genes, could therefore be of importance for reducing the incidence of mutations resulting from NHEJ mediated DNA repair, see chapter 2.2.2 in this thesis.

Hypothetically, the choice between NHEJ and HRR would mainly affect diploid cells in the G1 phase of the cell cycle since HRR is the preferred repair pathway in the S and G2 phases of the cell cycle, and only diploid cells have a homologous DNA template in the G1 phase (Kadyk and Hartwell, 1992; Mathiasen and Lisby, 2014; chapters 3.2.3 and 3.2.4 in this thesis). One way to test the effect of Rph1p on mutagenesis could be to construct a gene repair assay, where the generation of a frame-shift mutation within a coding region could restore gene function. In a diploid which is homozygous for the recessive mutant allele, the presence of *RPH1* would be expected to reduce the revertant frequency since the homologous mutant allele would preferentially be used as a template for HRR.

Alternatively, since the deletion of histone lysine demethylase activity of Rph1p was shown in publication II to significantly affect the expression of 45 genes, it would be interesting to study the effects of the *rph1-H235A* mutation

on gene expression in a *set2* background. Set2p is a histone lysine methylase targeting the same residue, H3K36, as Rph1p, and would therefore be expected to be epistatic over *rph1-H235A*, but presumably not over the *rph1* knockout.

Finally, the finding in publication II that the *rph1-H235A* mutation increased the expression of telomere proximal genes, but only when analysed as a group, suggests the possibility that H3K36 methylation influences a transcriptional regulation mechanism that is active close to telomeres. This mechanism could hypothetically involve the telomere proximate silencing conferred by the Sir complex (Aparicio et al., 1991). Therefore, it would also be interesting to investigate the effect of the *rph1-H235A* mutation on gene expression in a *sir2* background.

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