

Systems Biology of *Saccharomyces cerevisiae* Physiology and its DNA Damage Response

Fazio, Alessandro; Workman, Christopher; Nielsen, Jens; Heierhorst, Jörg

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Systems Biology of
Saccharomyces cerevisiae
Physiology and its DNA Damage
Response

CENTER FOR
SYSTEMS
BIOLOGY
ANALYSIS
AND
MODELING
CBS

Alessandro Fazio
Department of Systems Biology
Technical University of Denmark

A thesis submitted for the degree of
Philosophiæ Doctor (PhD)

December 2010

Assessment Committee

Prof. Lene Juel Rasmussen

Department of Cellular and Molecular Medicine
Københavns Universitet (KU)

Prof. Robert de Bruin

MRC Laboratory for Molecular Cell Biology
University College London (UCL)

Assoc. Prof. Uffe Hasbro Mortensen

Center for Microbial Biotechnology
Technical University of Denmark (DTU)

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Preface

This Ph.D. thesis was prepared at the Department of Systems Biology, the Technical University of Denmark (DTU); it presents a systems biology approach to *Saccharomyces cerevisiae* growth physiology and DNA damage response and consists of an introduction and four manuscripts written in the period 2007-2010.

The studies described in this work were carried out at the Center for Biological Sequence Analysis (CBS-DTU), the Center for Microbial Biotechnology (CMB-DTU) and the Biotechnology Department (Delft University of Technology) under the supervision of Assoc. Prof. Christopher T. Workman (CBS-DTU) and Prof. Jens Nielsen (Chalmers University of Technology, Goteborg). Moreover, part of the work was carried out at St Vincent's Institute of Medical Research (The University of Melbourne) under the supervision of Dr. Jörg Heierhorst.

The work was funded by the Technical University of Denmark; the external stay at St Vincent's Institute was co-funded by the Otto Mønsted's Fond and Oticon Fonden and the work described in Chapter 4 was part of the Yeast Systems Biology Network (YSBN) project (EC FP6 funding).

Kongens Lyngby, December 2010
Alessandro Fazio

Abstract

The yeast *Saccharomyces cerevisiae* is a model organism in biology, being widely used in fundamental research, the first eukaryotic organism to be fully sequenced and the platform for the development of many genomics techniques. Therefore, it is not surprising that *S. cerevisiae* has also been widely used in the field of systems biology during the last decade. This thesis investigates *S. cerevisiae* growth physiology and DNA damage response by using a systems biology approach.

Elucidation of the relationship between growth rate and gene expression is important to understand the mechanisms regulating cell growth. In order to study this relationship, we have grown *S. cerevisiae* cells in chemostat at defined growth rates and measured the transcriptional response. We have applied a complex experimental design, involving three factors: specific growth rate, oxygen availability and nutrient limitation. We have identified 268 growth rate dependent genes. These genes were used to identify key areas of the metabolism around which expression changes were significantly associated and we found nucleotide synthesis and ATP producing and consuming reactions. Moreover, by scoring the significance of overlap between growth rate dependent genes and known transcription factor (TF) target sets, we identified 13 TFs, involved in stress response, cell cycle and ribosome biogenesis, that appeared to coordinate the response at increasing growth rates. Therefore, in this study we have identified a more conservative set of growth dependent genes by using a multi-factorial experimental design. Moreover, new insights into the metabolic response and transcriptional regulation of these genes have been provided by using systems biology tools (Chapter 3).

One of the prerequisite of systems biology should be the standardization and reproducibility of experimental and analytical techniques, in order to allow the comparison of data generated in different laboratories. With the aim of addressing this aspect, we have collaborated in a large study involving ten laboratories, constituting the Yeast Systems Biology Network (YSBN). *S. cerevisiae* cultivations were performed in a single laboratory and samples were sent to the other partners. The experimental design involved two factors: strain (CEN.PK113-7D and YSBN2) and growth condition (batch and chemostat). Transcriptome was measured with four different platforms (Affymetrix, Agilent, qPCR and TRAC), metabolome was analyzed in seven

laboratories, using different protocols, and enzyme activities were determined in two different laboratories. The comparison of the analyses showed that reproducibility of the results was affected by the laboratory and the protocol used. Transcription and enzyme activity analyses gave consistent results, while metabolite level measurements showed some variability. Therefore, even though the source of biomass was unique, the reproducibility of data appeared to be a challenging task. Nevertheless, we were able to perform an integrative analysis and discover that the lower biomass yield of CEN.PK113-7D was due to higher protein turnover than YSBN2; this finding would not be achievable using a single omics dataset. Moreover, the generated datasets are a valuable resource for the yeast systems biology community (Chapter 4).

Upon DNA damage, *S. cerevisiae* cells respond activating the so-called cell cycle checkpoints that promote damage repair and viability. The activation of these checkpoints depends on kinase cascades and regulation of transcription is one of the responses elicited by checkpoint activation. Therefore, we have decided to investigate the transcriptional and phenotypic responses to the alkylating agent methyl methanesulfonate (MMS) of mutant strains carrying deletions of genes encoding protein kinases (Mec1, Tel1, Rad53, Dun1, Chk1, Alk1) and protein phosphatases (Ptc3, Pph3, Oca1) involved in DNA damage response (DDR). We have discovered a prominent role for Rad53, Mec1 and Tel1 in transcriptional response. Moreover, we have shown for the first time the important role of Oca1 at the transcriptional level. We have built a comprehensive network of the central DDR pathway by integrating data from different cellular levels and identified regulatory circuits involving key players of this pathway. Integration of transcriptional and phenotypic data allowed us to discover sets of genes whose expression levels correlate with growth rates upon MMS treatment. Finally, we have also investigated the role of non protein-coding RNAs in DNA damage response (Chapter 5).

When DNA damage is repaired, cells restart the cell cycle and resume growth. This process is called damage recovery. In *S. cerevisiae*, the molecular mechanism of recovery relies on dephosphorylation of Rad53 by protein phosphatases (PPs), that, in case of recovery from MMS-induced damage, are Ptc2, Ptc3 and Pph3. In order to elucidate the relationship between Rad53 and PPs, we have generated strains carrying mutations in Rad53 domains (SCD1 and FHA1) and deletion of genes encoding the PPs. Then, we have investigated the Rad53 phosphorylation status and the phenotype of these mutant strains. This study has allowed us to propose a role for the threonine 8 of Rad53-SCD1 domain and its Ptc2/3-mediated dephosphorylation during MMS recovery (Chapter 6).

Dansk resumé

Gæren *Saccharomyces cerevisiae* er en modelorganisme indenfor biologien, hvor den i vidt omfang bruges i grundlæggende forskning og er den første eukaryotiske organisme der er blevet fuldstændig sekvenseret. Ydermere har den været platform til udvikling af mange genteknologiske teknikker. Det er derfor ikke overraskende, at *S. cerevisiae* også har været særligt udbredt indenfor systembiologien i det seneste årti. Denne afhandling undersøger *S. cerevisiae*'s vækstoffysiologi og respons på DNA skader gennem en systembiologisk tilgang.

Klarlægningen af forholdet mellem vækstrate og genekspression er vigtig for forståelsen af mekanismerne der regulerer cellevæksten. For at undersøge dette forhold har vi dyrket *S. cerevisiae* i en kemostat med definerede vækstrater og målt den transkriptionelle respons. Vi har brugt et komplekst eksperimentelt design, der involverer tre faktorer: specifik vækstrate, ilt tilgængelighed og næringsbegrænsning. Vi har identificeret 268 vækstrateafhængige gener, som var uafhængige af de andre to eksperimentelle faktorer. Disse gener blev brugt til at identificere nøgleområder i metabolismen, hvor ændringerne i ekspressionen var signifikant associeret og vi fandt nukleotid- syntese og ATP producerende og konsumerende reaktioner. Endvidere, ud fra vækstrate afhængige gener og kendte transkriptions faktorer (TF) målgrupper tildeltes en score af signifikansen til overlappet af disse, hvoraf vi identificerede 13 TFs, som så ud til at koordinere responsen ved opadgående vækstkurver, at være involveret i stress responser, celle cyklus og ribosom biogenese. Derfor har vi i dette studie identificeret et mere konservativt sæt af vækstafhængige gener ved brug af et multi-faktoriellet eksperimentelt design. Endvidere er ny indsigt i metabolisk respons og transkriptionel regulering af disse gener blevet udredt ved hjælp af systembiologiske værktøjer (Kapitel 3).

En af forudsætningerne for systembiologien formodes at være standardiseret og reproducerbare eksperimentelle og analytiske teknikker, hvilket tillader sammenligning af data genereret i forskellige laboratorier. I et forsøg på at adressere dette aspekt, har vi samarbejdet i en stor undersøgelse, der involverer ti laboratorier, som tilsammen udgør Yeast Systems Biology Network (YSBN). Dyrkningen af *S. cerevisiae* blev fortaget i et enkelt laboratorium og prøverne blev derefter sendt ud til de andre partnere. Det eksperimentelle design involverede to faktorer: stammerne (CEN.PK113-7D and YSBN2) og vækstoffaktorerne (batch and kemostat). Transkriptomet blev målt på fire forskellige platforme (Affymetrix, Agilent, qPCR and TRAC), metabolomet blev analyseret i syv laboratorier ved brug af forskellige protokoller, og enzym- aktivitetene blev bestemt i to forskellige laboratorier. Sammenligningen af analyserne viste, at reproducerbarheden af resultaterne var berørt af laboratoriet og hvilken protokol der blev brugt, dette gjorde sig

især gældende for metabolit-niveauerne. Analyserne af transkriptomet og enzym-aktiviteten viste mere konsekvente resultater. Derfor, selvom kilden til biomassen var unik, viste reproducerbarheden af dataen sig, at være en udfordrende opgave. Ikke desto mindre var vi i stand til at udføre en integrativ analyse og finde frem til at det lavere udbytte af CEN.PK113-7D var grundet en højere proteinomsætning end YSBN2. Dette fund ville ikke kunne opnås ved et enkelt -omisk datasæt. Desuden er det genereret datasæt en værdifuld ressource for gærssystembiologiens samfund (Kapitel 4).

Ved DNA-skader, reagerer *S. cerevisiae* celler ved at aktivere de såkaldte celleyklus checkpoints, der fremmer reparationen af skader og levedygtighed. Aktivering af disse checkpoints afhænger af en kinase-kaskade og regulering af transkription er en af de responser, som fremkaldes ved checkpoint aktivering. Derfor har vi besluttet at undersøge den transkriptionelle og fænotypiske respons på det alkylende stof methyl methanesulfonate (MMS) i mutant stammer, der har gener der koder for protein kinaser slået ud (Mec1, Tel1, Rad53, Dun1, Chk1, Alk1) og protein fosfataser (Ptc3, Pph3, Oca1) der er involveret i responsen på DNA-skader (DDR). Vi opdagede, at Rad53, Mec1 og Tel1 spiller en fremtrædende rolle i transkriptionel respons. Desuden viste vi for første gang den vigtige rolle, som Oca1 har på transkriptionelt niveau. Vi har bygget et omfattende netværk af det centrale DDR system ved at integrere data fra forskellige cellulære niveauer og har identificeret regulerende kredsløb der involverer nøglespillere i dette system. Integreringen af transkriptionel og fænotypisk data tillod os at opdage sæt af gener, hvis ekspressionsniveau er korreleret, enten positivt eller negativt, med stigende vækstrater ved behandling med MMS. Sidst har vi undersøgt ikke-kodende RNAs rolle i DNAs respons på skader (Kapitel 5).

Når DNA-skader er repareret, genstarter celler celleyklussen og genoptager vækst. Denne proces er kaldt skade-genopretning. Hos *S. cerevisiae*, afhænger den molekylære mekanisme for genopretning, af defosforylering af Rad53 via protein fosfataser (PPs), som, hvis i tilfælde af bedring efter MMS-induceret skader er, Ptc2, Ptc3 og Pph3. For at belyse forholdet mellem Rad53 and PPs har vi genereret stammer der bærer mutationer i Rad53 domæner (SCD1 and FHA1) og deletioner i gener der koder for PPs. Derefter undersøgte vi fosforyleringsstatussen af Rad53 og fenotypen hos disse mutantstammer. Denne undersøgelse har givet os mulighed for at foreslå threonine 8 af Rad53-SCD1 domænet og dets Ptc2/3-medieret defosforylering som faktor i bedringen efter MMS behandling (Kapitel 6).

A Girardella, i Durani e i Capaci

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Life is the art of meeting

Vinicius De Moraes

My PhD life has had many ups and downs. Besides the scientific education, what have I really achieved after all? People and places... Yes, people and places that have come across my life making these three years unforgettable. People belong to places and viceversa. I belong to Chiomonte, Torino, Copenhagen, Delft and Melbourne. Each of these places is unique, like the people I met.

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Each one of these people knows the reasons why he/she is present in this list. Grazie!!

...

The fingers. What's the answer?

Oh, you're another one of those bright young fellows...
who always know the right answer, is that it?

Welcome to real life.

How many do you see?

There are four fingers, Arthur.

No, no, no. Look at me.

What?

Y-You're focusing on the problem.

If you focus on the problem, you can't see the solution.

Never focus on the problem. Look at me!

How many do you see?

No, look beyond the fingers.

How many do you see?

Eight.

Eight. Eight. Yes! Yes!

Eight's a good answer. Yes.

See what no one else sees.

See what everyone else chooses not to see...

out of fear and conformity and laziness.

See the whole world anew each day.

...

from PATCH ADAMS

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Abbreviations

ANOVA - analysis of variance	OD - optical density
ATP - adenosine triphosphate	ORF - open reading frame
BER - base excision repair	PCA - principal component analysis
Cdk - cyclin-dependent kinase	PCNA - proliferating cell nuclear antigen
cDNA - complementary DNA	PCR - polymerase chain reaction
DDR - DNA damage response	PK - protein kinase
dNTP - deoxyribonucleotide	PP - protein phosphatase
DSB - double strand break	PRR - post-replicative repair
ESR - environmental stress response	qPCR - quantitative PCR
FACS - fluorescent-activated cell sorting	RNR - ribonucleotide reductase
FC - fold change	RP - ribosomal protein
FDR - false discovery rate	RPA - replication protein A
FHA - forkhead associated	rRNA - ribosomal RNA
GC - gas chromatography	S/Ser - serine
GO - gene ontology	SCD - SQ/TQ cluster domain
HPLC - high-performance liquid chromatography	SGD - <i>Saccharomyces</i> genome database
HU - hidroxyurea	snRNA - small nuclear RNA
HR - homologous recombination	snoRNA - small nucleolar RNA
IR - infrared radiation	ssDNA - single-stranded DNA
KO - knockout	TCA - tricarboxylic acid cycle
LC - liquid chromatography	T/Thr - threonine
MMR - mismatch repair	TF - transcription factor
MMS - methyl methanesulfonate	TLS - translesion DNA synthesis
mRNA - messenger RNA	TOF - time of flight
MS - mass spectrometry	TRAC - transcript analysis with affinity capture
NADH - nicotinamide adenine dinucleotide	tRNA - transfer RNA
ncRNA - non-coding RNA	UV - ultraviolet radiation
NER - nucleotide excision repair	WT - wild type
NHEJ - non-homologous end-to-end joining	YPD - yeast extract/peptone/dextrose
NMR - nuclear magnetic resonance	YSBN - yeast systems biology network

Genetic nomenclature (using *MEC1* as an example):

gene (*MEC1*), protein (Mec1), gene deletion (*mec1Δ*), gene mutation (*mec1-100*)

Manuscripts not included in the thesis

- PIDDOCKE MP, FAZIO A, VONGSANGNAK W, WONG ML, HELDT-HANSEN HP, WORKMAN CT, NIELSEN J AND OLSSON L. Revealing the beneficial effect of protease supplementation to high gravity fermentations using ‘-omics’ techniques. *Submitted to Microbial Cell Factories*
- PIDDOCKE MP, FAZIO A, VONGSANGNAK W, WONG ML, NIELSEN J AND OLSSON L. Transcriptional study of high gravity beer fermentations: the effect of glucose repression and nitrogen limitation. *In preparation*
- SOHONI SV, FAZIO A, WORKMAN CT, MIJAKOVIC I AND LANTZ AE. Synthetic promoter library for modulation of actinorhodin production in *Streptomyces coelicolor A3(2)*. *In preparation*
- KRAT C, FAZIO A, WORKMAN CT AND PETER M. Autophagy-dependent transcriptional changes upon nitrogen starvation in *Saccharomyces cerevisiae*. *In preparation*
- HOCH NC, CHEN ESW, FAZIO A, HAMMET A, PELLICOLI A, TSAI MD AND HEIERHORST J. Rad53 SQ/TQ cluster domain phosphorylation is required for replication of undamaged DNA. *In preparation*
- OLIVEIRA AP, WESTERGAARD SL, FAZIO A, MUSTACCHI R, OLSSON L, PATIL KR AND NIELSEN J. The regulatory role of Hxk2 extends beyond regulation of glucose repression. *In preparation*

Part I

Introduction

Chapter 1

Saccharomyces cerevisiae Growth Physiology

1.1 From baking to systems biology

The age-old usage of the yeast *Saccharomyces cerevisiae* in baking has given rise to its common name, baker's yeast. *S. cerevisiae* is a unicellular fungus that exists in either diploid or haploid state. Scientific research with *S. cerevisiae* has been going on since late 18th century. The first studies investigated the fundamental principles of fermentation, pure cultures and cytology, the latter greatly improved by the development of the electron microscope in 1930. While much of the structural composition of the yeast cell had been explored, little was known about metabolism and pathways, not to mention regulatory mechanisms, governing gene expression and protein biosynthesis. In the mid 1800's, Louis Pasteur worked on quantitative differences concerning aerobic and anaerobic sugar utilization by yeast and his discoveries were later related to that phenomenon called glucose repression. The glycolytic pathway was mainly resolved during the first half of the twentieth century as being part of the fermentation pathway. From the 1930's to around the 1960's, knowledge of the main respiratory pathway and understanding of the tricarboxylic acid cycle (TCA cycle or the Krebs' cycle) were also developed. In relation to the respiratory pathway, the mitochondria were identified as site of cellular respiration. For an extensive description of the history of research on yeast, the reader is referred to the review series from J.A. Barnett.

In 1989, the major project of sequencing the genome of *S. cerevisiae* was initiated by the Yeast Sequencing Project, which involved more than 100 laboratories in Europe, USA, Canada and Japan. By 1996, the project was completed, making *S. cerevisiae* the first eukaryotic organism to be fully sequenced (Goffeau *et al.*, 1996). The knowledge of the genomic sequence has given researchers another very powerful tool to better understand the functions of the microorganism. This includes the ability to identify new genes and to infer gene regulation at the genome level. Additionally, access to the genomic sequence has been a key component in metabolic engineering of *S. cerevisiae* as a production organism in various processes. The strain used for systematic sequencing

was S288c (Mortimer & Johnston, 1986), which, however, has a defective *HAP1* gene making it incompatible with studies of mitochondrial and related systems (Gaisne *et al.*, 1999).

As described above, *S. cerevisiae* has a number of advantages that make it attractive as a model organism (Castrillo & Oliver, 2004). First, *S. cerevisiae* is unicellular and has many cellular components in common with higher eukaryotic organisms. This makes it possible to transfer knowledge obtained with this microorganism to more complex organisms (Mager & Winderickx, 2005). Second, many techniques for studying biology have been developed in *S. cerevisiae* thereby giving present researchers a wide range of tools to test and analyze the alterations they have conferred on the studied strains. For example, yeast has been at the forefront of the development of large scale genomic approaches. Essentially, all high-throughput functional genomics techniques and the underlying bioinformatics tools for analysis of the so-called ‘omes’ (e.g., transcriptome, proteome, metabolome, interactome) were originally developed using yeast as a model system. A complete set of *S. cerevisiae* deletion mutants for approximately all 6,000 open reading frames (ORFs) is available for the heterozygous diploid strains (Winzeler *et al.*, 1999). Third, *S. cerevisiae* can be simply cultivated at well controlled conditions, allowing studies of high reproducibility. Moreover, *S. cerevisiae* is an important microorganism used in large-scale industrial processes. Besides its employment in traditional fermentation industries, well-established fermentation and molecular biology techniques, together with its GRAS status (generally regarded as safe), make this organism particularly attractive for both modern applications and the development of new processes. The main current applications of this yeast include: baking, bulk and fine chemicals production (ethanol, glycerol, ergosterol), medical applications (recombinant proteins and peptides), beer brewing, wine and distilled beverages production, single cell proteins (food supplement and animal feed) and yeast extract (enzyme for food industry, component of microbial culture media, biochemical for research, vitamin supplement, food flavours).

Considering the aspects discussed above, it is not surprising that *S. cerevisiae* has also been the organism of choice for the development of the new field called systems biology (Chuang *et al.*, 2010; Ideker *et al.*, 2001; Kitano, 2002). Systems biology proposes a holistic view of the biological systems, as opposed to the reductionist view, which only considers single components of the systems. The aim of the systems biology approach is efficiently enunciated by the following statement: ‘*The reductionist approach has successfully identified most of the components and many of the interactions but, unfortunately, offers no convincing concepts or methods to understand how system properties emerge...the pluralism of causes and effects in biological networks is better addressed by observing, through quantitative measures, multiple components simultaneously and by rigorous data integration with mathematical models*’ (Sauer *et al.*, 2007)

The success of this approach has been possible because of the recent advancements in technology and bioinformatics that have permitted the accomplishment of high-throughput genome-wide studies and the development of complex analysis and modeling

tools. With respect to *S. cerevisiae*, the following is a list of important genome-wide studies:

- Subcellular localization (Huh *et al.*, 2003; Kumar *et al.*, 2002).
- Protein-protein interactions (Gavin *et al.*, 2002; Ho *et al.*, 2002; Ito *et al.*, 2001; Krogan *et al.*, 2006; Reguluy *et al.*, 2006; Tarassov *et al.*, 2008; Uetz *et al.*, 2000; Yu *et al.*, 2008).
- Genetic interactions (Costanzo *et al.*, 2010; Reguluy *et al.*, 2006; Tong *et al.*, 2001, 2004).
- Protein-DNA interactions (Harbison *et al.*, 2004; Lee *et al.*, 2002; Ren *et al.*, 2000).
- Post-translational modifications (PTMs), e.g. phosphorylation (Breitkreutz *et al.*, 2010; Chi *et al.*, 2007; Ficarro *et al.*, 2002; Fiedler *et al.*, 2009; Ptacek *et al.*, 2005) and acetylation (Choudhary *et al.*, 2009).
- Genome-scale metabolic model (Förster *et al.*, 2003; Herrgaard *et al.*, 2008; Nookaew *et al.*, 2008).

Most of the data provided by these studies has been stored in databases or tools freely available on the internet (Aranda *et al.*, 2010; Bader *et al.*, 2001; Bodenmiller *et al.*, 2008; Breitkreutz *et al.*, 2008; Ceol *et al.*, 2010; Fernandez-Ricaud *et al.*, 2007; Gauthier *et al.*, 2008; Jensen *et al.*, 2009; Kals *et al.*, 2005; Kelley *et al.*, 2004; King *et al.*, 2006; Koh *et al.*, 2010; Stark *et al.*, 2010; Wingender *et al.*, 2001; Xenarios *et al.*, 2002). The number of publications about yeast systems biology has been rapidly increasing over the past decade, with the goal of elucidating new aspects of *S. cerevisiae* metabolism and physiology (for example, see Herrgaard *et al.* (2006), Workman *et al.* (2006), Bradley *et al.* (2009), Moxley *et al.* (2009)). In the present thesis I will focus on the investigation of *S. cerevisiae* growth and DNA damage response from a systems biology perspective.

1.2 Growth

Growth is determined by the cellular sensing and responses to the external environment, which provides nutrient as well as different sources of stress. Through interconnected signaling pathways, nutrient availability affects cellular metabolism, transcriptional profile and developmental program. Nutrients are used inside the cells for energy production and synthesis of biomolecules, which allows cells to attain the critical size required to start a new cell cycle, hence to proliferate. However, growth is not the only option; indeed, according to the external conditions, quiescence, filamentous development and sporulation may also occur (**Figure 1.1**).

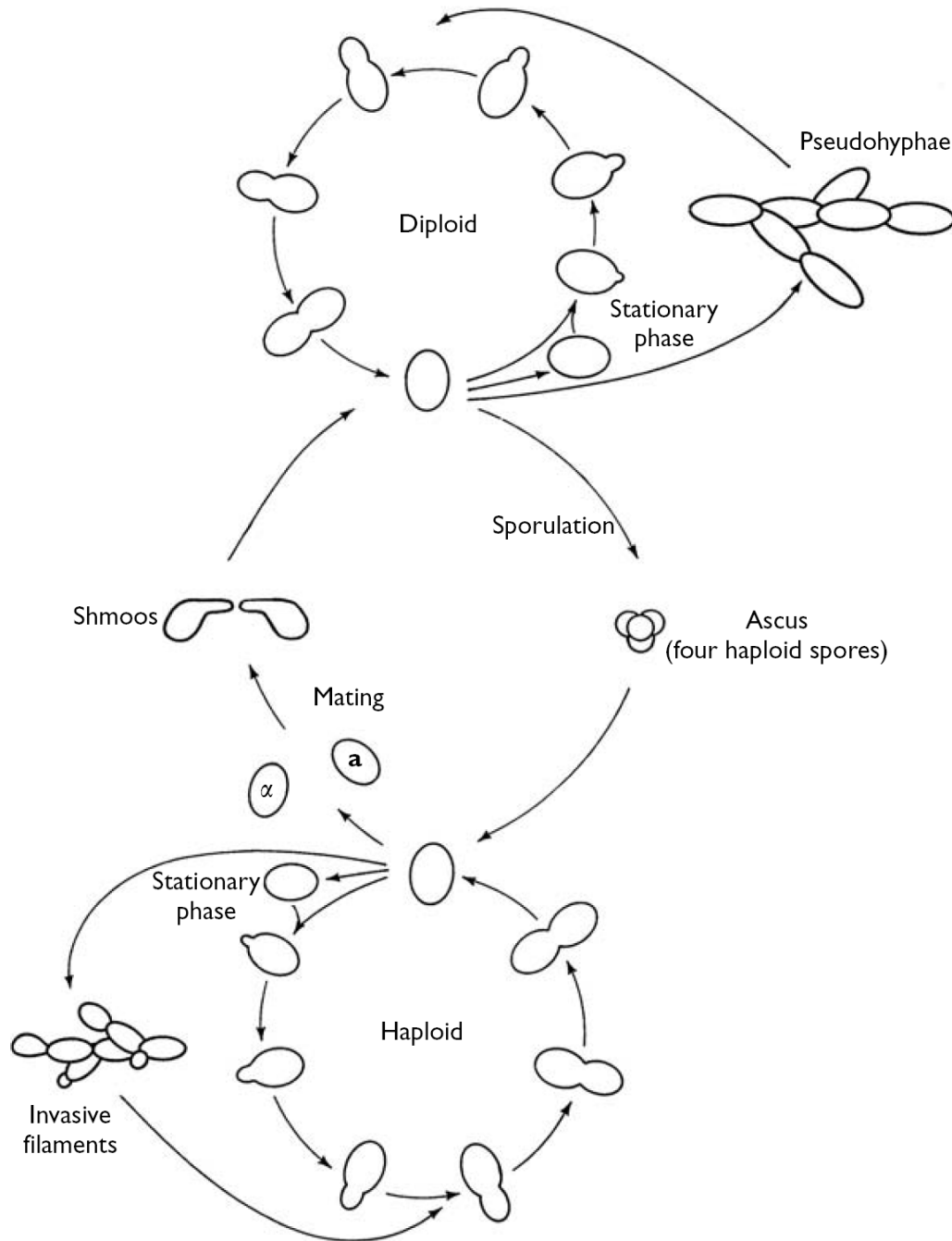


Figure 1.1: The life cycle of *Saccharomyces cerevisiae* - Figure taken from Dickinson & Schweizer (2004).

The networks that detect and signal the nature and amount of nutrients, which involve key regulators such as PKA, TORC1 and Snf1, are complex and not well understood yet and will not be described in this thesis; the reader is referred to a recent review for a detailed description (Zaman *et al.*, 2008). Instead, in order to introduce Chapter 3 and 4, an overview of *S. cerevisiae* metabolism, cell cycle and modes of cultivations is hereby presented. Moreover, DNA microarray will be described in this chapter, since this technology has been extensively used during the experimental work of this thesis.

1.2.1 Metabolism overview

Central carbon metabolism

Glucose is the main fuel molecule for cells and its catabolism provides free energy in the form of adenosine triphosphate (ATP). Extracellular glucose is sensed at the plasma membrane by the proteins Snf3 and Rgt2 (Santangelo, 2006). Glucose causes these sensors to generate an intracellular signal that alleviates the repression applied by the transcription factor Rtg1 and induces expression of several *HXT* genes encoding hexose transporters. These glucose transporters belong to the major facilitator superfamily (MFS) of transporters and act by energy-independent facilitated diffusion, with glucose moving down a concentration gradient (Ozcan & Johnston, 1999).

The main glucose metabolic pathway is glycolysis (or Embden-Meyerhof-Parnas pathway, EMP), that converts glucose to pyruvate with concomitant production of energy (ATP) and reductive equivalents (NADH). Glycolysis is connected to another important pathway, the pentose phosphate pathway, that provides NADPH and pentose sugars for anabolic reactions. *S. cerevisiae* is a facultative anaerobe and under aerobic condition, pyruvate is completely oxidized to CO₂ and H₂O through the TCA cycle and the mitochondrial electron transport chain (respiration), generating additional energy and reductive equivalents (NADH and FADH₂). In anaerobiosis, instead, pyruvate can be fermented to ethanol, with a lower net energy production respect to aerobic processes. Therefore, during fermentation the final electron acceptor is an organic molecule, instead of oxygen.

During exponential growth phase and chemostat cultivations (see section 1.2.3, page 11) at high dilution rates, the glycolytic flux is high and sugar fermentation occurs even under aerobic conditions. In this case, the metabolism is mixed (respiro-fermentative) and the end products are not only biomass and carbon dioxide, but also ethanol, glycerol and acetate. The aerobic alcohol fermentation is also referred to as the Crabtree effect. When cells are cultivated in carbon-limited chemostat cultivations at low dilution rates, the metabolism is fully respiratory.

S. cerevisiae can also grow on non-carbohydrate precursors like amino acids, glycerol and lactate, by converting these precursors to pyruvate and then to glucose through the gluconeogenesis pathway. Glycolysis is no longer operating towards pyruvate, but all of its enzymes, except the two catalyzing irreversible steps, now take part in the gluconeogenesis pathway, where the end-metabolite is glucose-6-phosphate, required for

building up the pool of sugar phosphates used for biosynthesis (e.g., glycogen, trehalose, nucleotides) and shunting through the pentose phosphate pathway. During growth on non-fermentable carbon sources (e.g., acetate, ethanol), there is another metabolic pathway operating in the cell: the glyoxylate shunt, that uses some of the reactions of the TCA cycle and two enzymes generally not present during respiro-fermentative growth (isocitrate lyase and malate synthase).

The presence of fermentable sugars affects the level of enzymes involved in the metabolism and physiology of *S. cerevisiae*; in particular, glucose affects the specific degradation pathways of other sugars (Carlson 1999). Glucose repression in *S. cerevisiae* involves complex mechanisms of glucose sensing and signal transduction that result in binding of repressor proteins to promoter regions of certain genes. Since glucose and fructose are the most favorable carbon sources, this mechanism switches off the uptake and metabolism of other sugars, when either of the two is present in the environment. Even though it is a well-studied regulatory phenomenon (Santangelo, 2006), glucose repression is not fully understood, as there are many signaling pathways involved that can in some cases cross-talk. Since glucose repression also negatively regulates genes involved in respiration it may be partly responsible for the Crabtree effect, because repression of respiration together with overflow metabolism may promote the onset of ethanol production in aerobic chemostat cultivations.

As described above, under aerobic conditions, glucose is mainly metabolized through the respiro-fermentative pathway leading to production of ethanol. Then, upon depletion of glucose in the surrounding media, *S. cerevisiae* changes metabolism and the ethanol can now be metabolized using the TCA cycle, the glyoxylate cycle and the mitochondrial electron transport chain: this is referred to as the diauxic shift (Brauer *et al.*, 2005; DeRisi *et al.*, 1997). This change in metabolism provides a competitive advantage for *S. cerevisiae* because few microorganisms are able to metabolize ethanol.

Central nitrogen metabolism

S. cerevisiae can use a broad range of nitrogen sources, but ammonium, glutamine and asparagine are able to support growth at higher growth rates than other amino acids and urea. The main nitrogen sensing transport systems are: Gpr1-Gpa2 G-protein coupled receptor, the specific ammonia transporter Mep2 and the amino acid permease (Gagiano *et al.*, 2002).

Similar to carbon metabolism, there are regulatory mechanisms that control expression of genes involved in nitrogen assimilation and utilization on a global level, such as the nitrogen catabolite repression (NCR) that prevents the usage of alternative nitrogen-sources when ammonium is present (Cooper, 2002). Although *S. cerevisiae* is able to use a wide range of nitrogen sources, it is necessary to convert them into either glutamate or glutamine, which act as starting points for the synthesis of nitrogen-containing macromolecules like amino acids and nucleotides. Glutamate and glutamine are synthesized by two cellular reactions that use free ammonium: glutamate dehydrogenase (converting α -ketoglutarate to glutamate) and glutamine synthetase (converting glutamate to glutamine). The alternative ammonium assimilation route consists of

glutamine synthase (GS) and glutamate synthase (GOGAT), the so-called GS-GOGAT system (Magasanik & Kaiser, 2002). After assimilation into glutamate and glutamine, ammonium can be transferred to other molecules through the activity of a class of enzymes called transaminases.

1.2.2 Cell cycle

Living cells multiply through a highly-regulated and orchestrated mechanism which implies the generation of a new daughter cell from an existing mother cell (Humphrey & Pearce, 2005; Nurse, 2000). In *S. cerevisiae*, division occurs by formation of a bud on the mother cell during the early stage of the cell cycle; this bud grows in size and receives all the components to sustain life as the cycle proceeds, until the new cell is released. In other yeast, like *Schizosaccharomyces pombe*, the division process implies growth by cell elongation at its ends and the appearance of a septum that separates evenly the two new cells.

Traditionally, the cell cycle has been divided into four phases which were defined according to morphological events visible in a microscope (Humphrey & Pearce, 2005; Nigg, 2001). It starts with the so-called gap one phase, or G_1 . During this phase, cells monitor external and internal environment and, if conditions are favorable, protein synthesis, organelle production and cell size increase take place in order to prepare cells for the subsequent DNA replication (synthesis phase, or S), where the DNA is exactly copied once. The replication machinery is recruited and a perfect copy of the genetic material is produced. Error may occur and cells have developed repair systems to ensure the stability of the genome (Branzei & Foiani, 2010; Kolodner *et al.*, 2002). In S phase, *S. cerevisiae* cells start to bud. The next phase, gap two or G_2 , aims at preparing the cell for the mitotic process, which occur during the M phase. This phase closes the cycle and ensures that the replicated genetic material is distributed equally between the mother and daughter cells, which are ready to start a new cycle, being again in G_1 phase. The M phase is divided into sub-phases: prophase, prometaphase, metaphase, anaphase, telophase and cytokinesis. Each of them is characterized by particular events describing the dynamic process involving the chromosomes and the mitotic spindle that leads to the physical separation of the two cells. In yeast the nuclear envelope does not break down as it happens in higher eukaryotes. During early G_2 phase the native and duplicated chromatin condense to form pairs of sister chromatids, that are held tightly together at the centromere in a X-like structure. In the meanwhile, the mitotic spindle, composed of microtubules originating from the distally located spindle pole bodies, has been formed and now binds to the kinetocore, a structure located inside the centromere. This binding makes the chromatids align along the equatorial plane of the nucleus and this step precedes the separation of the pairs of sister chromatids by the pulling action of the spindle microtubules. In this way, each set of chromatids is transported to opposite sides of the nuclear envelope and the G_2 phase is ended by cell separation (cytokinesis).

When a new cell is released, it will enter G_1 phase and grow until it reaches the critical cell size that is required to traverse the first checkpoint, called 'Start'. This

critical point is a growth and proliferation checkpoint in which the decision of executing DNA replication is made according to cell size and availability of nutrients (Johnston *et al.*, 1977; Jorgensen & Tyers, 2004; Jorgensen *et al.*, 2004; Zaman *et al.*, 2008). At the molecular level, commitment to a new cell cycle depends on the activation of the S-phase transcription factor complexes SBF and MBF, which are inhibited by Whi5 during G₁ check. Cdc28-G₁ cyclins (see below) phosphorylate Whi5 and alleviate its inhibition (Costanzo *et al.*, 2004; de Bruin *et al.*, 2004; Wagner *et al.*, 2009). G₁ cyclin levels appear to be the limiting-step in completing ‘Start’ (Jorgensen *et al.*, 2004; Schneider *et al.*, 2004).

Yeasts have developed complex mechanisms to survive and grow in nutrient limiting condition and to reset the metabolism when nutrients become abundant. An important distinction has to be made about the concepts of nutrient limitation and starvation. When one or more essential nutrients are limiting, cells will stall in G₁, while reprogramming the metabolism for usage of an alternative source; in this way cells are still capable of passing through the ‘Start’ checkpoint. When cells experience complete starvation for one or more nutrients, they will enter that quiescent, or stationary, phase of non-proliferation called G₀, where they can stay for long period of time before resuming growth as a consequence of improved environmental conditions. Quiescent cells present distinct morphological and biochemical differences respect to proliferating cells. It is important to note that nutrient sensing and growth regulation occur only during G₁ phase (Gray *et al.*, 2004; Werner-Washburne *et al.*, 1993; Winderickx *et al.*, 2003).

Tight control and regulation of cell cycle timing and execution is critical for cell viability and this regulation relies mostly on phosphorylation of key proteins by cyclin-dependent kinases (Cdk), which, together with cyclins, orchestrate the cycle transitions (Nigg, 2001). In *S. cerevisiae*, there exists only one Cdk (Cdc28) and its activity and specificity depend on the binding to different cyclins (Cln-Clb), whose levels oscillate during the cell cycle. Cdk-cyclin complexes ensure the unidirectional progression of the cell cycle by activating cyclins needed during the following phase and targeting for degradation proteins from the previous phase. Cdks are also positively regulated through phosphorylation by Cdk-activating kinases (Cak) and negatively regulated by Cdk inhibitors (Cki). Ckis and cyclins undergo ubiquitination and proteasome-mediated degradation, mediated by the SCF complex, during the G₁/S transition, and the anaphase-promoting complex (APF), during the metaphase/anaphase transition.

Therefore, the cell cycle is a delicate process, whose regulation relies on molecular events depending on the activity and levels of Cdks, cyclins, Caks and Ckis. Moreover, several checkpoints are present during the cell cycle to monitor DNA replication and repair of damaged DNA (see Chapter 2).

Given adequate nutrients, *S. cerevisiae* can grow as either a diploid or a haploid organism. One diploid cell is formed by the fusion (mating) of two haploid cells of opposite sex, or mating type (*a* and *alpha*). The mating is driven by the production of pheromones and specific cell-surface receptors. Diploid cells cannot mate, but, when growing in unfavorable conditions, they can produce spores through the process of meiosis. The mating type is determined by the presence of one of two alleles of a locus

called *MAT* (*MATa* or *MATalpha*), which encode *a* or *alpha* specific regulatory proteins determining transcription or repression of different set of genes. Diploid cells display a combination of these regulatory proteins. Even though for many laboratory strains the mating type is maintained during proliferation, some strains can switch repeatedly between *a* and *alpha* by replacement of the information present at the *MAT* locus through rearrangement with a flanking silent cassette encoding for the opposite mating type (Hidden *MAT* Left, *HML*, and Hidden *MAT* Right, *HMR*). Mating type switch is a gene conversion event initiated by the haploid-specific endonuclease HO active during the G₁ phase of the cell cycle. After cleavage by HO, exonucleases degrade the DNA at the *MAT* locus and the resulting gap is re-filled by copying the genetic information from the silent cassette of opposite mating type (*HMR* or *HML*).

1.2.3 Modes of cultivation

From an experimental point of view, cellular growth is generally monitored and measured according to two main modes of cultivation: batch and chemostat.

In batch cultures, saturating concentration of nutrients are supplied and the growth is quantified by measuring the number of living cells over time. During a yeast batch culture, distinct phases can be distinguished (**Figure 1.2**). When a rapidly fermentable sugar (e.g., glucose or fructose) is used as carbon source, yeast cells experience an initial adaptation phase of non-division called lag phase, followed by an exponential fermentative growth phase that slows down when sugar concentration becomes limiting. At this point, a second lag phase allows cells to reset the metabolism (diauxic shift) to respiration and resume growth using the products of the previous fermentation as carbon source (e.g., ethanol or acetate). When one or more essential nutrients become exhausted or a toxic compound accumulates, cells enter the stationary phase with the aim of surviving the starvation period. Eventually, there is a decline in living cell number during the death phase, often following an exponential decrease.

Growth during exponential phase is described by the following equation:

$$X(t) = X_0 \cdot e^{\mu t} \quad (1.1)$$

where, X is the number of cells, X_0 is the initial number of cells, μ is the specific growth rate and t is time. X can be the optical density (OD) of the culture and the parameter μ [h⁻¹] is estimated from an exponential fit of OD measurements. Growth in batch cultivations follows Monod kinetics:

$$\mu = \frac{\mu_{max} \cdot s}{s + K_s} \quad (1.2)$$

where s is the concentration of the limiting substrate, μ_{max} is the maximum growth rate and K_s is the substrate concentration value at which μ is equal to $\mu_{max}/2$. Batch cultivations starts with a value of s much higher than K_s , therefore μ approaches μ_{max} and it is constant for most of the fermentation period. When s drops into the range of K_s , μ decreases until complete stop when s becomes close to zero. Not only the growth

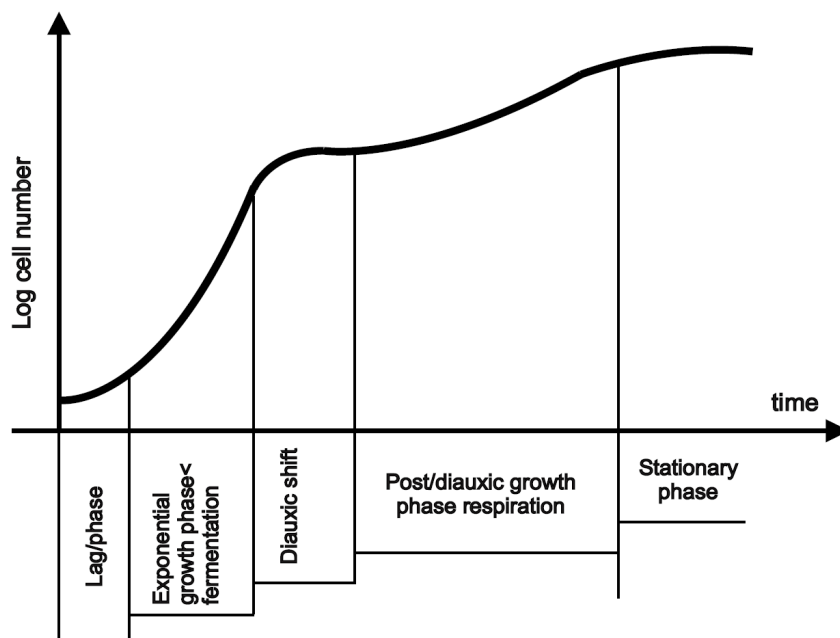


Figure 1.2: Typical phases of a fermentative yeast batch cultivation - Figure taken from Winderickx *et al.* (2003).

rate is changing, but also concentrations of nutrients and products as growth proceeds, and limited gas transfer and diffusion rate can cause temporal concentration gradients. All of these aspects are undesirable in quantitative studies.

The chemostat (Monod, 1950; Novick & Szilard, 1950) is a particular mode of operation for a vast majority of continuous stirred tank reactors (CSTR). In the chemostat, a specific growth rate is maintained by limiting the amount of an essential nutrient during the cultivation. A typical chemostat is composed of (1) two feed ports, one for liquid feed and one for gaseous feed, (2) a liquid reaction medium where the substrates are converted into biomass and metabolic products and (3) two exit ports, one for liquid effluent containing cells and products and one for exhaust gas, e.g. carbon dioxide, produced during the reaction. When the liquid feed rate v , the gas feed rate v_g , the reactor volume V and the concentrations of substrates in the liquid and gas feed streams are independent of time, that is all the input variables are constant, all the output variables, e.g. biomass and product concentrations, are expected to be constant. In these conditions, the reactor is said to operate in steady-state continuous mode. Therefore, the chemostat allows microbial cultivations under constant physico-chemical conditions.

As mentioned above, the variables v (liquid feed rate) and V (reactor volume) are important parameters and the ratio between them gives another important input variable, the dilution rate D , that is measured in units of reciprocal time, usually h^{-1} ,

and is the reciprocal of the holding time.

$$D = \frac{v}{V} \quad (1.3)$$

The value of D can be maintained as a constant by controlling either input variables or output variables. In a chemostat, a constant D can be attained by controlling the v and V values. When a steady-state is reached inside the bioreactor, it is assumed that the biomass and product concentrations in the effluent are identical to those found at any point in the reactor. It always takes time to reach a steady-state and usually five residence times ($5 \cdot D^{-1}$) are required. At steady-state, the dilution rate is equal to the specific growth rate μ . This offers the unique possibility to study metabolism and its regulation at a fixed and constant specific growth rate lower than μ_{max} under tightly defined nutritional conditions. Moreover, the very low residual concentration of the growth-limiting nutrient alleviates effects of catabolite repression and substrate toxicity.

Given the tight control of individual cultivation parameters, chemostat enables the analysis of combinatorial effect of experimental factors in order to investigate their hierarchical importance (Fazio *et al.*, 2008; Gutteridge *et al.*, 2010; Knijnenburg *et al.*, 2007; Tai *et al.*, 2005). Moreover, the reproducibility of chemostat cultivations allows inter-laboratory comparisons, provided that experimental procedures are standardized (see Chapter 4). This aspect is an important advantage for microarray studies (Bammeler *et al.*, 2005; Daran-Lapujade *et al.*, 2009; Hayes *et al.*, 2002; Piper *et al.*, 2002).

In both chemostat and batch cultivations, the cellular doubling time (T_d) is inversely proportional to the specific growth rate ($T_d = \ln(2)/\mu$) and this equation provides a direct link between growth and cell cycle.

1.2.4 Growth and gene expression

The vast majority of gene expression studies in *S. cerevisiae* has been performed in batch cultivations. However, the changing specific growth rate can interfere with the interpretation of the gene expression response to genetic mutations or perturbed environment. Chemostat cultivations allow the elimination of growth rate as a variable in comparative studies of strains or growth conditions. Nutrient availability exerts growth rate control by regulating ribosome biogenesis, through PKA and TORC1 signaling pathways, and, even before the advent of microarray technology (see section 1.3), a strong positive correlation was found between expression of ribosomal protein (RP) genes and specific growth rate (Kraakman *et al.*, 1993; Mager & Planta, 1991). From an energetic point of view, ribosome biogenesis is the most expensive cellular process (Warner, 1999) and protein translation by the ribosome provides a direct link between biomass increase (growth rate) and ribosome synthesis (Nomura, 1999).

Chemostat-based microarray studies have allowed a deeper understanding of the growth rate-dependent genes and it goes beyond the simple regulation of RP genes (Brauer *et al.*, 2008; Castrillo *et al.*, 2007; Fazio *et al.*, 2008; Gutteridge *et al.*, 2010; Regenber *et al.*, 2006). For example, the transcriptional response to various chemical

and physical stress conditions in batch cultivation, defined as the environmental stress response (ESR) (Gasch & Werner-Washburne, 2002; Gasch *et al.*, 2000), overlaps with the growth rate-dependent transcriptional response, because environmental stresses generally induce a reduction of μ_{max} . Indeed, nutrient availability, growth rate and stress response are interconnected, but the causal connection is not completely clear yet. Coordination of gene expression response and growth rate could be explained through either a feedback mechanism, where growth rate is sensed and feeds-back to modulate gene expression, or a feed-forward mechanism, where gene expression changes as a consequence of environmental parameters, independently of the actual growth rate (Levy & Barkai, 2009). Experimental evidences have supported the second model by showing that gene expression precedes growth rate changes and results solely from the perception of the external nutritional status (Levy *et al.*, 2007; Ronen & Botstein, 2006; Zaman *et al.*, 2009). More recently, a computational tool has been developed for the prediction of growth rate based on gene expression (Airoldi *et al.*, 2009). A deeper investigation of the transcriptional regulation of growth rate-dependent genes will be presented in Chapter 3.

1.3 The DNA microarray technology

The development of high-throughput technologies allowing simultaneous analysis of mRNA of virtually all genes in a selected organism has been a major advancement in molecular biology. Earlier methods for transcription analysis such as Northern blots allowed analysis of only a few genes at a time. Several methods for parallel or high-throughput analysis of gene expression or detection of differentially expressed genes have been tested and reported (Burns *et al.*, 1994; Liang & Pardee, 1992; Okubo *et al.*, 1992; Prashar & Weissman, 1996; Velculescu *et al.*, 1995; Zhao *et al.*, 1995). However, to date, the most successful methods for genome-wide transcription analysis are those applying DNA arrays. Spotted cDNA arrays, where cDNA, oligonucleotide or PCR products are spotted or synthesized on a glass slide, and high density oligonucleotide DNA arrays for whole genome transcription analysis (initially manufactured by Affymetrix) were introduced about fifteen years ago (Fodor *et al.*, 1993; Lockhart *et al.*, 1996; Schena *et al.*, 1995). The publication in 1996 of the genome sequence of *S. cerevisiae* (Goffeau *et al.*, 1996) gave a boost to the fabrication of whole-genome DNA arrays for this yeast and the first genome-wide transcription studies were possible (DeRisi *et al.*, 1997; Wodicka *et al.*, 1997). In the following years, the technology developed and emerged in more and more laboratories. Today DNA arrays are widely used and more genomes have been sequenced leading to transcription analysis of many different organisms. The use of DNA arrays for expression studies is based on the fundamental process of hybridization. DNA arrays are simply a surface (e.g. glass) bearing large sets of immobilized nucleic acid probe sequences at particular locations available for hybridization. RNA is extracted from a biological sample and labeled through several preparation steps. The mixture of labeled RNA is then applied to the DNA array under controlled conditions for optimal hybridization with the complementary immobilized

probes on the array surface. Then, fluorescent labeling is used to locate and quantify the binding of applied target sequences to their complementary sequences on the array by imaging with a laser scanner. The resulting scan can be processed to calculate an intensity value for each gene represented on the array using appropriate computer software.

Spotted DNA arrays are produced by using a robot spotter to apply small quantities of a probe to designated destinations on a coated substrate such as a glass slide. Usually, the arrays contain only one probe per transcript located in separated areas of the surface. Affymetrix technology differs from the others because 25 base-long oligonucleotide probes are synthesised directly on the array surface using a technique based on photolithography (McGall & Christians, 2002; Pease *et al.*, 1994). Other manufactures have emerged during the years (NimbleGen, Agilent and Illumina), using different technologies and different solutions with respect to number of features per array and probe length.

With respect to DNA microarray data analysis, several softwares have been developed and they are either commercially or freely available. One example of the latter is the statistical programming language R, which forms the basis for the Bioconductor project (Gentleman *et al.*, 2004, 2005). Bioconductor is an open source platform for bioinformatics and has become extensively used in microarray analysis because of the vast array of freely available tools ('packages'), e.g. *affy* (Gautier *et al.*, 2004) and *limma* (Smyth, 2004). DNA microarray analysis starts with the acquisition of intensity values from the scanned arrays, which, as first step, have to be preprocessed in order to eliminate systematic errors due to sample preparation, staining and scanning and make the comparison of expression levels of different arrays possible (Zakharkin *et al.*, 2005). This noise reduction is necessary before statistical testing can be applied to infer biological significance of the data. The preprocessing involves three main steps: (1) background correction, (2) normalization and (3) gene expression index calculation. Many different approaches have been proposed (Irizarry *et al.*, 2003; Quackenbush, 2002; Ritchie *et al.*, 2007; Wu & Irizarry, 2004) and one of the most used complete preprocessing algorithm is RMA (robust multi-array average) (Irizarry *et al.*, 2003).

In order to extract biological significance from the preprocessed data, experimental replicates and statistical tests are crucial (Churchill, 2002; Cui & Churchill, 2003; Storey & Tibshirani, 2003; Yang & Speed, 2002). Biological replicates are a necessity in order to carry out statistical tests and to confirm the significance of the observations. The most common applied statistical tests are the Student's *t*-test and *n*-way analysis of variance (ANOVA), when dealing with factorial designs. However, the assumptions of these tests (e.g., the normal distribution of the compared populations) are not always met and, therefore, different approaches are often considered (non-parametric tests and Bayesian statistics). When performing statistical tests on thousands gene expression values, it is very important to correct for multiple testing because running multiple tests increases the number of false positives proportional to the number of tests run. For instance, if a significance level of 0.05 is chosen, then about five false positives are expected for every one hundred genes tested (5% probability of encountering a

false positive). Therefore, it is necessary to apply corrections for multiple testing in order to redefine a new significance level. The Bonferroni correction has been widely used, but its stringency has led to the development of other methods, such as the False Discovery Rate (FDR) method (Benjamini & Hochberg, 1995) that aims at minimizing the number of false positives within the selected genes.

The classical applications of DNA microarrays have been the identification of significantly differentially expressed genes among two or more conditions (e.g., wild type versus knock-out strains), the understanding of (dis)similarities of gene expression levels among all samples (e.g., principal component analysis and clustering methods) and the classification of unknown samples according to gene expression profiles of predefined sample groups (Leung & Cavalieri, 2003). The first microarrays contained probes mainly for known or predicted open reading frames (ORFs), i.e. that part of the genome that is translated into proteins. With respect to *S. cerevisiae*, important studies were carried out about key cellular processes during the first years of the microarray era (Cho *et al.*, 1998; Chu *et al.*, 1998; DeRisi *et al.*, 1997; Spellman *et al.*, 1998). Later, new microarray designs have emerged to meet the experimenters' desire to investigate new aspects of the DNA biology. Tiling arrays, for example, probe the entire genome, without considering the position of the genomic features, and it allows the discovery of new transcribed regions ignored by the classical gene expression microarrays. In recent years, tiling arrays have been successfully applied to gain new insight into *S. cerevisiae* transcriptional programs (David *et al.*, 2006; Gagneur *et al.*, 2009; Granovskaia *et al.*, 2010; Gresham *et al.*, 2006; Huber *et al.*, 2006; Juneau *et al.*, 2007; Xu *et al.*, 2009; Zhang *et al.*, 2007). Moreover, microarrays have been used in studies of protein-DNA binding (chromatin immunoprecipitation and DNA microarray, ChIP-chip) (Harbison *et al.*, 2004; Iyer *et al.*, 2001; Lee *et al.*, 2002; Ren *et al.*, 2000), DNA methylation, genotyping and copy number variations. In this thesis, gene expression arrays have been used in the works included in Chapter 3 and 4, while Chapter 5 presents an application of tiling arrays.

Chapter 2

Saccharomyces cerevisiae DNA Damage Response

The first part of this Chapter will describe general concepts about *S. cerevisiae* DNA damage response (DDR), such as damage sensing, repair and cell cycle checkpoint activation. Later, S-phase checkpoint will be treated in more details, being important for the interpretation of Chapter 5 and 6. Finally, genome-wide and systems biology approaches to DDR will be discussed.

2.1 An overview

Genomes are constantly under the threat of exogenous and endogenous sources of DNA damage that might cause genomic instability (Zhou & Elledge, 2000). Therefore, sophisticated mechanisms to sense and react to these insults have been developed by cells in order to ensure viability and faithful transmission of genetic information (Friedberg *et al.*, 2006). To give an idea of the extent of the threat, in human cells thousands of purine residues are lost spontaneously every day (Nakamura *et al.*, 1998) and the intracellular metabolite S-adenosylmethionine can methylate adenine residues more than a thousand times per day (Rydberg & Lindahl, 1982). Moreover, the essential process of DNA replication is not error-free. Inadequate response to DNA damage or defects in DNA repair have been linked to ageing and disease states (Bartkova *et al.*, 2005; Bell *et al.*, 1999; Gayther *et al.*, 1998; Gorgoulis *et al.*, 2005; Kastan & Bartek, 2004; Lavin & Shiloh, 1997; Setlow *et al.*, 1969; Vogelstein & Kinzler, 2004). Which are the main sources of damage? As mentioned before, they can be grouped into two main categories:

- Endogenous DNA damage. It includes spontaneous alterations in DNA base chemistry caused by reactions of hydrolysis, oxidation and methylation (Lindahl & Barnes, 2000). Incorporation of incorrect bases during DNA replication, due to poor or defective proofreading function of the polymerase, can be also included in this category of damage.

- Exogenous, or environmental, DNA damage. It is caused by external chemical and physical agents, such as ionizing and UV radiation, non-polar compounds, alkylating, cross-linking, chemical and enzymatic agents

Cells possess many defense mechanisms to respond to DNA damage. The repair of the damaged DNA can occur according to different mechanisms. However, not all DNA damage is repaired, sometimes it is tolerated in order to enhance cellular survival and avoid potentially lethal consequences of DNA replication arrest (see section 2.3).

DNA repair and tolerance efficiency is facilitated by the so-called cell cycle checkpoints which delay or arrest the cell cycle in order to promote repair and tolerance processes in response to DNA damage. These checkpoints rely on complex molecular events and will be treated in more details in section 2.2. Finally, another biological response to DNA damage adopted by multicellular organism is the programmed cell death, or apoptosis, which operate when the level of mutation and genomic instability leads to unwanted phenotypes or unrecoverable genomic states.

The responses to DNA damaged have to be interpreted in the dynamic context of the cell because they intertwine with other processes like metabolism, replication, transcription and recombination, which determine the probability and efficiency of the response. The DNA-damage response is highly conserved among eukaryotic organisms and initially most of the research was conducted in fission and budding yeast (Elledge, 1996; Kolodner *et al.*, 2002). Many homologs of the yeast DDR components exist in mammalian cells (**Table 2.1**), even though the response is more elaborated because of the p53-dependent pathways (Harper & Elledge, 2007). This Chapter will focus on the DDR in the budding yeast *Saccharomyces cerevisiae*.

2.2 Cell cycle checkpoints

DNA damage checkpoints are defined as specific delays in cell cycle transitions due to the presence of damaged DNA, which typically has to be repaired before the cell cycle can restart. The concept of DNA damage checkpoint was introduced with the discovery of the G₂/M arrest after X-ray irradiation of *S. cerevisiae* cells, that required the *RAD9* gene (Weinert & Hartwell, 1988). In *S. cerevisiae* the main checkpoints occur at the G₁/S transition, during the S-phase and at the G₂/M transition (Elledge, 1996; Sanchez *et al.*, 1999; Sidorova & Breeden, 1997; Siede *et al.*, 1993; Weinert & Hartwell, 1988). There are two types of S-phase checkpoint: the DNA replication checkpoint, which responds to replication stress (Santocanale & Diffley, 1998), and the intra-S checkpoint, which responds to DNA damage (Paulovich *et al.*, 1997). Checkpoints work through a signal transduction process, which includes sensors, mediators, amplifiers, transmitters and downstream effectors, but this view is a simplification because some proteins, for example, can act as both sensors and effectors. Therefore, the DNA damage checkpoints should rather be seen as complex regulatory networks with feedback loops and threshold responses (Branzei & Foiani, 2005, 2009; Harrison & Haber, 2006; Kastan & Bartek, 2004; Nyberg *et al.*, 2002; Putnam *et al.*, 2009; Segurado & Tercero, 2009; Zhou & Elledge, 2000).

Table 2.1: Conserved *S. cerevisiae* and *H. sapiens* checkpoint proteins and their functions (Friedel *et al.*, 2009).

<i>S. cerevisiae</i>	<i>H. sapiens</i>	Function
RFA-ssDNA	RPA-ssDNA	Signal
Rad24-RFC	RAD17-RFC	Sensor (RFC-like complex, clamp loader)
Ddc1-Rad17-Mec3	RAD9-RAD1-HUS1	Sensor (9-1-1, DNA damage checkpoint clamp)
Dpb11	TOPBP1	Sensor (required for ATR activation)
Mre11-Rad50-Xrs2	MRE11-RAD50-NBS1	Sensor (MRX/MRN)
Mec1/Lcd1	ATR/ATRIP	Sensor (signaling kinase)
Tel1	ATM	Sensor (signaling kinase)
Pol ϵ	POL ϵ	Fork associated (leading strand polymerase)
Sgs1	BLM, WRN	Fork associated (RecQ helicase)
Tof1/Csm3	TIM/TIPIN	Fork associated
Ino80	INO80	Fork associated (chromatin remodeler)
Mrc1	Claspin	Mediator
Rad9	BRCA1/53BP1	Mediator
Rad53	CHK2	Effector (signaling kinase)
Chk1	CHK1	Effector (signaling kinase)

The core machinery of DNA damage checkpoints is the phosphoinositol-3-kinase-related kinase (PIKK) family members, which, in *S. cerevisiae*, are Mec1 (mitosis entry checkpoint protein 1), the homolog of human ataxia telangiectasia and Rad3-related (ATR), and Tel1 (telomere length regulation protein 1), the homolog of human ataxia telangiectasia mutated (ATM) (**Figure 2.1**).

These two kinases do not directly recognize the damaged DNA, but they are loaded onto the damaged sites by complexes recognizing the DNA damage and intermediates of repair processes. In fact, stretches of single-stranded DNA (ssDNA) are formed as a consequence of damaged DNA resection operated by repair process, and uncoupling of helicase and replicative polymerase during replication fork stall or collapse (Byun *et al.*, 2005; Sogo *et al.*, 2002). Mec1 is recruited by Lcd1, the homolog of human ATRIP, which recognizes ssDNA bound by replication protein A (RPA) (Zou & Elledge, 2003); Mec1 activation is dependent on the proliferating cell nuclear antigen (PCNA)-like checkpoint clamp Ddc1-Mec3-Rad17, loaded onto ssDNA by Rad24 (Majka & Burgers, 2007; Majka *et al.*, 2006), and on Dpb11 (Mordes *et al.*, 2008; Navadgi-Patil & Burgers, 2009). Intriguingly, co-localization of PCNA and Mec1/Lcd1 complexes is sufficient to activate the checkpoint even in the absence of damage (Bonilla *et al.*, 2008). In contrast, Tel1 binds the DNA end-binding Mre11-Rad50-Xrs2 (MRX) complex (Nakada *et al.*, 2003).

Mec1 and Tel1 do not share complete redundancy. Tel1 plays an important role in telomere length maintenance and responds mainly to double strand breaks (DSBs) during G₁ (Ritchie *et al.*, 1999); Mec1 is more important during S and G₂ phases and responds to a wide range of DNA damage (replication stress, base adducts, UV-

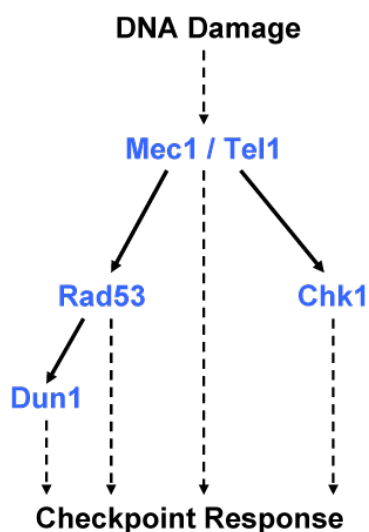


Figure 2.1: Overview of the kinase cascade activating the cell cycle checkpoints upon DNA damage - The kinases have both redundant and specific effects depending on the type of DNA damage. Solid lines indicate phosphorylation events, while dotted lines indicate regulation.

induced nucleotide damage and DSBs) (Cimprich & Cortez, 2008; Ira *et al.*, 2004). In some cases Tel1 can induce formation of ssDNA and Mec1 activation (Grenon *et al.*, 2006; Mantiero *et al.*, 2007). Both Mec1 and Tel1 phosphorylate several downstream substrates preferentially on serine-glutamine or threonine-glutamine (SQ/TQ) motifs, often found in SQ/TQ cluster domains (SCDs) (Kim *et al.*, 1999; Traven & Heierhorst, 2005). Checkpoint proteins also often contain modular phosphoprotein-binding domain, such as FHA (forkhead associated) and BRCT (BRCA1 C-terminal) (Mohammad & Yaffe, 2009). Nevertheless, not all the substrates of Mec1 and Tel1 are known (Chen *et al.*, 2010; Smolka *et al.*, 2007). Two important targets are Chk1 and Rad53 kinases, whose activations depend on the adaptor proteins Mrc1 and Rad9 (Melo & Toczyski, 2002). Mrc1, a component of the replication fork that binds the polymerase epsilon, participate in Mec1-dependent activation of Rad53 in response to replication stress (Alcasabas *et al.*, 2001), while Rad9 is responsible for Rad53 and Chk1 activation in response to DNA damage (Gilbert *et al.*, 2001; Sanchez *et al.*, 1999).

One of the most important target of Rad53 is the kinase Dun1, which gets activated by binding to phosphorylated Rad53 through its own FHA domain (Bashkirov *et al.*, 2003; Chen *et al.*, 2007; Lee *et al.*, 2008a). The main function of Dun1 is to regulate the nucleotide pool inside the cells by activating the transcription of ribonucleotide reductase (Rnr) subunit genes and by targeting for degradation the Rnr inhibitor Sml1 (suppressor of mec1 lethality 1) (Zhao & Rothstein, 2002) (see page 28). Moreover, Dun1 is important to prevent gross chromosomal rearrangements (Myung *et al.*, 2001).

This kinase cascade activated upon damage to DNA aims at propagating the signal generated at the chromatin level to diffusible protein kinases, which facilitate the repair

of the damage. The pathway is more complex than the provided description, since Mec1, Tel1, Rad53 and Chk1 have many targets, and also because protein phosphatases have a prominent role in the DNA damage response, as it will be described in Chapter 6 (Bakkenist & Kastan, 2004).

2.3 DNA repair

Checkpoint response helps the cell in tuning the repair mechanism by integrating the specificity of the lesion with the cell cycle position. Generally checkpoint activation leads to cell cycle arrest, but sometimes the lesion is repaired rapidly and no arrest is needed; sometimes cells wait to be in a specific cell cycle phase before taking care of the damage repair (Branzei & Foiani, 2008; Lazzaro *et al.*, 2009). Sources of DNA damage have been previously introduced and now the most common types of DNA lesions and repair mechanisms will be briefly described. The main typologies of damage to DNA are:

Double strand breaks These are cuts to one or both strands of a chromosome (Harrison & Haber, 2006). The most common DSB-inducing agent is ionizing radiation (IR) and the damage can be repaired by two mechanisms: non-homologous end-to-end joining (NHEJ) and homologous recombination (HR). The first one implies direct ligation of the halves of the broken molecule without requirement for homology, while HR requires homology between the region surrounding the lesion and a donor sequence. DSBs are recognized by the MRX complex, thus leading to activation of Tel1, and, if the break is not rapidly rejoined, the response to DSBs produces DNA intermediates containing ssDNA.

UV-induced lesions Ultraviolet light can induce the production of photoproducts (e.g., cyclobutane pyrimidine dimers) that can be removed by direct reversal, activity of photolyase or nucleotide excision repair (NER) mechanisms. The latter generates 30-nucleotide ssDNA gap in the region containing the lesion, which is then refilled by polymerases. When the UV dosage is low, NER is sufficient to repair the damage, while, at high UV dosage, ssDNA fragments, produced by the action of helicase/nuclease, trigger the activation of checkpoint.

Base alterations Upon exposure to endogenous or exogenous sources of damage, the formation of alkylated, deaminated and oxidized bases is repaired by base excision repair (BER). Some studies suggest that BER is linked to checkpoint activation through interaction with the PCNA-related checkpoint clamp (Chang & Lu, 2005).

Mispairing When DNA replication errors escape the proofreading activity of polymerases, base mismatches might occur and cells respond through mismatch repair (MMR) (Li, 2008). Moreover, base mispairing may be caused by endogenous and exogenous agents and reactive species; for example, MMR has been shown to be involved in repair of damage by SN₁ methylating agents (Stojic *et al.*, 2004).

MMR only targets the newly synthesized DNA strand and, if the lesion is on the parental strand, the futile repair attempt will produce ssDNA gap, sufficient to activate checkpoint response (Mojas *et al.*, 2007).

When the DNA repair mechanisms do not manage to efficiently repair the lesion, cells enter S-phase with damaged DNA and this causes stalling of the replication fork in proximity of the lesion, because polymerase cannot act at this site. In order to complete replication, cells can utilize a DNA-damage tolerance pathway or post-replicative repair (PRR) in order to give repair mechanisms the opportunity to act in subsequent cell cycles (Branzei & Foiani, 2007, 2008; Friedberg, 2005). Therefore, damage tolerance allows cells to continue DNA replication beyond the damaged sites. PRR can be error-free when the damage is efficiently repaired by template switch, a recombination-like mechanism that switches to the undamaged strand as template. Sometimes damage tolerance can lead to incorporation of mutations (mutagenesis), as it happens during the process of translesion DNA synthesis (TLS), which employs polymerase with low fidelity and are prone to misincorporation of nucleotides. This mechanism is important from an evolutionary point of view, since it is source of spontaneous mutations and new genetic information.

In summary, DNA repair mechanism are linked to the activation of checkpoint, presumably through a common intermediate (ssDNA), and the checkpoint response ensure modulation of repair and other signal generation (**Figure 2.2**), as described in the next section. However, it is believed that most of DNA lesions do not trigger checkpoint activation and are repaired by constitutively active repair pathways.

2.4 Checkpoint targets

Many checkpoint targets have been identified by traditional and mass spectrometry based studies and often results do not overlap, probably because of methodological issues (Chen *et al.*, 2010; Matsuoka *et al.*, 2007; Smolka *et al.*, 2006, 2007; Stokes *et al.*, 2007). Verification of the targets is not always easy, because the phosphorylation can be cell-cycle or damage dependent, and some residues can be redundant. Nevertheless, it has been possible to classify checkpoint response targets in few categories (Putnam *et al.*, 2009).

Cell cycle targets The main role of checkpoint responses is to arrest the cell cycle upon damage by, for example, preventing progression through mitosis (Clémenson & Marsolier-Kergoat, 2006; Liang & Wang, 2007; Sanchez *et al.*, 1999) and interfering with the activation of origins of replication through phosphorylation of Dbf4 (Duncker & Brown, 2003).

DNA repair targets Unlike the transcriptional SOS response in bacteria (Sutton *et al.*, 2000), yeast cells possess post-transcriptional regulation of DNA repair protein; for example Rad53 phosphorylates Rad55, which has a role in HR (Herzberg *et al.*, 2006) and Dun1 phosphorylates Nej1 (NHEJ) (Ahnesorg & Jackson, 2007).

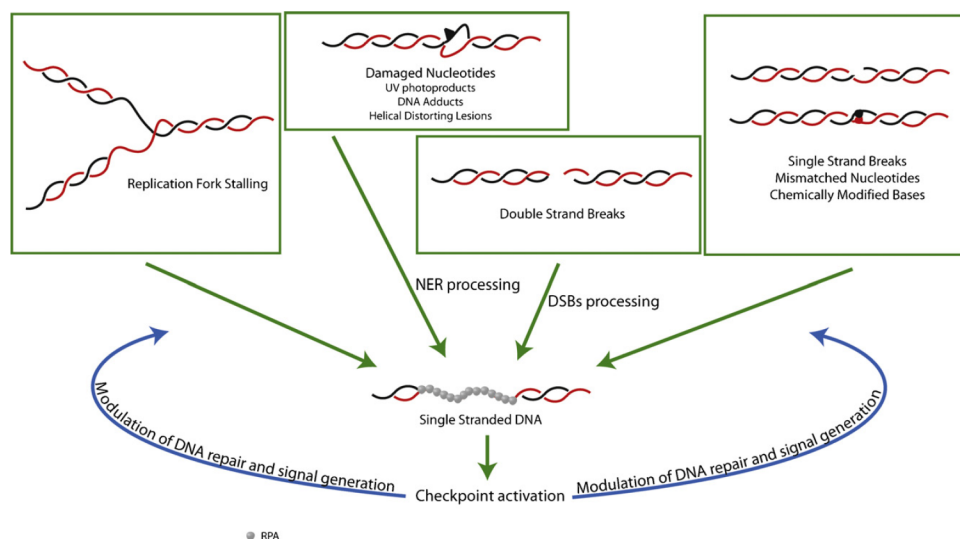


Figure 2.2: The role of DNA repair intermediates in DNA damage checkpoint activation - Different DNA lesions trigger different repair mechanisms that converge to a common signal transduction cascade. The most common repair intermediates are fragments of ssDNA covered with RPA. Upon activation, checkpoint modulate repair and regulate cell cycle progression. Figure taken from Lazzaro *et al.* (2009).

Chromatin targets The Serine 219 of the histone variant H2A is a known target of Mec1/Tel1 (Downs *et al.*, 2000; Redon *et al.*, 2003) and the phosphorylated form, γ -H2A, interacts with Rad9 (Hammett *et al.*, 2007) and chromatin remodelling complexes (e.g., Ino80 and Swr1), that promote repair (Fillingham *et al.*, 2006; Schleker *et al.*, 2009). Moreover, checkpoint activation promotes the maintenance of the acetylation of lysine 56 of histone H3 (Celic *et al.*, 2006).

Cytoplasmic targets Checkpoint response is not limited to the nucleus, but it also affects morphology and cytoplasmic targets, like septins (Enserink *et al.*, 2006; Smolka *et al.*, 2006).

Transcriptional targets Many genes are regulated in response to DNA damage and some examples are: ribonucleotide reductase subunit (*RNR*), 3-methyladenine DNA glycosylase (*MAG1*, involved in BER), photolyase (*PHR1*), NER (*RAD2*, *RAD7*, *RAD16*, *RAD23*, *SNM1*), HR (*RAD51*, *RAD54*), DNA-damage inducible (*DDI1*) and cyclin (*CLN1*, *CLN2*) genes. Many transcriptional studies have been performed to investigate the DNA damage response in yeast (reviewed by Fry *et al.* (2005) and Fu *et al.* (2008)) and one striking observation is that there is low agreement between transcriptionally regulated genes and damage resistance-conferring genes (Birrell *et al.*, 2002). This observation indicates that the yeast response is different than the SOS response in bacteria (Sutton *et al.*, 2000), where the repair factors, which are found in operons, are de-repressed under the action

of a master repressor (LexA). The DNA-damage response at the transcriptional level will be treated in greater details in Chapter 5.

2.5 S-phase checkpoint

Before describing the molecular events which determine the checkpoint response during S-phase, it is useful to briefly describe the DNA replication process and one of the damaging agents experimentally used to induce S-phase checkpoint response, namely methyl methanesulfonate (MMS).

2.5.1 DNA replication

Cells replicate DNA during S-phase through a complex and tightly regulated process which ensure that the genetic information is copied exactly once with high fidelity (**Figure 2.3A**). In yeast, replication starts at defined sequences called autonomously replicating sequences (ARS) (Brewer & Fangman, 1987; Raghuraman *et al.*, 2001), while, in bacteria, only one origin is present in the genome. Replication is a delicate process because origins of replication must fire only once every S-phase, and in a sufficient number to permit the timely replication of all the chromosomes.

The first step of DNA replication is the loading of the pre-replicative complex (pre-RC), consisting of the helicase complex Mcm2-7, the ORC complex, Cdc6 and Cdt1, onto one ARS (Diffley, 2004). Since the replication is a bi-directional process, it is likely that two Mcm2-7 complex are loaded at the same time in opposite orientations. Cdk activity makes sure that pre-RC can be formed exclusively during G₁, thus preventing re-replication (Nguyen *et al.*, 2001). Origins do not fire at the same time and require the interaction with several additional factors (e.g., Mcm10, Cdc45, GINS, Sld2, Sld3 and Dpb11) to form the pre-initiation complex, pre-IC. DNA unwinding generate ssDNA regions which are bound by RPA, and polymerase alpha (Pol α), which contains an intrinsic primase activity, is believed to prime both strands; later, polymerase epsilon (Pol ϵ) and delta (Pol δ) act on the leading and lagging strand, respectively. Addition of several other proteins leads to the constitution of the replication progression complex (RPC), which includes also the checkpoint mediators Dbp11, Tof1 and Mrc1. Besides controlling pre-RC formation, Cdk activates replication forks by phosphorylating several targets (e.g., Sld2-3).

2.5.2 Methyl methanesulfonate

Alkylating agents are electrophilic compounds that are attracted to the nucleophilic centres of DNA bases. They are used in the treatment of some types of cancer (Helleday *et al.*, 2008) and methyl methanesulfonate (MMS) has been used for many years as a DNA damaging agent in repair studies. It is an SN₂ type agent and mainly methylates guanine and adenine to N⁷-methylguanine (7MeG) and N³-methyladenine (3MeA) to induce base mispairing and replication block (Beranek, 1990; Pegg, 1984). N³-methyladenine is toxic and inhibits DNA synthesis *in vitro* (Larson *et al.*, 1985).

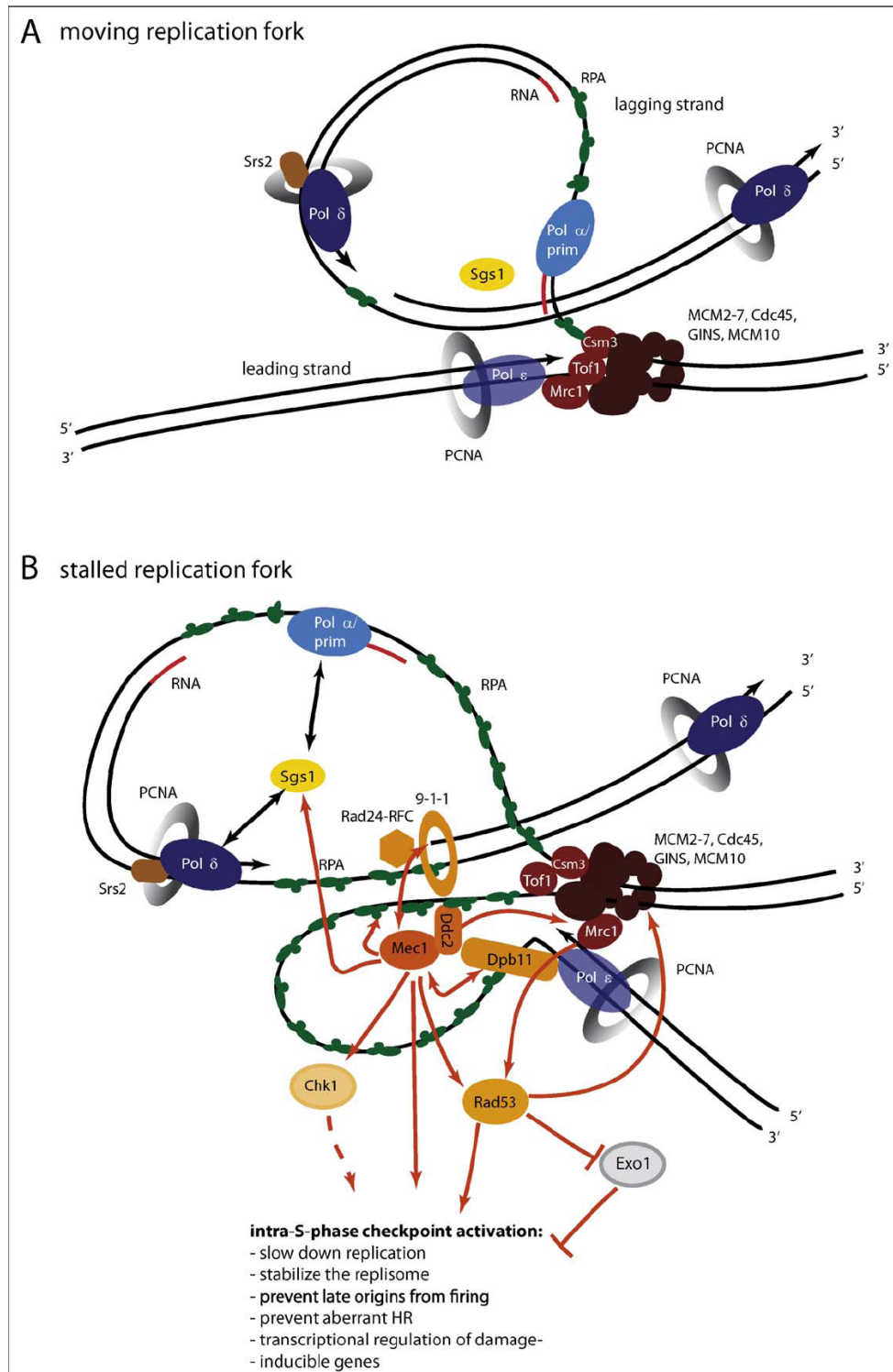


Figure 2.3: Model of a (A) moving and (B) stalled replication fork in *S. cerevisiae* - For a description, see section 2.5.1 and 2.5.3; arrows indicate regulation and interaction among proteins/complexes (Friedel *et al.*, 2009)

The MMS damage is mainly repaired by BER and DNA alkyltransferases (Lindahl & Wood, 1999). Mag1 3MeA DNA glycosylase has a central role in repair because it starts the BER pathway by cleaving the bond between the methylated base and the deoxyribose of the sugar phosphate backbone (Begley *et al.*, 2000; Wyatt *et al.*, 1999). MMS has been used to study S-phase checkpoint (Paulovich & Hartwell, 1995; Tercero & Diffley, 2001), even though the mechanisms that allow cells to replicate alkylated DNA are not well understood. When replication forks collide with alkylated bases, they stop their progression and the checkpoint response is activated to stabilize the forks. Homologous recombination and DNA damage tolerance pathways promote fork restart, once the lesion is repaired, and bypass of unrepaired region. Therefore, the coordinated action of checkpoint, recombination and DNA tolerance preserves the integrity of replicating chromosomes (Lopes *et al.*, 2006; Vázquez *et al.*, 2008; Xiao *et al.*, 1996).

2.5.3 Replication fork stalling and checkpoint activation

During S-phase, helicase (Mcm2-7) and polymerases (Pol ϵ and δ) have to work in a close association and uncoupling of their actions causes replication forks to slow down or stall. This event can be a consequence of natural barriers formed by DNA-binding proteins and DNA structures, active transcription sites, replication slow zones, replication terminal sites, agents blocking progression of the helicase (e.g., cross linking agents) or progression of the polymerase (Labib & Hodgson, 2007; Mirkin & Mirkin, 2007). Experimentally, replication fork pausing can be induced by hydroxyurea (HU) treatment, that limits the level of dNTP pools, or by intra-S damage with MMS (Tercero & Diffley, 2001). Stalled forks generally maintain association with the replisome, but forks will collapse if the replisome dissociates as a consequence of DSBs or prolonged pausing due to inefficient removal of protein complexes. When forks stall because of one of the above causes, generation of RPA-coated ssDNA is responsible for the activation of Mec1 (see **Figure 2.3B** and section 2.2) (Majka & Burgers, 2007; Mordes *et al.*, 2008).

The signal transduction is amplified by the intervention of mediators, like Rad9 and Mrc1. Rad9 is important for the Mec1-dependent activation of Rad53, a kinase that has a pivotal role in checkpoint response and whose phosphorylation is sufficient and necessary for checkpoint activation (Branzei & Foiani, 2006; Pellicoli & Foiani, 2005). Rad9 is thought to act as a scaffold to promote Rad53 autophosphorylation (Gilbert *et al.*, 2001). In response to replication stress (e.g., dNTP depletion by HU treatment), amplification of the signal depends mainly on Mrc1, which becomes phosphorylated in many residues by Mec1 (Alcasabas *et al.*, 2001; Osborn & Elledge, 2003). Mec1, through Rad9, also activates Rad53-paralogue Chk1 (Sanchez *et al.*, 1999), which cooperates with Rad53 in the G2/M arrest and may have a role during replication stress response when Rad53 is missing (Segurado & Diffley, 2008). Not always lesions or replication stress lead to the formation of ssDNA and checkpoint activation; in some situations, such as natural pause sites at rDNA replication fork barrier, the response is checkpoint-independent and requires additional factors (Mirkin & Mirkin, 2007; Tour-

riere & Pasero, 2007).

Checkpoint responses during S-phase produce multiple effects that help maintain cell viability. Forks unable to re-start replication after damage removal ('collapsed' forks) (Lopes *et al.*, 2001) and replication slow regions (fragile sites) (Cha & Kleckner, 2002) are prone to chromosome rearrangements and translocations, leading to genomic instability (Kolodner *et al.*, 2002; Nyberg *et al.*, 2002) and, in higher eukaryotes, cancer predisposition and development (Vogelstein & Kinzler, 2004). Wild type cells are able to resume replication after HU or MMS treatment, while *mec1* Δ and *rad53* Δ show lethality in the presence of replication stress, with *mec1* Δ being more sensitive to HU and MMS than *rad53* Δ (Desany *et al.*, 1998; Lopes *et al.*, 2001; Tercero & Diffley, 2001; Tercero *et al.*, 2003).

The main function of checkpoint kinases during replication stress is to promote repair and facilitate replication through the mechanisms described below (**Figure 2.4**) (Branzei & Foiani, 2005, 2009; Friedel *et al.*, 2009; Segurado & Tercero, 2009; Zegerman & Diffley, 2009).

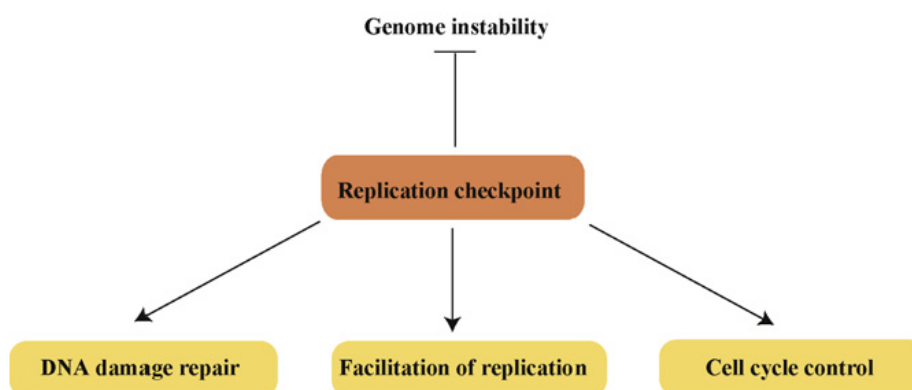


Figure 2.4: The role of replication checkpoint in maintaining genome stability and cell viability - Figure taken from Branzei & Foiani (2009)

Transcriptional response

Activation of Mec1 and Rad53 results in the activation of another kinase, Dun1, that induces the transcription of genes involved in DNA repair and ribonucleotide biosynthesis (Huang *et al.*, 1998; Zhou & Elledge, 1993). Another target of Rad53 is Swi6, the shared subunit of the heteromeric transcription factors SBF and MBF, required for the transcription of genes at the G₁/S transition (Sidorova & Breeden, 1997, 2003). Many genes are regulated in response to DNA damage (see Chapter 5), even though transcription seems to have a minor role in the recovery of stalled forks after HU treatment (Tercero *et al.*, 2003).

Regulation of ribonucleotide reductase

Ribonucleotide reductase complex is composed of two large regulatory (Rnr1/Rnr3) and two small catalytic (Rnr2/Rnr4) subunits. This enzyme catalyzes the rate-limiting step in nucleotide (dNTPs) formation, that is, the reduction of NTPs to dNTPs (Nordlund & Reichard, 2006). During the unperturbed S-phase, dNTP level increases because of enhanced transcription of *RNR* genes after phosphorylation of the transcriptional repressor Rfx1 (Crt1) (Elledge & Davis, 1990), degradation of the *RNR* inhibitor Sml1 and Dif1-dependent re-distribution of Rnr2/Rnr4 to the cytoplasm, where Rnr1/Rnr3 are localized (Yao *et al.*, 2003). All of these pathways are dependent on Dun1 activity (Huang *et al.*, 1998; Lee *et al.*, 2008b; Zhao & Rothstein, 2002). Upon DNA damage, dNTP level increases 6-8 fold (Chabes, Georgieva *et al.* 2003). Deletion of *MEC1* or *RAD53* causes lethality, which can be suppressed by increasing the dNTP pools. This can be achieved by up-regulation of *RNR*, deletion of *SML1*, *RFX1*, *DIF1* or *HUG1*. Nevertheless, increased nucleotide levels do not suppress the DNA damage sensitivity of *mec1* Δ or *rad53* Δ , suggesting additional role for Mec1 and Rad53 other than increasing dNTP pools. Instead, damage sensitivity of the *dun1* Δ cell is suppressed by *SML1* deletion, suggesting that an important role of Dun1 is to increase dNTP pools. High dNTP levels are beneficial for survival, because it reduces spontaneous fork stalling and enhance TLS.

Inhibition of late origin firing

Origin activation follows a temporal program throughout S-phase and replication origins are generally divided into early and late firing (Raghuraman *et al.*, 2001; Shirahige *et al.*, 1998). Since the replication fork is required to activate the checkpoint response (Tercero *et al.*, 2003), early origins have to be fired in order to activate the kinase cascade, which, in turn, inhibits the firing of late origins (Santocanale & Diffley, 1998; Santocanale *et al.*, 1999; Shirahige *et al.*, 1998). This event occurs between the pre-RC and the pre-IC formation, presumably by inhibition of Cdk and/or Cdc7/Dbf4 (Dahmann *et al.*, 1995; Jares *et al.*, 2000). Inhibition of late origins is important during response to DNA damage because it may increase the efficiency of DNA repair, prevent depletion of dNTP pool, needed for TLS, and protect particular chromosome structure (e.g., telomeres). However, inhibition of origin firing only has little contribution to cell survival after S-phase DNA damage (Tercero *et al.*, 2003). A new model proposes that, after replication block by HU, late origin firing is not specifically inhibited, but, instead, the entire S-phase is delayed according to the speed of replication fork progression (Alvino *et al.*, 2007).

Intra-S repair

During replication stress response, replication can be re-initiated downstream the lesions by repriming events on both strands, producing internal gaps (Branzei & Foiani, 2007; Heller & Marians, 2006; Langston & O'Donnell, 2006). These gaps can be re-filled by the post-replicative repair mechanisms (see section 2.3). Execution of PRR

appears to be regulated by the modification state (ubiquitination and sumoylation) of the PCNA-related clamp; in fact, translesion DNA synthesis depends on Rad6/Rad18-mediated mono-ubiquitination of PCNA (Hoege *et al.*, 2002). This example shows the interdependency between checkpoint and PRR (Barbour *et al.*, 2006).

Stabilization of stalled replication forks

Stabilization of replication forks is believed to be the crucial function in cell survival of the checkpoint kinases and it is genetically separable from the late origin inhibition (Tercero *et al.*, 2003). A high proportion of DNA remains unreplicated in *mec1* Δ and *rad53* Δ cells and this may account for the elevated lethality of these checkpoint mutants after exposure to MMS (Tercero & Diffley, 2001). MMS significantly slows down replication fork rate in both wild-type and mutant cells, as a consequence of physical impediment by methylated DNA or intermediates formed during DNA processing (Paulovich & Hartwell, 1995; Vázquez *et al.*, 2008). Furthermore, HU treatment of *rad53* Δ cells reduces the percentage of replication bubbles, with accumulation of unusual DNA structures (collapsed forks) and the failure in DNA replication completion is irreversible, even after HU removal (Lopes *et al.*, 2001). Fork stalling does not depend on the checkpoint kinases which, instead, play an important role in completion of genome replication when the block is removed. Completion of replication does not require firing of additional origins, indicating that stalled forks are re-started. Fork stabilization is mainly achieved through phosphorylation of replicative proteins (Cobb *et al.*, 2003; Lucca *et al.*, 2004; Ohouo *et al.*, 2010).

Checkpoint kinases Mec1, Rad53 and Chk1 present both distinct and redundant roles in fork stabilization (**Figure 2.5**) and targets of Mec1 and Rad53 have been identified in two recent phosphoproteomic studies (Chen *et al.*, 2010; Smolka *et al.*, 2007).

When cells are exposed to MMS, one of the main roles of Rad53 is preventing the accumulation of aberrant DNA structures at stalled forks (e.g., reversed fork and excessive ssDNA) produced by the action of exonuclease Exo1. Deletion of *EXO1* completely suppresses the damage sensitivity of *rad53* Δ cells and allows completion of DNA replication, while this suppression is not found in *rad53* Δ cells blocked with HU (Cotta-Ramusino *et al.*, 2005; Segurado & Diffley, 2008). However, the mechanism used by Exo1 to affect fork integrity is not known. It is important to consider that replisome instability in checkpoint mutants has been described only in HU-treated cells (Cobb *et al.*, 2003, 2005; Lucca *et al.*, 2004), while MMS-treated *rad53* Δ *exo1* Δ cells maintain functional forks, suggesting an ability to maintain stable replisomes without Exo1 after MMS treatment (Segurado & Diffley, 2008). Unlike Rad53 deletion, deletion of *EXO1* in a *mec1* Δ background does not suppress *mec1* Δ sensitivity or prevent replication fork collapse. Not surprisingly, *mec1* Δ cells are considerably more sensitive to HU and DNA-damaging agents than *rad53* Δ cells (Desany *et al.*, 1998). These observations suggests Mec1 has a different role in stabilizing stalled forks, independent of Rad53 (Segurado & Diffley, 2008). Indeed, Mec1 is important for keeping replication polymerases engaged (Cobb *et al.*, 2003) and, while Mec1/Lcd1 accumulates at stalled forks, Rad53 does

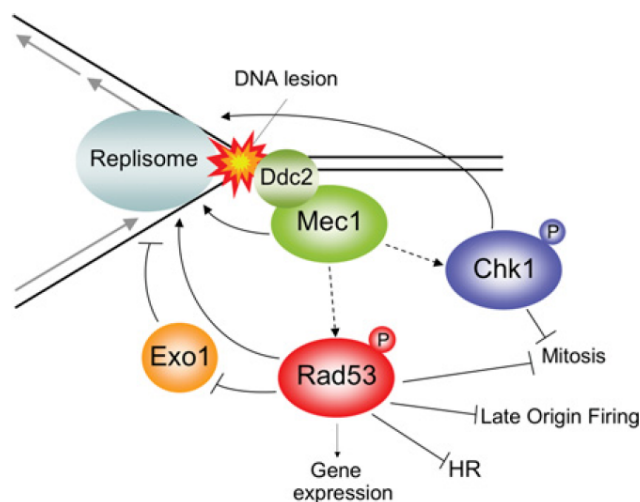


Figure 2.5: Interplay of Mec1, Rad53 and Chk1 during replication fork stabilization - Figure taken from Segurado & Tercero (2009).

not (Cobb *et al.*, 2005). Therefore, Mec1 is not only a sensor of damage, according to the traditional definition, but it also acts as an effector kinase during DNA damage response. Despite the low sensitivity to MMS and HU of *chk1* Δ cells (Sanchez *et al.*, 1999), a role for Chk1 in replication fork stability has been proposed. In fact, since Exo1-dependent suppression in *rad53* Δ cells requires Chk1 (Segurado & Diffley, 2008), Chk1 and Rad53 may have redundant roles that provide safety mechanisms to ensure cell viability.

Therefore, replication fork stabilization enables the resumption of DNA synthesis once the stress is removed, since the *de novo* assembly of pre-RC is not possible during S-phase. Moreover, prevention of unscheduled recombination helps fork stabilization (Lambert *et al.*, 2007; Lisby *et al.*, 2004).

2.6 Checkpoint inactivation: recovery and adaptation

Thus far, emphasis has been given to the checkpoint activation. The inactivation of the response is equally important, even though considerably less is known. DNA damage checkpoint inactivation can occur either after repair of the damage (recovery or return to homeostasis) or in the presence of persistent damage (adaptation or desensitization) (Clémenson & Marsolier-Kergoat, 2009).

During recovery, generally the most upstream component of the checkpoint cascade is inactivated by constitutively active or regulated inhibitors and this event leads to inactivation of the whole pathway. Therefore, recovery allows return to optimal growth under unperturbed conditions. During adaptation, cells decrease the response to a stimulus after prolonged exposure to it and the inactivation can hit any of the

components of the pathway. Adaptation is very important for unicellular organisms because it avoids reproductive death or lysis induced by permanent cell cycle arrest, and it preserves viability at the expense of mutation accumulation. In other words, adaptation can be seen as a final attempt at survival after yeast cells have exhausted all other repair options. Moreover, cell cycle restart allows the repair of the damage by phase-specific mechanisms (Branzei & Foiani, 2008; Galgoczy & Toczyski, 2001).

Inactivation can be brought about by inhibitors inactivating the checkpoint components or preventing their activation, as well as, by proteolytic degradation; this view is, however, simplistic because multiple regulators can act on the same component. Since checkpoint activation relies on phosphorylation cascades, it is plausible to believe that protein phosphatases (PPs) have an important role in the inactivation process, as demonstrated in many studies (Bakkenist & Kastan, 2004; Heideker *et al.*, 2007). Studies of recovery and adaptation required experimental techniques that would allow the monitoring of damaged DNA structures; the hallmark for this kind of studies in *S. cerevisiae* was the design of a system for controlled formation of DSBs by the endonuclease HO (Keogh *et al.*, 2006; Lee *et al.*, 1998; Toczyski *et al.*, 1997; Vaze *et al.*, 2002). The other important tool to investigate checkpoint component inactivation is the Western Blot technique, that allows the monitoring of the phosphorylation status in function of the protein electrophoretic mobility (see Chapter 6).

Direct inhibitors of DDR kinases will be the focus of Chapter 6, with particular emphasis on Rad53 inactivation. No inhibitors targeting Mec1/Tel1 have been reported so far, even though in human cells the PP2A phosphatase has been shown to regulate ATM phosphorylation (Freeman *et al.*, 2010; Goodarzi *et al.*, 2004). Also proteins involved in DNA and chromatin metabolism play important roles in checkpoint inactivation. For example, in response to a DSB, phosphorylated H2A, which recruits the chromatin remodeling complex Ino80 (Fillingham *et al.*, 2006; van Attikum *et al.*, 2004), is dephosphorylated by the phosphatase Pph3 (Keogh *et al.*, 2006). Finally, in response to an irreparable HO cut, another proposed inactivation mechanism is the inhibition of a Rad53 activator by protein kinase Cdc5 (Toczyski *et al.*, 1997; Vidanes *et al.*, 2010).

2.7 Global response to DNA damage

Up to this point, the checkpoint response has been mainly described at the level of events regarding the DNA. In order to have a comprehensive view of the cellular response, a global systems analysis of the coordinated processes responding to DNA damage is needed (Begley *et al.*, 2004; Fry *et al.*, 2005). Such a global response is required because cellular components other than DNA are subject to lesions (RNA, proteins, lipids, carbohydrates and metabolites) (Boffa & Bolognesi, 1985).

S. cerevisiae has been the organism of choice for systematic high-throughput studies because of the availability of genetic tools that allowed the construction of the gene deletion collection (Giaever *et al.*, 2002; Winzeler *et al.*, 1999). In the context of DNA damage response, the main high-throughput approaches are described below (Davidson

& Brown, 2009).

- Chemical-genetic approach (or genomic phenotyping), which evaluates the sensitivity of gene deletion mutants to a given chemical. Initially, experiments were performed in solid media containing the compound of interest, but then pooled-strain competitive-growth strategies were developed (Aouida *et al.*, 2004; Begley *et al.*, 2002, 2004; Bennett *et al.*, 2001; Birrell *et al.*, 2001; Brown *et al.*, 2006; Cejka & Jiricny, 2008; Chang *et al.*, 2002, 2006; Game *et al.*, 2003; Hanway *et al.*, 2002; Haugen *et al.*, 2004; Hillenmeyer *et al.*, 2008; Lee *et al.*, 2005; Parsons *et al.*, 2004; Pierce *et al.*, 2007; Ross-Macdonald *et al.*, 1999; Stefanini *et al.*, 2010).
- Synthetic genetic analysis (SGA) approach, which analyzes the genetic interactions among pairs of deleted genes by comparing the phenotype of the double mutant to those of the single mutants (reviewed in Dixon *et al.* (2009)). SGA allowed the screening of genetic interactions on a genome-scale (Costanzo *et al.*, 2010; Tong *et al.*, 2001; Tong & Boone, 2006; Tong *et al.*, 2004). Several SGA approaches have been directed to the study of the DNA damage response (Collins *et al.*, 2007; Lin *et al.*, 2008; Pan *et al.*, 2004, 2006).
- Spontaneous genomic instability screens, which couples the gene deletion collection with the introduction of markers for the identification of specific phenotypes (Measday *et al.*, 2005; Yuen *et al.*, 2007).
- Proteomics approach, mainly based on mass-spectrometry (Bennetzen *et al.*, 2010; Chen *et al.*, 2010; Matsuoka *et al.*, 2007; Smolka *et al.*, 2007; Stokes *et al.*, 2007).
- Transcriptional profiling (see Chapter 5).

2.7.1 Systems biology studies

The increasing amount of available genome-wide datasets in yeast has allowed the integration of information from different cellular levels and the construction of a framework on which information can be overlaid (Beyer *et al.*, 2007). This systems biology approach has complemented and enriched the transcriptional and phenotypic data, making it possible to build comprehensive and elaborated networks of macromolecules involved in the DDR (Begley *et al.*, 2002, 2004; Collins *et al.*, 2007; Pan *et al.*, 2006; Said *et al.*, 2004; StOnge *et al.*, 2007; Tan *et al.*, 2008; Workman *et al.*, 2006). Some of the most significant studies are hereby reviewed.

Integration of phenotypic data with protein interaction network led to the discovery of six main multi-protein hubs involved in damage response, whose functions include DDR, cytoskeleton remodeling, RNA and protein metabolism, chromatin remodeling, protein degradation and vacuole function, chromosome segregation and cell division (Begley *et al.*, 2002). Moreover, subcellular localization data allowed the identification of toxicity modulating hot spots inside the cell. After treatment with MMS, the identified hot spots, besides the nucleus, were the endosome, microtubule and vacuolar

membrane; it was proposed that H⁺-ATPase pump acidifies the vacuole to promote the ideal environment for damaged macromolecule degradation (Begley *et al.*, 2004). Another study employed a graph theory-based analysis to identified proteins modulating the effects of different agents and several complexes were found, some already linked to damage response, and some unexpected, such as the nuclear pore complex and RNA polymerase II holoenzyme (Said *et al.*, 2004).

Comparison of genetic interaction profiles has been used to predict functional association of DDR genes to build a DNA integrity network (Pan *et al.*, 2006). The diploid synthetic lethality analysis with microarrays (dSLAM) approach, a variation of SGA that maps synthetic lethal interactions by using microarray (Pan *et al.*, 2004), was used to identify functional modules in the checkpoint response and reveal the organization of the DDR complex network, according the notion that genetic interactions often occur among functionally related genes, but are rare among genes belonging to the same complex or pathway. For example, in one module *MEC1* was grouped with *LCD1* and *RAD53*, confirming the interconnected roles of the proteins encoded by these genes. Moreover, the PCNA complex was found in a different module, consistently with the notion that Mec1 and PCNA act as independent DNA damage sensors (Melo *et al.*, 2001).

Collins *et al.* (2007) studied interactions among genes involved in chromosome biology, quantifying both aggravating and alleviating interactions (epistatic miniarray profile, EMAP). Genetic data made it possible to reveal the organization of protein complexes and to assign proteins to pathways. This study was extended by integrating genetic and physical interaction data to gain a unified map of protein complex modules and functional links among them (Bandyopadhyay *et al.*, 2008). Combination of chemical-genetic and quantitative genetic interaction data regarding genes conferring resistance to MMS, showed the power of this approach in dissecting the homologous recombination pathway (StOnge *et al.*, 2007). Workman *et al.* (2006) integrated transcription factor binding profiles, gene expression and protein interaction to produce a system-level view of the DDR. For the first time, the protein-DNA interactions in MMS-treated yeast cells were interpreted within the context of physical interactions. Using the chromatin immunoprecipitation-DNA microarray assay (ChIP-chip) and gene expression profiles, the authors identified TF-gene pairs showing strong evidence of regulation following DNA damage, that is, TF-gene pairs showing differential expression under normal versus damaging conditions, which was lost after deletion of the TF ('deletion buffering'; this kind of analysis has been applied in the work described in Chapter 5). After adding physical interaction data, a network of the yeast DDR was constructed, including new regulatory circuits connecting the DNA repair to other processes, such as cell cycle, stress response, lipid and nucleotide metabolism. Finally, a recent study has integrated growth phenotypes, promoter binding profiles and gene expression profiles in order to elucidate the role of the YAP transcription factor family during DDR (Tan *et al.*, 2008).

The described global studies have provided new insight into the biological responses to DNA damage in yeast. However, the current networks are based on high-throughput

experiments carried out, in most of the cases, under basal conditions and, therefore, do not represent a comprehensive picture of the dynamic and complex interactions that manifest upon stress. Indeed, protein-protein and protein-DNA interactions may change drastically when a DNA damaging agent is added, as demonstrated by Workman *et al.* Moreover, networks should be complemented with information regarding the post-translation modifications (PTMs) of proteins (e.g., phosphorylation and acetylation), because in many cases the function of a protein depends on its modifications, as it has been described in the phosphorylation cascade governing the checkpoint activation. Nevertheless, with respect to the DDR network analysis, large dataset obtained under damaging conditions are becoming available: protein-protein interactions (Ho *et al.*, 2002), phosphoproteome (Chen *et al.*, 2010; Smolka *et al.*, 2007), protein-DNA interactions (Tan *et al.*, 2008; Workman *et al.*, 2006) and genetic interaction (Batenchuk *et al.*, 2010; Lee *et al.*, 2005; Pan *et al.*, 2006). In Chapter 5, we have used some of these datasets and adopted a systems biology approach in order to provide new insights into the DNA damage response to MMS.

Part II

Manuscripts

Chapter 3

Research article

Open Access

Transcription factor control of growth rate dependent genes in *Saccharomyces cerevisiae*: A three factor design

Alessandro Fazio^{†1,2}, Michael C Jewett^{†1,4}, Pascale Daran-Lapujade³, Roberta Mustacchi¹, Renata Usaite¹, Jack T Pronk³, Christopher T Workman² and Jens Nielsen^{*1,5}

Address: ¹Center for Microbial Biotechnology, Department of Systems Biology, Technical University of Denmark, Building 223, DK-2800, Kgs. Lyngby, Denmark, ²Center for Biological Sequence Analysis, Department of Systems Biology, Technical University of Denmark, Building 208, DK-2800 Kgs. Lyngby, Denmark, ³Kluyver Centre for Genomics of Industrial Fermentation and Department of Biotechnology, Delft University of Technology, Julianalaan 67, 2628 BC, Delft, The Netherlands, ⁴Department of Genetics, Harvard Medical School, Boston, MA 02115, USA and ⁵Department of Chemical and Biological Engineering, Chalmers University of Technology, SE- 412 96, Gothenburg, Sweden

Email: Alessandro Fazio - alfa@cbs.dtu.dk; Michael C Jewett - mcjewett@genetics.med.harvard.edu; Pascale Daran-Lapujade - P.A.S.Daran-Lapujade@tudelft.nl; Roberta Mustacchi - rm@bio.dtu.dk; Renata Usaite - ru@bio.dtu.dk; Jack T Pronk - J.T.Pronk@tudelft.nl; Christopher T Workman - workman@cbs.dtu.dk; Jens Nielsen^{*} - nielsenj@chalmers.se

^{*} Corresponding author [†]Equal contributors

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Abstract

Background: Characterization of cellular growth is central to understanding living systems. Here, we applied a three-factor design to study the relationship between specific growth rate and genome-wide gene expression in 36 steady-state chemostat cultures of *Saccharomyces cerevisiae*. The three factors we considered were specific growth rate, nutrient limitation, and oxygen availability.

Results: We identified 268 growth rate dependent genes, independent of nutrient limitation and oxygen availability. The transcriptional response was used to identify key areas in metabolism around which mRNA expression changes are significantly associated. Among key metabolic pathways, this analysis revealed *de novo* synthesis of pyrimidine ribonucleotides and ATP producing and consuming reactions at fast cellular growth. By scoring the significance of overlap between growth rate dependent genes and known transcription factor target sets, transcription factors that coordinate balanced growth were also identified. Our analysis shows that Fhl1, Rap1, and Sfp1, regulating protein biosynthesis, have significantly enriched target sets for genes up-regulated with increasing growth rate. Cell cycle regulators, such as Ace2 and Swi6, and stress response regulators, such as Yap1, were also shown to have significantly enriched target sets.

Conclusion: Our work, which is the first genome-wide gene expression study to investigate specific growth rate and consider the impact of oxygen availability, provides a more conservative estimate of growth rate dependent genes than previously reported. We also provide a global view of how a small set of transcription factors, 13 in total, contribute to control of cellular growth rate. We anticipate that multi-factorial designs will play an increasing role in elucidating cellular regulation.

Background

Regulation of cell growth is of crucial importance for the survival of all living cells. Much effort, therefore, has focused on understanding the mechanisms that control how cells achieve balanced growth, e.g. control of the cell cycle and biosynthesis of cellular building blocks. To date, DNA microarray technology [1,2] has had a considerable impact in defining causal relationships between different growth conditions and the transcriptional response of cells. A number of previous studies in *S. cerevisiae* have focused on the genome-wide transcriptional response of cells to nutrient limitation [3-5], oxygen availability [6-8] and growth rate (Table 1).

To identify growth rate dependent genes, two major requirements must be met. First, the specific growth rate of the culture (h^{-1}) must be controlled. This is necessary to eliminate variability that is inherent in dynamic batch cultivation [7,9-11]. The general approach for obtaining constant specific growth rate is through continuous i.e. chemostat cultivation. Here the specific growth rate is kept constant by continuously feeding a culture with fresh nutrients having one limiting reagent at a specific dilution rate (D). The dilution rate is adjusted to obtain different specific growth rates. Second, it is also important to measure gene expression patterns over a range of specific growth rates. By studying factors in addition to specific growth rate (e.g. nutrient limitation), growth rate dependent genes that are independent of environmental factors can be identified.

Previous works have suggested that growth rate has a tremendous influence on the yeast transcriptional program. Specifically, Regenberg *et al.* [12] described more than 2400 growth rate dependent genes and proposed a role for the chromosomal location in the regulation of these

genes. Castrillo *et al.* [13] adopted a systems biology approach to investigate the effect of growth rate at the transcriptome, proteome and metabolome levels. They identified about 900 genes whose expression is growth regulated and concentrated, in particular, on the role of the TOR complex 1. More recently, Brauer *et al.* [14] determined that transcript levels of more than one quarter of all yeast genes are linearly correlated with growth rate. While growth rate dependent genes have been identified from single factor studies [12] and two factor designs, such as growth rate and nutrient limitation [13,14], multi-factor designs, such as the approach presented here, are expected to identify growth rate dependent genes that are more independent of the specific growth conditions.

Here we carried out a three factor design to dissect the role of growth rate on the transcriptional program of yeast. The three factors were specific growth rate, nutrient limitation (carbon/nitrogen limitation), and oxygen availability. For the specific growth rate, multiple levels, i.e. 0.03, 0.1 and 0.2 h^{-1} were evaluated. In the context of growth rate studies, the effect of oxygen availability has not yet been considered. Beyond identifying growth rate dependent genes independent of nutrient limitation and oxygen availability, we sought to use recently developed systems biology tools to distinguish transcription factors (TFs) that may coordinate and regulate the processes that control cellular growth (e.g. cell cycle period, protein biosynthesis, and energy metabolism).

Results and discussion

A three-factor design to investigate growth rate dependent genes

To study the growth-rate related transcriptional response in *S. cerevisiae* CEN.PK113-7D, we applied a systems approach that integrated transcriptome measurements

Table 1: Studies of growth rate effect on transcriptional response in *Saccharomyces cerevisiae*

Study	Strain	Cultivation Mode	Limiting Nutrient ^a	O ₂ Availability	D (h ⁻¹)	Array type
Hayes <i>et al.</i> (2002)	FY1679 ^b	Batch/Chemostat	C/N	Aerobic	0.1–0.2	Membrane/Glass slide
Pir <i>et al.</i> (2006)	BY4743 ^c	Chemostat	C/N	Aerobic	0.1–0.2	Affymetrix Yeast S98 GeneChip
Regenberg <i>et al.</i> (2006)	CEN.PK113-7D ^d	Chemostat	C	Aerobic	0.02-0.05-0.1-0.2-0.25-0.33	Affymetrix Yeast S98 GeneChip
Castrillo <i>et al.</i> (2007)	FY1679 ^b	Chemostat	C/N/P/S	Aerobic	0.07-0.1-0.2	Affymetrix Yeast S98 GeneChip
Brauer <i>et al.</i> (2008)	DBY10085 ^d DBY9492 ^d DBY9497 ^d	Chemostat	C/N/P/S/U/L	Aerobic	0.05-0.1-0.15-0.2-0.25-0.3	Agilent Yeast V2 (Cy3/Cy5)
This Study	CEN.PK113-7D ^d	Chemostat	C/N	Aerobic/Anaerobic	0.03-0.1-0.2	Affymetrix Yeast S98 GeneChip

^a C, carbon; N, nitrogen; P, phosphorus; S, sulfur; U, uracil; L, leucine

^b Isogenic to S288C

^c S288C-derived

^d CEN.PK-derived

with data from protein-DNA interaction networks. A $2 \times 2 \times 3$ factorial design was pursued resulting in 12 different growth conditions (Fig. 1), which have been investigated in triplicate. Specifically, steady-state conditions were chosen to perturb (a) specific growth rate (equal to the dilution rate D), (b) nutrient limitation, and (c) oxygen availability. Each factor comprised at least two levels: (a) $D = 0.03/0.1/0.2 \text{ h}^{-1}$, (b) carbon/nitrogen limitation, and (c) aerobiosis/anaerobiosis. Because the specific growth rate (μ) equals the dilution rate (D) in our chemostat experiments, the selected range covers cell doubling time ($T_2 = \ln(2)/\mu$) between 3.5 and 23.1 h.

We first collected genome-wide transcription profiles from each steady-state using the Affymetrix GeneChip platform. To reduce data dimensionality and explore the data structure, Principal Components Analysis (PCA) was applied to the normalized microarray data (Fig. 2). Three main principal components were observed, comprising 69% of the variance (see Additional file 1). Strikingly, the PCA projections revealed that the three main principal components segregate the data along the three factors of our factorial design. The factor giving the greatest variance was oxygen availability (O-A split along PC1). The second largest source of variability was observed for nutrient limitation (C-N split along PC2), followed by dilution rate (growth rate split along PC3; Fig. 2A-C). While PC1 shows a clear separation between aerobic and anaerobic conditions, PC2 only distinctly separates the carbon and nitrogen limited conditions for the aerobic case. This is

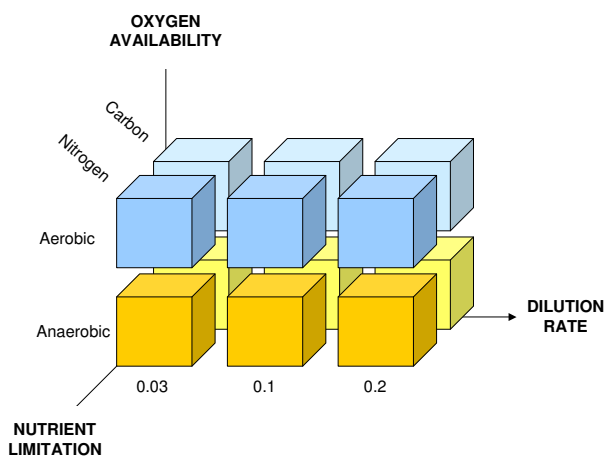


Figure 1
Experimental design. Each block represents one of the 12 possible combinations among the three experimental factors (oxygen availability, nutrient limitation and dilution rate). Each experiment was carried out in triplicate, therefore a total of 36 different cultivations were performed. Dilution rate values are given in h^{-1} .

probably due to the fact that in the absence of oxygen only fermentative metabolism is possible, while both respirofermentative (N-limitation) and fully respiratory (C-limitation) metabolism may occur in aerobic conditions. The third factor, specific growth rate, also shows good groupings, although not as distinct as for the other factors (Fig. 2B-D). This is consistent with the transcriptome data from Castrillo *et al.* [13], in which C-limited cultivations were strongly segregated from the other nutrient limited conditions. Notably, the high reproducibility of the replicates demonstrates the quality of our data.

Functional analysis of the 268 growth rate dependent genes

To quantitatively reveal which genes had significantly changed expression, MicroArray Analysis of Variance (MAANOVA) was carried out by using mixed-model and Fs test (see Methods and Additional file 2). This test permitted the discovery of genes showing significant transcriptional changes with respect to each considered factor (specific growth rate, nutrient limitation and oxygen availability). Table 2 shows the number of differently expressed genes for each of the three factors at different cut-off q -values. At a false discovery rate (FDR) of 2%, which was selected for further analysis, a total of 268 growth rate dependent genes were identified as significantly changed. To group genes with common expression profiles over the dilution rate range, the selected gene lists were clustered using hierarchical clustering (Fig. 3). Of the 268 significantly changed genes, 114 genes were up-regulated with increasing growth rate and 154 genes were down-regulated with increasing growth rate (see Additional file 3). The significantly changed genes are linearly correlated (either negatively or positively) with increasing growth rate (see Additional file 1). Consistent with the PCA analysis, the factor showing the most prominent segregation was oxygen availability. It is possible that this result, in part, reflects the distribution of experimental effort (see Methods).

To determine significantly enriched Gene Ontology (GO) process terms within the up-regulated and down-regulated growth rate dependent gene clusters, we used the Saccharomyces Genome Database (SGD)-GO tools (significance at $P \leq 0.01$; see Additional file 3). Among genes up-regulated with increasing growth rate, biosynthetic processes were the most significantly enriched (Table 3). In particular, genes involved in ribosome biogenesis and assembly, translation, and protein biosynthesis were over-represented. Nearly half of the up-regulated genes (53/114) encoded for components of the ribosome complex. These results suggest that faster growing cells build biomass more efficiently and are consistent with previous reports [12-15].

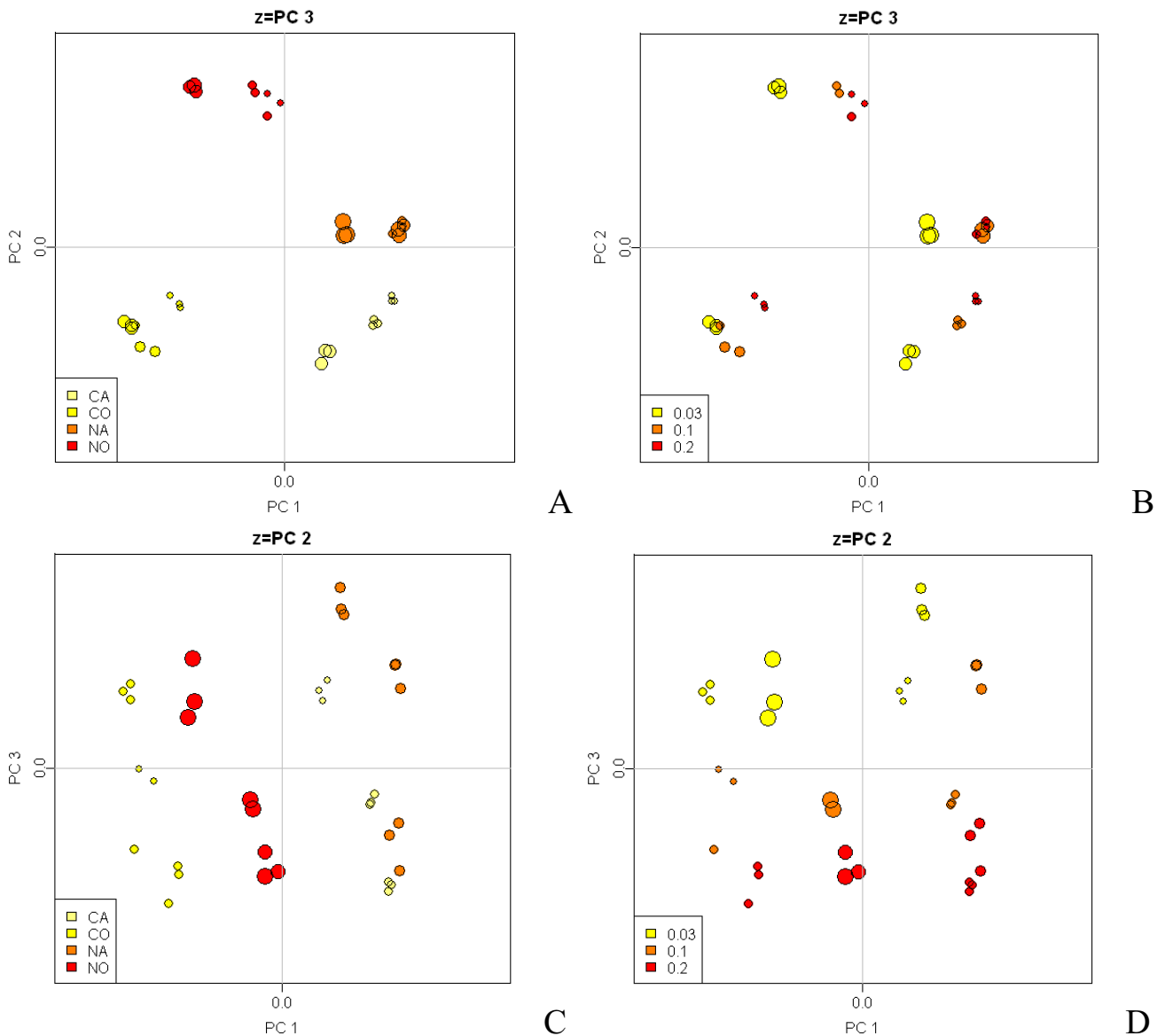


Figure 2
PCA projection of samples in the first 3 PC dimensions. Plots A and B show PC dimensions 1 versus 2 as the x- and y-axis and spot size as PC3 in the z-axis. Plots C and D show PC1 vs PC3 and highlight the segregation due to the dilution rate factor in PC3. Color in A and C represents a different combination of these two factors: NO, aerobic nitrogen-limited culture; CO, aerobic carbon-limited culture; NA, anaerobic nitrogen-limited culture; CA, anaerobic carbon-limited culture. Colors in B and D show the dilution rates 0.03, 0.1 and 0.2 h⁻¹.

Among the 154 down-regulated genes, the most over-represented GO terms were response to stress, carbohydrate metabolic process, and catabolic process (Table 3). More specifically, genes encoding proteins involved in ER associated protein catabolism (*HRD3*), vacuole homeostasis (*FAB1*, *GGA1*), ubiquitin cycle (*APC9*, *RTT101*, *UBC8*) and ubiquitin-dependent protein catabolism (*MET30*, *RPN4*, *RPN14*, *YFL006W*) show lower expression levels at higher specific growth rates. *RPN4*, for example, regulates

cellular levels of the proteasome [16,17]. While gene expression required for protein synthesis increases with increasing growth rate, gene expression required for protein degradation decreases. It is tempting to speculate that increased protein degradation processes at lower growth rates, typically under sub-optimal conditions, is a survival mechanism designed to more efficiently re-use possible resources.

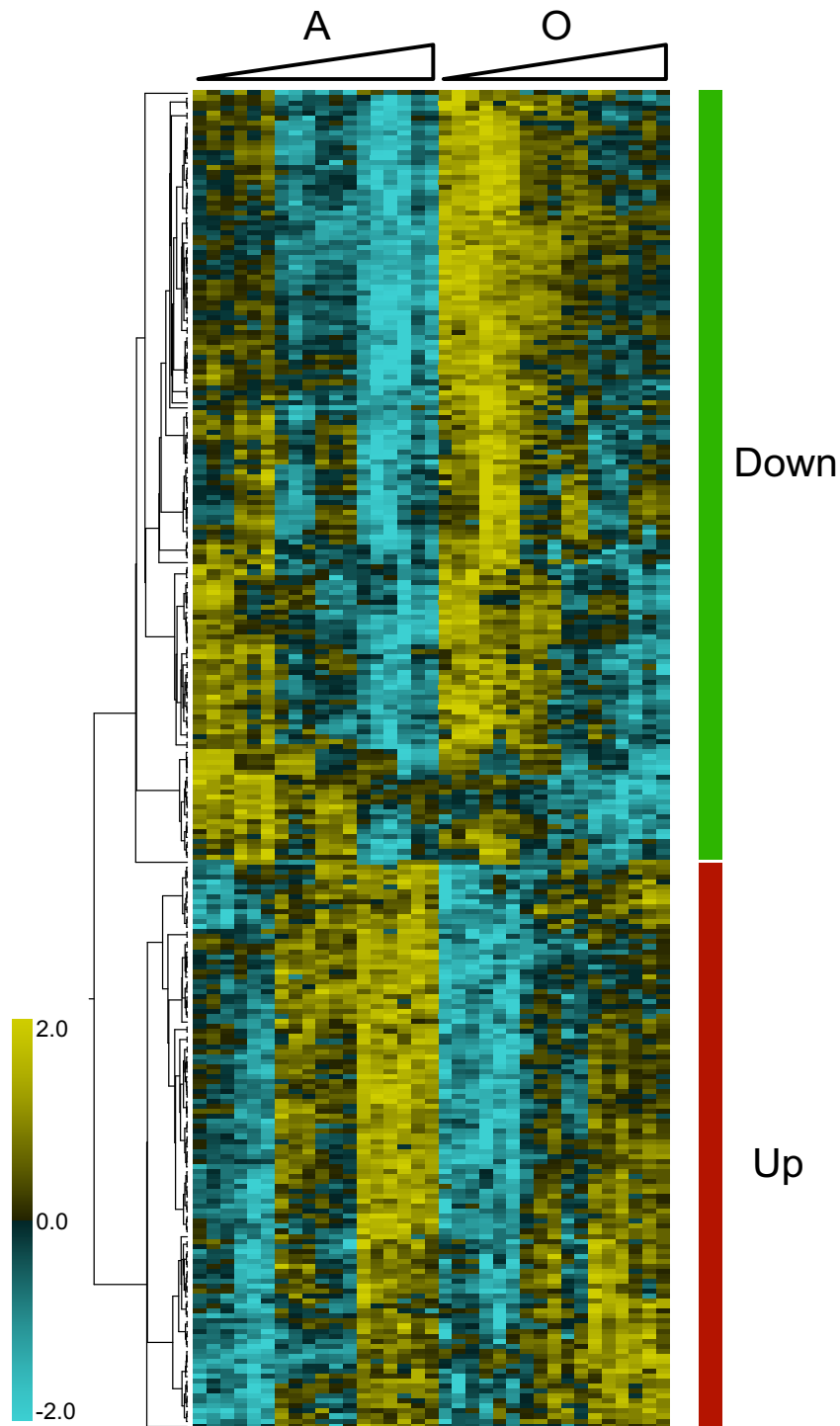


Figure 3
Hierarchical clustering of growth rate dependent genes. The columns represent the experiments and the left hand side of the cluster refers to the anaerobic (A) dataset, while the right hand side to the aerobic (O) dataset. The columns are ordered at increasing dilution rate values (0.03 - 0.1 - 0.2 h⁻¹), as indicated by the triangles at the top of the clusters. The rows represent the 268 growth rate dependent genes and the two main clusters of up- and down-regulated genes with increasing *D* are shown. The scale of the color bar is based on z-score.

Table 2: The number of significantly changed genes (MAANOVA analysis) at different q-value threshold values

q-value threshold	0.01	0.02	0.03	0.04	0.05
Dilution rate	0	268	494	720	938
Nutrient limitation	373	504	579	642	698
Oxygen availability	1208	1933	2038	2355	2594

Strikingly, 11% of down-regulated genes have kinase activity (only 2.8% of yeast genes have kinase activity according to SGD), suggesting a possible role for phosphorylation in regulating the growth rate response. In addition, down-regulated genes having an unknown biological process (22.7%) or function (35.1%) were over-represented. The lack of annotation may be a result of these genes being expressed weakly under the rapid growth conditions used in most microarray experiments [12].

To identify metabolites in yeast around which mRNA expression changes are significantly associated, we applied the Reporter Metabolite Algorithm [18] (see Methods). The most significant Reporter Metabolites are listed in Table 4. These metabolites participate in diverse

metabolic pathways from nucleotide and amino acid metabolism, to phospholipid synthesis and the pentose phosphate pathway. Orotate, for example, is involved in the *de novo* synthesis of pyrimidine ribonucleotides. A closer look revealed that *URA5*, whose gene product catalyzes orotate phosphoribosyl transferase, was among the significantly up-regulated genes with increasing growth rate. *URA5* is not regulated by pathway intermediates and our analysis suggests that transcriptional control of this critical enzyme involved in DNA synthesis helps to mobilize resources necessary for growth. It is striking that ATP, which participates in more reactions than any other metabolite [19], is among the most significant Reporter Metabolites. This result suggests that gene expression of enzymes involved in ATP production and consumption reactions is significantly regulated over changes in specific growth rate. In summary, the Reporter Metabolite results highlight the broad impact that growth rate has across metabolism.

Transcription factor control of growth rate dependent genes

To identify and score TFs that might regulate the processes that control cell growth, we scored the significance of overlap between the 268 growth rate dependent genes and

Table 3: GO annotation based on the Biological Process ontology for growth rate dependent genes

GO Term	Gene hits	Cluster frequency	P-value
<i>Up-regulated Genes (114)</i>			
cellular biosynthetic process	61	53.5%	1.58E-21
translation	51	44.7%	8.27E-21
biosynthetic process	66	57.9%	1.61E-20
macromolecule biosynthetic process	55	48.2%	4.28E-19
cellular protein metabolic process	59	51.8%	3.95E-11
protein metabolic process	60	52.6%	4.11E-11
cellular macromolecule metabolic process	60	52.6%	4.78E-11
primary metabolic process	90	78.9%	7.37E-11
gene expression	62	54.4%	1.29E-10
cellular metabolic process	91	79.8%	4.39E-10
metabolic process	92	80.7%	9.58E-10
cellular process	101	88.6%	8.12E-07
macromolecule metabolic process	77	67.5%	8.86E-07
ribosome biogenesis and assembly	21	18.4%	4.20E-04
ribosomal subunit assembly	8	7.0%	6.90E-04
ribosome assembly	8	7.0%	2.82E-03
ribonucleoprotein complex biogenesis and assembly	21	18.4%	5.59E-03
<i>Down-regulated Genes (154)</i>			
cellular carbohydrate metabolic process	18	11.8%	2.90E-04
carbohydrate metabolic process	18	11.8%	1.14E-03
macromolecule catabolic process	22	14.4%	1.86E-03
response to stress	26	17.0%	6.33E-03
catabolic process	24	15.7%	9.30E-03
energy reserve metabolic process	7	4.6%	9.34E-03

Gene hits indicate the number of genes in the clusters of up-/down-regulated genes belonging to that particular GO term; the value is also given as percentage (cluster frequency). P-values are provided as a score of significance (cut-off ≤ 0.01).

Table 4: Reporter Metabolite analysis

Reporter Metabolites	Number of neighbors	P-value
Orotate	3	7.10E-04
D-Mannose 6-phosphate	5	9.71E-04
Spermidine	3	1.68E-03
alpha, alpha-Trehalose	4	3.30E-03
5-Phospho-alpha-D-ribose 1-diphosphate	17	5.15E-03
1-(5'-Phosphoribosyl)-5-amino-4-imidazolecarboxamide	4	5.22E-03
D-Ribose 5-phosphate	18	7.42E-03
Dolichyl beta-D-mannosyl phosphate	7	7.60E-03
FAD	2	9.45E-03
1-Phosphatidyl-D-myo-inositol 4,5-bisphosphate	3	9.99E-03
beta-D-Glucose	3	1.00E-02
ATP	113	1.02E-02
5'-Methylthioadenosine	2	1.05E-02
alpha-D-Glucose 6-phosphate	11	1.19E-02
O-Phospho-4-hydroxy-L-threonine	2	1.26E-02
N6-(L-1,3-Dicarboxypropyl)-L-lysine	2	1.37E-02
Glycogen	4	1.42E-02
Urea-1-carboxylate	1	1.69E-02
(S)-Dihydroorotate	2	1.75E-02
2-Phenylacetamide	1	1.82E-02
Phenylacetic acid	1	1.82E-02
Indole-3-acetamide	1	1.82E-02
Indole-3-acetate	1	1.82E-02
(S)-1-Pyrroline-5-carboxylate	1	1.82E-02
L-1-Pyrroline-3-hydroxy-5-carboxylate	1	1.82E-02
trans-4-Hydroxy-L-proline	1	1.82E-02

Reporter Metabolite analysis [18] identifies metabolites around which the most significant transcriptional changes occur. The number of neighbors indicates the number of genes whose products catalyze a reaction involving that particular metabolite. The algorithm took as input the MAANOVA analysis referring to dilution rate effect. The P-value gives a measure of significance and all results < 0.02 are reported.

known TF target sets [20,21] (Table 5, hypergeometric test at $P < 0.01$). In total, this analysis revealed 13 TFs having significantly enriched target sets (Fig. 4) for genes up-regulated with increasing growth rate. Fhl1, Rap1, Sfp1, and Yap5 are involved in regulating ribosomal protein gene expression. Ace2 and Swi6 participate in cell cycle regulation. Yap1, Yap6, Smp1, and Pdr1 are involved in stress response and signaling. Bas1 is involved in amino acid and nucleotide biosynthesis, while Stb4 and Gat3 have unclear roles. The connectivity of TFs with enriched targets demonstrates how the global response of growth rate dependent genes may be controlled (Fig. 4). Sin4, Rap1, Swi6, and Swi4 appear to coordinate the response by linking protein synthesis, the cell cycle, and the stress response. No significant TFs were found when the same TF analysis was performed for the down-regulated genes.

Fhl1, Rap1, and Sfp1 were the TFs with the greatest enrichment of growth-rate dependent target genes (Table 5). These TFs are all involved in ribosomal protein (RP) gene transcription. There are 138 RP genes in yeast, and their expression accounts for more than 50% of the RNA pol II dependent transcription [22]. Rap1 participates in ribosomal gene expression [23-25] and is involved in moving nucleosomes from a certain region of chromatin in order

to allow Fhl1 and Ifh1 to trigger RP gene transcription [26-29].

Ace2 and Swi6 are known cell cycle regulators [30] and our TF enrichment analysis suggests a role for these two TFs in controlling growth rate, which remains a hypothesis. Swi6 is part of the two heterodimeric transcriptional regulators SBF (Swi4/Swi6) and MBF (Mbp1/Swi6) [31], that act in the early cell cycle (G1 phase). Ace2, instead, plays an important role during the M phase. Previously, the effect of Ace2 on the length of G1 phase has been reported by Laabs *et al.* [32], who demonstrated that a G1 specific delay in yeast daughter cells is due to this TF. Little is known about Stb4 (SGD classifies Stb4 as having an unknown biological process): it binds to Swi5 [33] and a two-hybrid screen [34] found that it binds to Sin3. We hypothesize that identification of Stb4 as a principal regulating TF in our study, and the close association of it with Swi6 and Ace 2 (Fig. 4), may hint at a possible role for Stb4 in regulating the cell cycle.

Highlighting the importance of both protein biosynthesis and cell cycle progression in controlling growth rate, Sfp1 was also identified in the TF enrichment analysis. Jorgensen *et al.* [35] suggested that Sfp1 activates RP gene

Table 5: Transcription factor target set enrichment results

TFs	Log10(p-value)	Overlap	Set1	Set2	Background
Harbison <i>et al.</i> (YPD), p < 0.01					
FHL1	-28.44	42	114	213	5636
RAP1	-16.52	42	114	414	5636
GAT3	-9.68	23	114	179	5636
SMPI	-4.45	17	114	180	5636
YAP5	-4.1	16	114	168	5636
PDR1	-3.48	15	114	164	5636
Harbison <i>et al.</i> (Other), p < 0.01					
FHL1 (rapa)	-27.96	42	114	220	5636
FHL1 (sm)	-24.83	44	114	294	5636
FHL1 (H ₂ O ₂ -Hi)	-16.35	30	114	189	5636
RAP1 (sm)	-13.11	37	114	392	5636
SFPI (sm)	-8.44	18	114	118	5636
Beyer <i>et al.</i> SLL > 4					
FHL1	-27.43	51	114	379	5636
RAP1	-20.29	34	114	196	5636
SFPI	-18.9	28	114	129	5636
STB4	-17.91	29	114	153	5636
SWI6	-16	42	114	430	5636
YAP6	-15.16	32	114	242	5636
YAPI	-14.47	35	114	314	5636
ACE2	-10.97	32	114	335	5636
BASI	-10.64	22	114	147	5636

Target sets defined by Harbison *et al.* [21] ChIP-chip study. p-values < 0.01 for YPD and other growth conditions are indicated (rapa: rapamycin, sm: sulfometuron methyl, H₂O₂-Hi, hydrogen peroxide 4 mM). Sets were also analyzed for Beyer *et al.* [20] derived target sets using sum of log-likelihood (SSL) > 4.

transcription by influencing the nuclear localization of Fhl1 and Ifh1. The TOR and PKA pathways, previously identified [13] as critical in controlling growth rate, participate in keeping Sfp1 in the nucleus [36]. Sfp1 also modulates cell cycle progression in the late G1 phase (Start) by controlling cell size in eukaryotic cells [37,38]. Cell cycle progression in the late G1 phase (Start) is dependent on the attainment of a critical cell size and critical translation rate [39].

Several identified TFs with significantly enriched targets are primarily involved in the stress response. Yap1 regulates the expression of oxidative stress response genes [40]. Chua *et al.* [41] have indicated that Yap1 overexpression induces genes involved in translation and tRNA metabolism. Yap6 is known to have a role in salt tolerance [42] and recently Steinfeld *et al.* [43] have indicated a role in regulation of sugar transport. Pdr1 is a zinc finger transcription factor whose target genes carry out ABC transport, other transport, and membrane lipid and cell wall biosyntheses [44]. We have previously proposed a role for Pdr1 in DNA damage response process and showed that Yap5 and Swi5 targets overlap significantly with Pdr1 targets in absence of the damaging agent [45].

In summary, the Reporter Metabolite and TF enrichment analyses both support the conclusion that in yeast changes in growth rates are associated with the regulation of protein synthesis, the cell cycle, and the stress response. For example, four TFs involved in regulation of protein synthesis genes are identified. In agreement, the Reporter Metabolite analysis identifies ATP. Thus, genes encoding products that catalyze reactions involving ATP, and one of the most energy intensive processes of the cell, are observed as being significantly changed. In addition, identification of cell cycle regulators is consistent with results from Reporter Metabolite analysis suggesting that regulation of metabolic pathways of DNA synthesis (the *de novo* synthesis of pyrimidine ribonucleotides) have significant transcriptional changes.

Comparison with previous growth rate studies

Compared with earlier studies on the influence of the specific growth rate on global transcription, our analysis provides a much more moderate estimate of the number of growth rate dependent genes. This is likely due to two main reasons. First, the three-factor design employed here de-emphasizes genes that might be significant when oxygen availability is not considered. Second, the statistical methods and significance thresholds among the studies

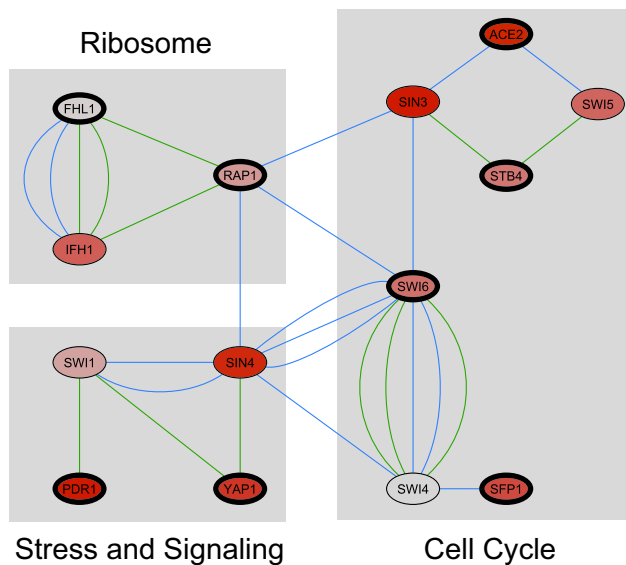


Figure 4
Network of TFs regulating the genes up-regulated with increasing dilution rate. Nodes with thicker outlines contain the TFs found in our analysis (YAP5, YAP6, SMP1, GAT3 and BAS1 do not map into this network). The connectivity among nodes is based on the interactions stored at BioGRID database [58] and the interaction types can be divided in two groups: (1) genetic interactions, which can be detected by dosage rescue, synthetic rescue, synthetic growth defect, synthetic lethality, phenotypic enhancement and phenotypic suppression (blue edges); and (2) protein interaction, detected by affinity capture-MS, affinity capture-western, reconstituted complex and two-hybrid (green edges). See Additional file 3 for details about these interactions. Moreover, nodes are colored according to the expression levels of the genes encoding the TFs and a grey-red scale is used (red color indicates higher expression levels). In this network, the TF expression values from experiments at 0.2 h⁻¹ are depicted. No significant differences in TF expression values were observed at different dilution rates (see Additional file 1). The network was drawn by using Cytoscape [59].

are different. Our previous study [12], for example, found the largest number of growth rate dependent genes (~2400). However, that study used a newly developed consensus clustering algorithm to group similar genes that correlated with growth rate [46]. As another illustration, Castrillo *et al.* [13] identified about 900 growth rate dependent genes by performing analysis of covariance (ANCOVA) and applying a *q*-value threshold of ≤ 0.05 for significance. At this threshold, their results are consistent with our findings (978 genes, *q*-value ≤ 0.05 ; see Table 2). The number of genes specifically overlapping between the work of Castrillo *et al.* and this study at a *q*-value threshold of 0.05 is 315. Using our more conservative cut-off, the overlap is 119.

Given differences between experimental designs and approaches for determining growth rate dependent genes, it is perhaps not surprising that few common genes are observed among our results and the three previous studies (see Additional file 1). Specifically, 21 up-regulated genes and 10 down-regulated genes were shared (Table 6 and 7). Among the common up-regulated genes, 11 were involved in translation (mostly RP genes) and 3 in sphingolipid biosynthesis (*FEN1*, *SUR4*, *URA7*). Of common down-regulated genes, 3 had unknown process (*YDR262W*, *YMR090W*, *YOL153C*) and 4 were involved in regulation of the enzyme fructose-1,6-bisphosphatase, Fbp1 (*PFK26*, *VID28*, *VID30*, *YLR345W*). Despite only a small overlap of specific genes among studies, significantly enriched GO Biological Process terms identified the same overarching biological changes. Considering the substantial variation between the different studies, our multi-factorial dataset is valuable for obtaining robust answers from queries on the effect of growth on transcription of different genes. Due to our multi-factorial design, our dataset is also valuable for evaluation of e.g. the effect of nutritional state independent of growth rate and oxygen availability.

Conclusion

By increasing the number of experimental factors, we have identified a more conservative set of growth-rate dependent genes. Specifically, our analysis has identified 268 specific growth rate dependent genes. Results of a gene function analysis were found to be in agreement with previous studies [12-14]. New insight into the regulation of growth rate regulated genes has also been provided. Specifically, 13 TFs have been identified as related to genes whose transcripts level increased with increasing growth rate and 8 of these are connected in a map of regulatory pathways supported by known protein-DNA interactions. Supported by the Reporter Metabolite analysis, the TFs that coordinate growth rate dependent genes are primarily involved in protein synthesis, the cell cycle, and the stress response. Strikingly, down-regulated genes with increasing growth rate did not show common regulation, likely due to the high percentage of uncharacterized genes. We have shown that multi-factor designs, combined with a systems biology approach, can enhance our knowledge about yeast responses to growth rate. This approach will be valuable for studying any other environmental or genetic factor of interest.

Methods

Strain and chemostat cultivations

The reference laboratory strain *S. cerevisiae* CEN.PK113-7D (*MATa*) [47] was grown in well controlled 2 liter jacketed chemostats (Braun Biotech and Applikon) with a constant working volume of 1.0 liter. Cultivations were carried out (in triplicates) in aerobic/anaerobic and car-

Table 6: Common up-regulated genes among growth rate studies

ORF	Gene Name	Description
YBL039C	URA7	Major CTP synthase isozyme (see also URA8), catalyzes the ATP-dependent transfer of the amide nitrogen from glutamine to UTP, forming CTP, the final step in de novo biosynthesis of pyrimidines; involved in phospholipid biosynthesis
YBR189W	RPS9B	Protein component of the small (40S) ribosomal subunit; nearly identical to Rps9Ap and has similarity to E. coli S4 and rat S9 ribosomal proteins
YBR191W	RPL21A	Protein component of the large (60S) ribosomal subunit, nearly identical to Rpl21Bp and has similarity to rat L21 ribosomal protein
YCR034W	FEN1	Fatty acid elongase, involved in sphingolipid biosynthesis; acts on fatty acids of up to 24 carbons in length; mutations have regulatory effects on 1,3-beta-glucan synthase, vacuolar ATPase, and the secretory pathway
YDL083C	RPS16B	Protein component of the small (40S) ribosomal subunit; identical to Rps16Ap and has similarity to E. coli S9 and rat S16 ribosomal proteins
YDR064W	RPS13	Protein component of the small (40S) ribosomal subunit; has similarity to E. coli S15 and rat S13 ribosomal proteins
YDR144C	MKC7	GPI-anchored aspartyl protease (yapsin) involved in protein processing; shares functions with Yap3p and Kex2p
YDR321W	ASPI	Cytosolic L-asparaginase, involved in asparagine catabolism
YEL040W	UTR2	Cell wall protein that functions in the transfer of chitin to beta(1-6)glucan; putative chitin transglycosidase; glycosylphosphatidylinositol (GPI)-anchored protein localized to the bud neck; has a role in cell wall maintenance
YER009W	NTF2	Nuclear envelope protein, interacts with GDP-bound Gsp1p and with proteins of the nuclear pore to transport Gsp1p into the nucleus where it is an essential player in nucleocytoplasmic transport
YGL076C	RPL7A	Protein component of the large (60S) ribosomal subunit, nearly identical to Rpl7Bp and has similarity to E. coli L30 and rat L7 ribosomal proteins; contains a conserved C-terminal Nucleic acid Binding Domain (NDB2)
YKL081W	TEF4	Translation elongation factor EF-1 gamma
YLR186W	EMG1	Protein required for the maturation of the 18S rRNA and for 40S ribosome production; associated with spindle/microtubules; nuclear localization depends on physical interaction with Nop14p; may bind snoRNAs
YLR325C	RPL38	Protein component of the large (60S) ribosomal subunit, has similarity to rat L38 ribosomal protein
YLR372W	SUR4	Elongase, involved in fatty acid and sphingolipid biosynthesis; synthesizes very long chain 20-26-carbon fatty acids from C18-CoA primers; involved in regulation of sphingolipid biosynthesis
YML036W	CGI121	Protein involved in telomere uncapping and elongation as component of the KEOPS protein complex with Bud32p, Kae1p, Pcc1p, and Gon7p; also shown to be a component of the EKC protein complex; homolog of human CGI-121
YML063W	RPS1B	Ribosomal protein 10 (rp10) of the small (40S) subunit; nearly identical to Rps1Ap and has similarity to rat S3a ribosomal protein
YMR318C	ADH6	NADPH-dependent medium chain alcohol dehydrogenase with broad substrate specificity; member of the cinnamyl family of alcohol dehydrogenases; may be involved in fusel alcohol synthesis or in aldehyde tolerance
YOL040C	RPS15	Protein component of the small (40S) ribosomal subunit; has similarity to E. coli S19 and rat S15 ribosomal proteins
YOL120C	RPL18A	Protein component of the large (60S) ribosomal subunit, identical to Rpl18Bp and has similarity to rat L18 ribosomal protein; intron of RPL18A pre-mRNA forms stem-loop structures that are a target for Rnt1p cleavage leading to degradation
YPL144W	YPL144W	Putative protein of unknown function; green fluorescent protein (GFP)-fusion protein localizes to the cytoplasm; null mutant is viable, exhibits shortened telomeres

bon/nitrogen limited conditions, at 30°C with a stirrer speed of 800 rpm, pH of 5.0 (maintained by automatic addition of 2 N potassium hydroxide) and dilution rates of 0.03, 0.1 and 0.2 h⁻¹. Aerobic conditions were maintained by sparging the cultures with air (1.0 L min⁻¹) and the concentration of dissolved oxygen was measured with Mettler Toledo polarographic electrode. Anaerobic conditions were maintained by sparging the medium reservoir and the fermentor with pure nitrogen gas (0.5 L min⁻¹). Moreover, oxygen diffusion was minimized by using norprene tubing and butyl septa. The bioreactors were fitted with cooled condensers (2 – 4°C) and the off-gas was led to a gas analyzer (INNOVA and NGA 2000 Rosemount) to measure the content of CO₂ and O₂. Steady-state was reached when at least five residence times had passed since starting the continuous cultivation and carbon dioxide evolution, dry weight measurements, and HPLC measurements of extracellular metabolites were constant.

The experimental work was divided into two efforts. Aerobic cultivations were carried out in the laboratory of Jens Nielsen. Anaerobic cultivations were carried out in the laboratory of Jack T. Pronk. Considerable effort was invested in standardizing the strain, growth conditions, sampling protocols, and analytical procedures. Our groups previously published a report that concluded that microarray experiments in our laboratories were excellently comparable [7]. Triplicate cultivations were carried out for each set of conditions to reduce bias that might unexpectedly arise and to account for biological variance.

Media

The medium composition was as previously described by Tai *et al.* [8]. For N-limited cultivations, residual glucose concentration in the chemostat was targeted to 17 ± 2 g L⁻¹. This was to sustain glucose repression at the same level in all cultivations. The mineral medium composition for

Table 7: Common down-regulated genes among growth rate studies

ORF	Gene Name	Description
YOL153C	YOL153C	Hypothetical protein
YLR345W	YLR345W	Similar to 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase enzymes responsible for the metabolism of fructose-2,6-bisphosphate; mRNA expression is repressed by the Rfx1p-Tup1p-Ssn6p repressor complex; YLR345W is not an essential gene
YGR070W	ROM1	GDP/GTP exchange protein (GEP) for Rho1p; mutations are synthetically lethal with mutations in rom2, which also encodes a GEP
YMR090W	YMR090W	Putative protein of unknown function with similarity to DTDP-glucose 4,6-dehydratases; green fluorescent protein (GFP)-fusion protein localizes to the cytoplasm; YMR090W is not an essential gene
YDR262W	YDR262W	Putative protein of unknown function; green fluorescent protein (GFP)-fusion protein localizes to the vacuole and is induced in response to the DNA-damaging agent MMS; gene expression increases in response to Zymolase treatment
YGL121C	GPG1	Proposed gamma subunit of the heterotrimeric G protein that interacts with the receptor Grp1p; involved in regulation of pseudohyphal growth; requires Gpb1p or Gpb2p to interact with Gpa2p
YIL107C	PFK26	6-phosphofructo-2-kinase, inhibited by phosphoenolpyruvate and sn-glycerol 3-phosphate, has negligible fructose-2,6-bisphosphatase activity, transcriptional regulation involves protein kinase A
YGR087C	PDC6	Minor isoform of pyruvate decarboxylase, key enzyme in alcoholic fermentation, decarboxylates pyruvate to acetaldehyde, regulation is glucose- and ethanol-dependent, involved in amino acid catabolism
YIL017C	VID28	Protein involved in proteasome-dependent catabolite degradation of fructose-1,6-bisphosphatase (FBPase); localized to the nucleus and the cytoplasm
YGL227W	VID30	Protein involved in proteasome-dependent catabolite degradation of fructose-1,6-bisphosphatase (FBPase); shifts the balance of nitrogen metabolism toward the production of glutamate; localized to the nucleus and the cytoplasm

the N-limited cultivations was (amounts per liter): $(\text{NH}_4)_2\text{SO}_4$ 1 g, KH_2PO_4 3 g, K_2SO_4 5.3 g, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 0.5 g, Trace Metal Solution 1 mL, antifoaming agent 0.05 mL and vitamin solution 1 mL. The mineral medium composition for the C-limited cultivations was (amounts per liter): $(\text{NH}_4)_2\text{SO}_4$ 5 g, KH_2PO_4 3 g, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 0.5 g, Trace Metal Solution 1 mL, antifoaming agent 0.05 mL and vitamin solution 1 mL. The inlet glucose concentration was ca. 11 and 25 g L⁻¹ for aerobic and anaerobic experiments, respectively. Moreover, anaerobic cultivation medium was supplemented with Tween 80/ergosterol solution (1.25 mL/L).

Analytical methods

The concentration of biomass at steady-state was determined on a dry weight basis by filtering 5 mL of culture through a pre-weighed 0.45 µm nitrocellulose filter (Gelman Sciences, Ann Arbor, MI). The filter was washed with distilled water, dried in a microwave oven at 150 W for 15 minutes and finally weighed to determine its increase in dry weight. Culture samples (10 mL) for determination of extracellular glucose, succinate, glycerol, acetate, ethanol and pyruvate concentrations were immediately filtered through a 0.2 µm filter (Osmonics, Minnetonka, MN, USA) and the filtrate was stored at -20°C for further analysis. The metabolite concentrations were determined by high pressure liquid chromatography using an Aminex HPX87H column (Biorad) kept at 65°C and eluted at 0.6 mL per minute with H_2SO_4 . Pyruvate was detected spectrophotometrically by a Waters 486 Tunable Absorbance Detector at 210 nm. Glucose, succinate, glycerol, acetate and ethanol were detected by a Waters 410 Differential Refractometer.

RNA sampling and isolation

Samples for RNA isolation from aerobic cultivations were taken by rapidly sampling 20 mL of culture into a tube with 35–40 mL of crushed ice in order to decrease the sample temperature to below 2°C in less than 10 seconds. Cells were then centrifuged (4500 rpm at 0°C for 3 minutes), instantly frozen in liquid nitrogen and stored at -80°C until further use. Sampling for RNA isolations from anaerobic cultivations was performed as described by Piper *et al.* [7].

Total RNA was extracted using FastRNA Pro RED kit (QBiogene, Inc, USA) according to manufacturer's instructions after partially thawing the samples on ice. RNA sample integrity and quality was assessed prior to hybridization with an Agilent 2100 Bioanalyzer and RNA 6000 Nano LabChip kit.

Probe preparation and hybridization to arrays

Messenger RNA extraction, cDNA synthesis and labeling, as well as array hybridization to Affymetrix Yeast Genome S98 arrays, were performed as described in the Affymetrix users' manual [48]. Washing and staining of arrays were performed using the GeneChip Fluidics Station 450 and scanning with the Affymetrix GeneArray Scanner.

Microarray gene transcription analysis

Affymetrix Microarray Suite v5.0 was used to generate CEL files of the scanned DNA microarrays. These CEL files were preprocessed by using gcrma and affy packages [49,50] available in Bioconductor. Raw data was background corrected by using gcrma package and normalized by using qspline method [51]. Probe summarization was

made using only the perfect match (PM) values and median polish settings [52].

Principal Components Analysis (PCA) was performed in order to elucidate the relative importance of the three factors characterizing our experimental design: oxygen availability, nutrient limitation and dilution rate. To select genes whose expression levels were related to these factors, MicroArray Analysis of Variance (MAANOVA) was performed with a mixed model ANOVA with the fixed factors 'oxygen', 'nutrient' and 'dilution rate' and a single random factor, 'sample', representing the biological replicates [53]. Among the various F-tests, the so called F_s was chosen [54] and the q -value method was used to correct for multiple testing [55], which was shown to be less conservative than the FDR methodology described by Benjamini & Hochberg [56]. The threshold of significance was set at 0.02 for a false discovery rate of 2%. MAANOVA is available as a package in Bioconductor and details of the code are given in Additional file 2. Subsequently, in order to group genes with common expression profiles over the dilution rate range, the selected gene lists were clustered using hierarchical clustering (unweighted pair-group average with a non-centric Pearson correlation based distance) and the Gene Ontology of the generated clusters was investigated [57].

Reporter Metabolite analysis

Using the entire gene expression data set, we applied the Reporter Metabolite Algorithm [18] with a newly reported genome-scale metabolic model of yeast (Nookaew *et al.*, submitted). More specifically, the genome-scale model was converted to a bipartite undirected graph. In this graph, each metabolite node has as neighbors the enzymes catalyzing the formation and consumption of the metabolite. The transcriptome data were mapped on the enzyme nodes using the significant values of gene expression. The normal cumulative distribution function was used to convert the p -values to a Z-score. Each metabolite was assigned the average score of its k neighboring enzymes, and this score was then corrected for the background by subtracting the mean and dividing by the standard deviation of average scores of 10,000 enzyme groups of size k selected from the same data set. These corrected scores were then converted back to P values by using the normal cumulative distribution function and the most significant metabolites, Reporter Metabolites, were ranked.

Transcription factor enrichment analysis

For the genes that were found to be differentially transcribed due to growth rate, we investigated if the set of up- and/or down-regulated genes were enriched for regulation by specific transcription factors. Definitions of transcription factor target sets (protein-DNA interactions) were

derived from two different data sources [20,21] at p -value threshold 0.01 for the Harbison *et al.* study and sum of log-likelihood threshold 4 for the Beyer *et al.* study. The hypergeometric test was performed for each TF in each of these 2 set definitions versus the up- and down-regulated genes and the resulting p -values were Bonferroni adjusted.

Abbreviations

FDR: False Discovery Rate; GO: Gene Ontology; MAANOVA: MicroArray ANalysis Of Variance; PCA: Principal Components Analysis; RP: Ribosomal Protein; SGD: *Saccharomyces* Genome Database; TF: Transcription Factor.

Competing interests

The authors declare that they have no competing interests.

Additional material

Additional file 1

Supplementary Figures and Tables. Additional figures and tables about the PCA analysis, consensus cluster analysis and the comparison among the four growth rate studies (Regenberg *et al.*, [12]; Castrillo *et al.*, [13]; Brauer *et al.*, [14]; Fazio *et al.*, [present study]).

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Additional file 2

Supplementary Methods. Details of the R code used for the analysis of CEL files.

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Additional file 3

Gene Lists, Gene Ontology and TF Interactions. This *.xls file contains 7 worksheets: (1) full annotation of the 114 (FDR 2%) growth rate-dependent genes up-regulated at increasing dilution rates; (2) full annotation of the 154 (FDR 2%) growth rate-dependent genes down-regulated at increasing dilution rates; (3) full annotation of the nutrient limitation dependent genes (FDR 2%); (4) full annotation of the oxygen availability dependent genes at (FDR 2%); (5–6) Gene Ontology analysis (Process, Function, Component) of the up/down-regulated growth rate genes performed by using GO Term Finder and GO Slim Mapper available at the *Saccharomyces* Genome Database (SGD) website; (7) Detailed description of the interactions of the transcription factor network presented in figure 4 of the paper.

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Chapter 4

Experimental systems biology: lessons from an integrated, multi-laboratory study in yeast

André B. Canelas², Nicola Harrison⁵, Alessandro Fazio³, Jie Zhang¹, Juha-Pekka Pitkänen⁶, Joost van den Brink², Barbara M. Bakker⁷, Lara Bogner⁸, Jildau Bouwman⁷, Juan I. Castrillo⁵, Ayca Cankorur¹⁰, Pramote Chumnanpuen¹, Pascale Daran-Lapujade², Duygu Dikicioglu¹⁰, Karen van Eunen⁷, Jennifer C. Ewald⁴, Joseph J. Heijnen², Betül Kirdar¹⁰, Ismo Mattila⁶, Femke I. C. Mensonides⁷, Anja Niebel⁸, Merja Penttilä⁶, Jack T. Pronk², Matthias Reuss⁸, Laura Salusjärvi⁶, Uwe Sauer⁴, David Sherman⁹, Martin Siemann-Herzberg⁸, Hans Westerhoff⁷, Johannes de Winde², Dina Petranovic¹, Stephen G. Oliver⁵, Christopher T. Workman³, Nicola Zamboni⁴, Jens Nielsen^{1*}

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¹Department of Chemical and Biological Engineering, Chalmers University of Technology, SE-41296 Goteborg, Sweden

²Department of Biotechnology, Kluyver Centre for Genomics of Industrial Fermentation, Delft University of Technology, Julianalaan 67, 2628 BC Delft, The Netherlands

³Center for Biological Sequence Analysis, Department of Systems Biology, Technical University of Denmark, DK-2800 Kgs. Lyngby, Denmark

⁴Institute for Molecular Systems Biology, ETH Zürich, CH-8093 Zürich, Switzerland

⁵Cambridge Systems Biology Centre & Department of Biochemistry, University of Cambridge, Sanger Building, 80 Tennis Court Road, Cambridge CB2 1GA, UK

⁶VTT Technical Research Centre of Finland, PO Box 1000, FI-02044 VTT, Espoo, Finland

⁷Department of Molecular Cell Physiology, Vrije Universiteit Amsterdam, De Boelelaan 1085, 1081 HV Amsterdam, The Netherlands

⁸Institut für Bioverfahrenstechnik, University of Stuttgart, Allmandring 31, D-70569 Stuttgart, 23 Germany

⁹LaBRI, Laboratoire Bordelais de Recherche en Informatique, UMR CNRS 5800, France

¹⁰Department of Chemical Engineering, Bogazici University, Bebek 34342, Istanbul, Turkey

* Corresponding author: Professor Jens Nielsen (nielsenj@chalmers.se)

Abstract

Despite much progress in the field of systems biology, this field is often held back by difficulties in obtaining comprehensive, high-quality, quantitative datasets. The yeast *Saccharomyces cerevisiae* serves as an excellent model organism in the field of systems biology. It is a widely used model organism for fundamental research and the development of omics technologies was pioneered using yeast as a model organism. Besides being an important model organism *S. cerevisiae* serves as an industrial work horse in the production of fuels, chemicals, food ingredients and pharmaceuticals, and with the current focus on biofuels and sustainability there is much interest in harnessing this yeast as a general platform cell factory. Here we undertook an inter-laboratory effort to generate high-quality quantitative data for a very large number of cellular components in yeast using transcriptome and metabolome analysis. We ensured the high-quality of the experimental data by evaluating a wide range of sampling and measurement techniques. The data were generated for two different yeast strains, each growing under two different growth conditions and based on integrated analysis of the high-throughput data we hypothesize that differences in growth rates and yields on glucose between the two strains are due to differences in protein metabolism.

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There are many definitions and interpretations of systems biology, but most involve mathematical modelling, high-throughput (or omics) analysis, mapping of interactions between cellular components, and quantification of dynamic responses in living cells (Barrett *et al.*, 2006; Brent, 2004; Bruggeman & Westerhoff, 2007; Ideker *et al.*, 2001; Kitano, 2002). In most cases the objective of systems biology is to obtain a quantitative description of the biological system under study, and this quantitative description is ideally in the form of a mathematical model that can be used to simulate the operations of the biological system. Even though some mathematical modelling concepts rely only on limited datasets, (e.g. flux balance analysis) most systems biology efforts will require large sets of high-quality experimental data that enable, for example, to discriminate between different model structures. Generation of such data is therefore the core of many studies that use the systems biology approach. However, the infrastructure and know-how needed to generate the large number of different data required for advanced systems biology studies (e.g. transcriptomics, proteomics, metabolomics) is normally beyond the capabilities of a single lab. There is therefore a trend towards multi-lab collaboration projects and the establishment of curated databases that contain high-quality datasets (Reguly *et al.*, 2006).

In order to ensure proper documentation of experiments, some effort has been directed also at establishing protocol formats, such as MIAME (Minimum Information About a Microarray Experiment) for DNA array experiments (Brazma *et al.*, 2001), MIAPE (Minimum Information About a Proteomics Experiment) and PRIDE (PROteomics IDentification) for proteome analysis (Martens *et al.*, 2005; Taylor *et al.*, 2007),

protocols for microbial metabolome analysis (van der Werf *et al.*, 2007), and even protocols for documentation of mathematical models such as MIRIAM (LeNovere *et al.*, 2005) (Minimum Information Requested In the Annotation of biochemical Models). Even though these protocol formats aim to ensure proper documentation of the actual experiments, there is still a need for consolidation of applied experimental conditions and procedures, in order to allow the generation of increasingly large, coherent datasets for the same organism or strain, that will eventually represent a rich resource for advanced mathematical modelling and contribute to our understanding of the living cell.

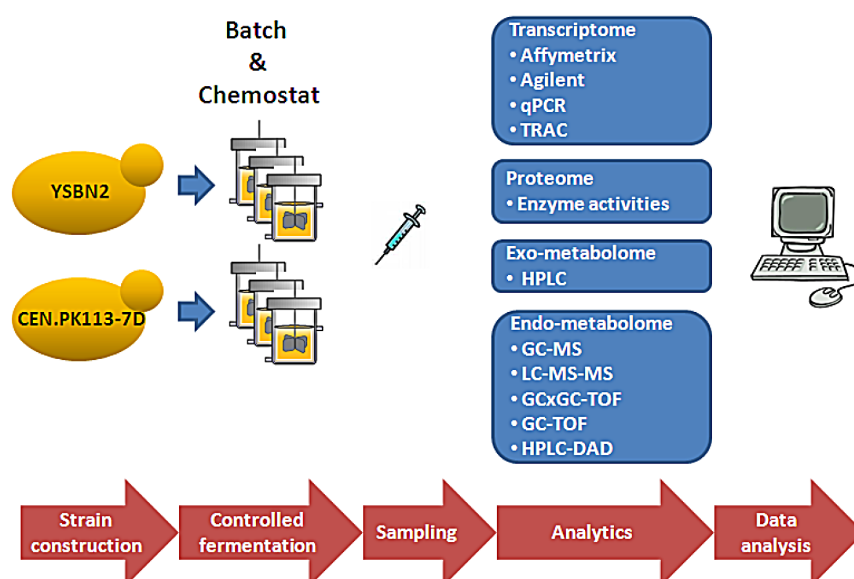


Figure 4.1: Overview of the systems biology pipeline used in this study - Two different haploid yeast strains are grown at two different conditions and samples are taken for several kinds of analysis. In the experiment described here, two yeast strains were used: YSBN2, that is closely related to the originally sequenced yeast strain S288c (for strain construction see Supplementary 1) and CEN.PK113-7D, that is a widely used yeast strain for physiological studies and industrial applications. Each strain was grown in bioreactors, both under substrate excess growth conditions (batch) and also under glucose-limited conditions (chemostat) (see Supplementary 2 for details). These fermentations were carried out in biological triplicates at a single location (TU Delft) and by using highly controlled bioreactors it was possible to obtain reproducible measurements of key physiological parameters, such as specific growth rate, nutrient uptake rates and product formation rates (see Supplementary 3). A fast sampling protocol was carefully designed (see Supplementary 4) taking into account the large amounts of samples needed as well as the turnover of the different types of molecules to be analyzed. After sampling and conditioning the different samples were shipped to the different laboratories involved in this project for analysis of the transcriptome, the metabolome and enzyme activities. Finally the results were collected and integrated, resulting in a very thorough phenotypic characterization of the two strains.

The Yeast Systems Biology Network (YSBN) therefore undertook a major effort on consolidating and comparing experimental conditions, procedures and protocols applied for the experimental part of yeast systems biology in 10 different European laboratories, and at the same time performed a comparative analysis of different quantitative analytical methods. This has resulted in establishment of a well documented experimental systems biology pipeline that is illustrated in **Figure 4.1**. The pipeline allows for the comparison of different yeast strains or the comparison of a single yeast strain grown under different conditions. Here we evaluated the pipeline by comparing two different yeast strains grown at two different conditions in bioreactors, namely a traditional batch culture (nutrient excess) and a glucose-limited chemostat culture (specific growth rate controlled by the rate of supply of the limiting nutrient, glucose). The generated data will represent valuable reference data for the two strains and two standard conditions and hence advance the field of yeast systems biology. Furthermore, we were interested to evaluate whether high-throughput data can be used to generate a hypothesis for explaining differences in overall phenotypes, i.e. growth rate and biomass yield on glucose, and hence be used to direct further experimental work.

The first step in the establishment of our experimental systems biology pipeline was to find appropriate yeast strains that would be of interest. In the yeast community a range of different yeast strains are being employed, with the strain series BY, W303 and CEN.PK being the most frequently used (van Dijken *et al.*, 2000). The BY strain series is a derivative of the originally sequenced strain S288c, for which there is a complete gene knockout collection. The CEN.PK strain series is used widely in physiological studies and, thanks to its rapid growth, it is also often used for metabolic engineering studies (van Dijken *et al.*, 2000). Physiological studies are generally best performed with prototrophic strains, whereas the BY series strains carry a number of auxotrophies that may cause problems for quantitative studies of the cellular physiology. There was hence a need to generate prototrophic strains in the S288c background that nonetheless carried some genetic markers that would permit checks against contamination, in large-scale or long-term cultures, and facilitate subsequent genetic crosses (for instance of evolved derivatives). For this reason, two diploid strains, YSBN1 and YSBN2, were generated from FY1 and FY2, two uracil auxotrophic strains that are direct derivatives of S288c (Winston *et al.*, 1995). FY2 is the strain from which the BY strain series (Brachmann *et al.*, 1998) and, hence, the strain from which the complete knockout collection (Giaever *et al.*, 2002) was derived. YSBN1 and YSBN2 are prototrophic strains that carry drug-resistance markers inserted into their genomes at a phenotypically neutral site (Baganz *et al.*, 1998; Oliver *et al.*, 1998) (Supplementary 1).

In order to evaluate the newly constructed strain we performed a detailed comparative analysis of the YSBN2 strain with the widely used strain CEN.PK113-7D. Each strain was grown in batch cultures, which were sampled in the mid-exponential growth phase (on glucose). After the diauxic shift and the ethanol growth phase the cultures were used to initiate chemostat cultivations, which were sampled after steady-state conditions were achieved. The fermentations were carried out in triplicate with each strain and each condition, resulting in a total of 12 samplings. The detailed cultivation proto-

cols are given as Supplementary 2. The experiments were conducted in well controlled bioreactors in a single laboratory, which ensured a very high degree of reproducibility (Supplementary 3), and samples were then shipped to different laboratories for analysis (see Table 4 in Supplementary 4). From the initial analysis it is interesting to observe that the maximum specific growth rate of the CEN.PK strain is significantly higher than that of the YSBN strain, by approximately 25%, whereas its biomass yield under carbon-limited conditions (chemostat) is significantly lower, by approximately 10%. Thus, one could speculate whether CEN.PK ability to grow faster when resources are abundant has come at the expense of efficiency in carbon and energy utilization under nutrient limitation.

Our analysis involved sampling for analysis of: 1) mRNA, using DNA arrays (Affymetrix and Agilent), qPCR and TRAC (TRanscript analysis with Affinity Capture) (Rautio *et al.*, 2006); 2) enzyme activities, using optimized and ‘*in vivo*-like’ assays; and 3) endometabolome, using several analytical platforms including LC-MS/MS, GC-TOF, GCxGC-TOF, NMR, HPLC-DAD and enzymatic analysis. Table 1 in Supplementary 4 gives an overview of the sampling procedure, which was designed taking into account the large number of samples needed and the fast turnover of some molecules to be analyzed (e.g. intracellular metabolites). Fast sampling and parallel sample processing required a team of 5 people, of which 3 carried out the same key steps in all 12 samplings. Videos demonstrating the sampling are available at www.sysbio.se/supp. Detailed protocols of the sampling and processing methods are given in Supplementary 4.

Transcripts were measured using a range of different methods. Genome wide analysis was performed using both the Affymetrix and the Agilent platforms (Supplementary 5). qPCR was performed in two different laboratories and was used to quantify the expression of a number of selected genes (Supplementary 6 and Supplementary 7). TRAC analysis was also performed, as it allows for multiplex detection of mRNA targets simultaneously from a large number of samples (Supplementary 8). Comparison of mRNA levels determined in four different labs using the four different analytical methods shows a good overall consistency (Figure 5 in Supplementary 5). qPCR analysis performed in one of the labs was used to check the consistency of 33 genes that were found to have significantly changed expression based on an ANOVA analysis of the Affymetrix data. This analysis shows very clearly that when high-quality platforms for genome-wide transcription analysis are used one obtains equally quantitative information as by qPCR (Table 7 in Supplementary 7), both in terms of significance and changes in expression level (Figure 5 in Supplementary 5). The TRAC analysis also showed a fairly good consistency with the DNA array data, but about 15% of the analyzed transcripts had a very poor correlation, based on analysis of the four different samples (2 strains x 2 conditions), and another 15% had a Pearson correlation coefficient lower than 0.85 (Table 2 in Supplementary 8). We did not find a linear correlation between the Affymetrix and the Agilent data (Figure 1 and Figure 5, Supplementary 5), but this can be explained by the use of a different dynamic scanning range by the Agilent scanner. However, the significant genes identified by ANOVA analysis using the two

platforms were fairly consistent, i.e. out of a total of 410 transcripts found to be significantly changed in response to growth conditions (including both strains and both array platforms) 241 transcripts were found consistently by both platforms (Figure 2 in Supplementary 5). Thus, we conclude that both platforms are equally strong, but in light of the large datasets available already with the Affymetrix we recommend the use of this platform as this will allow for further expansion of the already large database of Affymetrix based transcriptome data.

We also performed activity measurement of key glycolytic enzymes in cell extracts. Measurements carried out in two different laboratories yield a good overall consistency in the data (Figure 1 and 2, in Supplementary 9). In connection with the experiment we also evaluated a new approach to quantify enzyme activities using assay conditions designed to better represent *in vivo* conditions, as opposed to the typical approach of using optimal conditions for each enzyme (Figure 3 in Supplementary 9). We found that with the '*in vivo*-like' assays the range of enzyme activities was in the same order of magnitude as the glycolytic flux, which was not observed using traditional analysis of glycolytic enzyme activities.

Metabolome samples were analyzed independently by seven participating labs according to their own standard operating procedures (see Supplementary 9). A total of four variations in extraction protocol, five analytical platforms (LC-MS/MS, GC-TOF, GCxGC-TOF, enzymatic, HPLC-DAD), and three internal standard strategies were used in different combinations. The interlab comparison shows that absolute concentration estimates from different labs can vary by up to 3-fold, even for identical sample processing. Interestingly, relevant metabolite ratios (e.g. mass action ratios) based on measurements from different labs were comparable. Furthermore, when comparing only the relative concentration differences between growth conditions or strains, all labs deliver a surprisingly consistent picture. Since this redundancy was obtained with heterogeneous preparation protocols and orthogonal analytical methods we conclude that, even with the current state-of-the-art metabolomics, ratio-metric measurements still have much higher confidence than absolute estimates.

Integrated data analysis started with looking at the exo-metabolome data, which provided information about the gross phenotype. Through measurements of all key nutrients and metabolites being secreted or released into the medium (including analysis of the gas-phase) it was possible to obtain very precise measurements of the overall metabolic fluxes and these are summarized in **Figure 4.2A**. Under substrate-excess growth conditions (batch cultivation) it was observed that the CEN.PK113-7D strain exhibited a $\geq 20\%$ higher glucose uptake rate, which was accompanied by a higher flux towards ethanol. The specific carbon dioxide production not associated with ethanol production was the same in the two strains, despite the fact that the specific oxygen uptake rate was more than 2-fold higher in the CEN.PK113-7D strain compared to the YSBN2 strain. This suggests that the higher oxygen uptake rate allowed for a reduced glycerol production by the CEN.PK113-7D strain, as overflow towards glycerol production under fermentative growth conditions serves as an alternative NADH sink. The higher glycolytic flux in the CEN.PK113-7D strain was not associated with

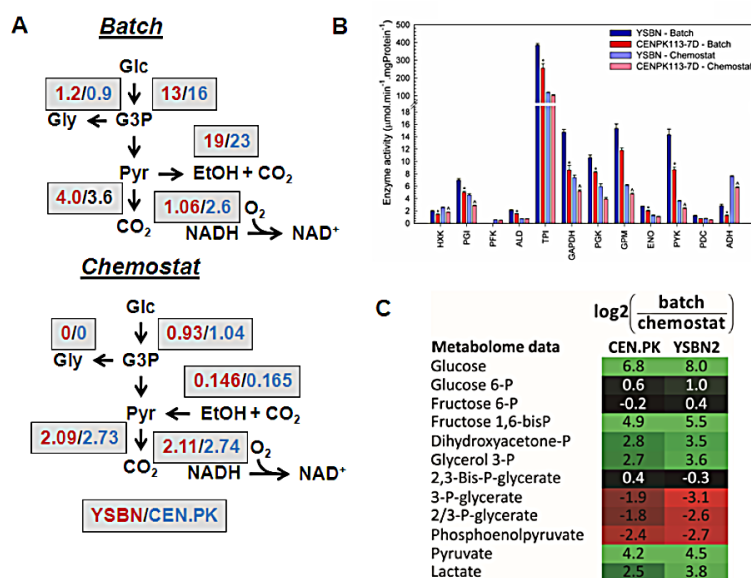


Figure 4.2: Overall fluxes in the strains CEN.PK113-7D and YSBN2 in batch and chemostat cultivations - (A) From measurements of the concentrations of biomass, glucose, ethanol and glycerol in the medium and of carbon dioxide and oxygen in the exhaust gas, the overall exchange rates can be calculated (all data are given in Supplementary 2). From the overall fluxes it is observed that the CEN.PK113-7D strain (blue data) has a higher glucose uptake rate, a higher flux towards ethanol and a higher oxygen uptake rate, whereas the carbon dioxide production rate is about the same for the two strains (the flux towards CO₂ is corrected for the amount of CO₂ formed in relation to ethanol production). However, YSBN2 has a higher glycerol production rate in the batch fermentation. In the chemostat culture the biomass yield is higher for the YSBN2 strain (red data) and this is accompanied by higher specific fluxes in the CEN.PK113-7D strain. (B) The higher glycolytic flux in the CEN.PK113-7D strain at substrate excess growth conditions (batch) is not associated with increased activities of glycolytic enzymes. For practically all of the glycolytic enzymes there was a higher activity in the YSBN2 strain compared with the CEN.PK113-7D strain (results were confirmed by measurements in two independent laboratories, see Supplementary 8). In the comparison between growth conditions, the higher glycolytic flux in batch culture compared with the chemostat is associated with higher enzyme activities for practically all glycolytic enzymes in both strains. However, this increase of more than 10-fold in glycolytic flux is accomplished with relatively modest changes in enzyme activities, pointing to a substantial element of metabolic regulation. (C) This is supported by measurement of glycolytic metabolites, where it is found that for both strains there is an increase in most of the glycolytic metabolites in the batch culture compared with the chemostat (green bars indicate increased metabolite levels, see Supplementary 11 for more details). Thus, the dramatic increase in glycolytic flux in yeast when there is a shift from glucose limitation to glucose excess is due partly to an increase in the activity of the glycolytic enzymes, but also due to a kinetic effect resulting in increased levels of most glycolytic metabolites. This finding is consistent with earlier more detailed studies on glycolysis (Daran-Lapujade *et al.*, 2007; Postmus *et al.*, 2008; Rautio *et al.*, 2006; van Hoek *et al.*, 2000). The metabolite data, however, do not provide any insight into the differences in fluxes between the two strains, as there is very small differences in metabolite levels between the different strains, and most differences indicate slightly higher metabolite concentrations in the YSBN2 strain, which has lower fluxes.

a higher activity of glycolytic enzymes or large differences in the levels of glycolytic intermediates (**Figure 4.2B-C**), which indicates that the difference in glycolytic flux is due to regulation at the metabolic level. In contrast, in both strains we found higher enzyme activities under batch conditions than in the chemostat. However, since the enzyme activity level was only about 50% lower in the chemostat compared with the batch cultures, it is clear that enzyme activities can not alone describe the almost 10-fold lower glycolytic flux in the chemostat. This indicates that the adjustment of the glycolytic flux to fermentative conditions is to a large extent determined by changes in the levels of metabolic intermediates and effectors, i.e. flux control is primarily at the metabolic level. This observation is compatible with a general pattern of metabolite-dominated regulation in the central metabolism of yeast (Daran-Lapujade *et al.*, 2007; Postmus *et al.*, 2008; van Hoek *et al.*, 2000). Finally, the higher activity of glycolytic enzymes in the batch cultures is associated with increased expression of several glycolytic genes (*PFK1*, *TPI1*, *ENO2*, *TDH2*, *TDH3*, *PYK1*, *PDC1*, *ADH1*) (Supplementary 6). However, the slightly higher enzyme activities found in the YSBN2 strain (compared to CEN.PK113-7D) are not associated with higher expression of the respective genes.

Although there is no direct correlation between fluxes, enzyme activities and intracellular metabolite levels, it is striking that under batch conditions the levels of practically all amino acids were noticeably higher in CEN.PK113-7D than in the YSBN2 strain. This led us to perform a more detailed analysis of the gene expression data. Using methods for integrative analysis (Patil & Nielsen, 2005; van den Brink *et al.*, 2008) (see Supplementary 11), we calculated enriched GO-terms for the transcripts differing significantly between the two strains at both growth conditions, as well as reporter metabolites (Patil & Nielsen, 2005), and reporter transcription factors (Oliveira *et al.*, 2008). These methods allow for identification of transcriptional hot-spots in metabolic networks, i.e. metabolites around which there are large transcriptional changes, and transcription factors that drive key transcriptional responses. The results of this analysis are shown in **Figure 4.3**. For the batch cultures the analysis points to a clear effect on amino acid transport, with several amino acid transporter genes being differentially expressed between the two strains (most transporters being expressed at a higher level in YSBN2). Also there is a distinct nitrogen catabolite repression response in the YSBN2 strain in the batch culture with Gln3 and Gat1 being identified as reporter transcription factors, i.e. transcription factors that control a set of genes that have significant transcriptional changes. Thus, there are clearly differences in how the two strains control amino acid biosynthesis. YSBN2 expresses many amino acid transporters even when growing on minimal medium (without amino acid supplementation) and CEN.PK113-7D is able to maintain higher intracellular amino acid pools. The latter may be important for ensuring efficient loading of the tRNAs needed for protein biosynthesis, which in turn could be the basic explanation for the higher specific growth rate of CEN.PK113-7D.

We could not identify a direct explanation for the higher glucose uptake of CEN.PK113-7D and the differences in overall fluxes through the central carbon metabolism between the two strains. As mentioned previously, several studies have shown that there is

Batch				Chemostat			
GO terms ¹	Reporter metabolite	Reporter TF	ORFs	GO term	Reporter metabolite	Reporter TF	ORFs
Response to pheromone		CBK1, FUS3	FUS3, FUS1, STE5, MFA1, STE2, AGA2, BAR1, PRM10, FAR1, STE18, SST2, STE4	protein catabolism		RPN4	PIM1, PRE7, VID24, UMP1, RPN6, CDC48, RPT3, RPN9, RAD23, PRB1, RPN11, PRE4, SCL1, RPT6, UFD1, PUP2, ECM29, RPN1, PRE3, UBX6, LAP4, PRE8, PRE5, YDJ1, PUP1, RPT4, RPN8, PRE2, RPN7, SUE1
Sterol biosynthesis	(R)-Mevalonate, Episterol	MOT3	ERG28, ERG26, ERG3, HMG1, ERG13, ERG2, CYB5, MVD1	amino acid biosynthesis	Cys, Gly, Asn, Leu	GCN4	TRP4, SAM2, GLY1, HIS1, ARG5,6, AGX1, MET13, STR3, SER2, ARG3, BAT2, ALT1, PUT1, SAM1, ECM40, LEU4, ARG1, ARG8, LEU9, ASN1
Amino acid transport	Leu, Phe, Ile, Val, Tyr, ALAxt, Asn, GLNxt, Cys, METxt, Trp, Gly, THRxt, PROxt, HISxt *	GLN3, GAT1	BAP3, CAN1, AGP2, BAP2, PUT4, SAM3, DIP5, AGP1, GAP1	heterocycle metabolism			URA7, HEM12, TRP4, ARO10, ADE8, SAM2, HEM2, SDT1, ADE5,7, PDC6, MTD1, SAM1, HMX1, ADH6, AAH1, BIO3, ADE2, COX10
				proteolysis		RPN4	PIM1, PRE7, VID24, UMP1, PRD1, RPN6, CDC48, RPT3, RPN9, RAD23, RPN11, PRE4, SCL1, RPT6, UFD1, PHB2, PUP2, RPN1, PRE3, UBX6, PRE8, YTA12, PRE5, YDJ1, PUP1, RPT4, RPN8, PRE2, RPN7
Trehalose metabolism	α,α'-Trehalose 6-P, α,α-Trehalose	TPS2	NTH1, TPS2, UGP1, TSL1, PGM2	sterol biosynthesis	5-Diphosphomevalonate		ERG26, ERG25, ERG1, NCP1, ERG7, ERG9, ERG20, ERG27, ERG6, HMG1, ERG13, ERG5, ERG2, ERG12, ERG8, CYB5, NSG2, ERG24, MVD1
				allantoin metabolism	Allantoin		DUR1,2, DAL1, DAL4, DAL2, DAL7, DAL3
				cytokinesis		CBK1	BOI2, DSE1, SCW11, MYO1, DSE2, CTS1, DSE4

Figure 4.3: Overview transcriptome analysis results obtained from the comparison between CEN.PK113-7D and YSBN2 strains - Representative GO terms, reporter metabolites, reporter TF and ORFs that were significantly changed between the two strains in the two different growth conditions. The color indicates the directional change (in red, genes being expressed higher in the YSBN2 strain; in green, genes being expressed lower in the YSBN2 strain). Complete lists are given in Supplementary 12. The representative GO terms were identified using hypergeometric tests on the genes that had significant differences in expression for the two strains. The reporter metabolites, calculated using the algorithm of Patil & Nielsen (2005), indicate locations in the metabolism around which there are large transcriptional differences between the two strains. The reporter TFs, calculated using the algorithm of Oliveira *et al.* (2008), indicate transcription factors for which there are significant changes in expression of the genes they are controlling. Finally the significant ORFs indicate representative examples of ORFs with significantly changed expression between the two strains. Abbreviation of amino acids follow standard nomenclature. Addition of xt marks that only the extracellular amino acid was identified as a reporter metabolite, whereas for those marked without xt both the intra- and extracellular amino acid was found to be a reporter metabolite.

complex regulation of the glycolytic flux, and that over-expression of glycolytic genes in yeast does not result in an increased flux through this pathway (Schaaff *et al.*, 1989; Smits *et al.*, 2000). A hypothesis for the the higher glucose uptake of CEN.PK113-7D could, however, come from the differences in protein biosynthesis in the two strains. Protein synthesis is expensive in terms of Gibbs free energy provided in the form of ATP, and the increased glycolytic flux in the CEN.PK113-7D strain comes around from a pull of ATP and other co-factors needed for biomass production. Changes in protein biosynthesis, protein catabolism and proteolysis in the two strains could also explain the lower biomass yield of CEN.PK113-7D in chemostat culture compared with the YSBN2 strain. Thus, we find GO terms on protein catabolism and proteolysis and a large number of genes associated with these terms were significantly higher expressed in the CEN.PK113-7D strain compared with the YSBN2 strain. This is clearly linked to the transcription factor Rpn4, that is regulating the 26S proteasome. This indication of increased protein turn-over in CEN.PK113-7D is also consistent with our finding of a Gcn4 response in that strain. Gcn4 is a transcription factor that positively regulates the transcription of a large number of genes which encode for amino acid biosynthetic enzymes. In fact, a large number of these genes are also indentified as being significantly higher expressed in CEN.PK113-7D compared with YSBN2. Taken together, these observations strongly point to higher amino acid and protein biosynthesis, as well as increased protein degradation in CEN.PK113-7D (see **Figure 4.4**). Since amino acid synthesis and polymerization represent the most energetically costly processes in biomass formation, a higher rate of protein turn-over in the CEN.PK113-7D strain could certainly explain the lower biomass yield. This provides a molecular hypothesis for the general statement made above; the CEN.PK113-7D strain has a more efficient machinery for rapid protein biosynthesis and it can thereby grow faster, but the effect of this is a less efficient utilization of the carbon and energy source at limited conditions. This is consistent with thermodynamic analysis of microbial growth, where it is found that there is trade off between the Gibbs free energy dissipation as a driving force for growth and the biomass yield (von Stockar *et al.*, 2006). The changes observed in the CEN.PK113-7D strain are summarized in **Figure 4.4**, where it is illustrated that increased activity of protein catabolism and proteolysis results in the formation of a futile cycle where there is a net consumption of ATP, and this imposes a requirement for increased catabolism and respiration that ensures the supply of ATP.

Finally it can be mentioned that we observed that, at both growth conditions, there was a higher expression of genes involved in sterol biosynthesis in CEN.PK113-7D, and this is consistent with findings that the level of ergosterol is significantly higher in CEN.PK113-7D than in the S288c strain, to which the YSBN2 strain is closely related (data not shown).

In conclusion we demonstrate that through integrative analysis of comprehensive datasets it is possible to suggest molecular explanations for observed phenotypes that would not be possible from a single omics dataset. Thus, systems biology analysis can be used to provide a hypothesis for differences in gross phenotypes such as growth rate and biomass yield on glucose, and through follow-up studies where specific molecular

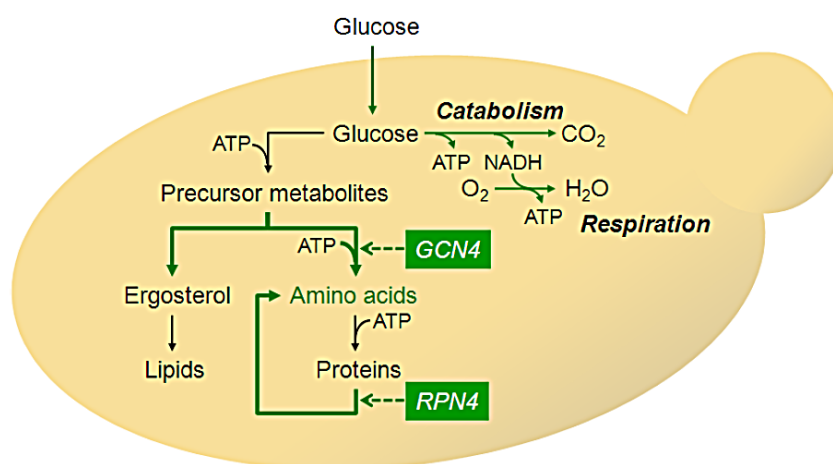


Figure 4.4: Summary of changes in metabolism in the YSBN2 strain compared with the CEN.PK113-7D strain in chemostat culture - It is seen that there is down-regulation of amino acid biosynthesis, protein catabolism and proteolysis in the YSBN2 strain compared with the CEN.PK113-7D strain (pathways that are transcriptionally down regulated are marked in green bold line). This is likely to result in less futile cycling of amino acids in the YSBN2 strain, with a reduced net consumption of ATP associated with biomass production. This results in a higher biomass yield as there is a more efficient utilization of the ATP generated in the catabolism (decreased flux as marked by green thin line) and hence relatively more glucose can be shunted towards biomass production.

processes are analyzed, the provided hypothesis can be evaluated resulting in novel biological insight. Furthermore, our interlaboratory comparison of different methods and the detailed protocols provided, allows implementing the appropriate analytical platform in connection with systems biological studies of yeast in different laboratories. Finally, we are confident that our interlaboratory comparison of different experimental methods for omics analysis provides very useful reference datasets for two yeast strains, and these reference datasets will allow further advancement of yeast systems biology. This holds in particular in connection with further use of the newly-constructed YSBN strains that represent a valuable resource for the yeast systems biology community as they are prototrophic (thus suitable for physiological studies) and yet closely related with the widely used BY-strain series.

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Supplementary material

The supplementary material section is too extensive to be included in this thesis. It can be found at the following website: www.sysbio.se/supp.

Chapter 5

The role of protein kinases and phosphatases in the transcriptional response to DNA damaging agent MMS in *Saccharomyces cerevisiae*

Alessandro Fazio¹, Tejal Joshi¹, Honey Polur¹, Laurent Gautier^{1,2} and Christopher T. Workman^{1*}

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¹*Center for Biological Sequence Analysis, Department of Systems Biology, Technical University of Denmark, DK-2800 Kgs. Lyngby, Denmark*

²*DTU Multiassay Core (DMAC), Technical University of Denmark, DK-2800 Kgs. Lyngby, Denmark*

* *Corresponding author: Assoc. Prof. Christopher T. Workman (workman@cbs.dtu.dk)*

Abstract

Saccharomyces cerevisiae responds to DNA damage by activating specific cell cycle checkpoints, that arrest/delay cell cycle phases in order to promote DNA repair and ensure viability. The activation of checkpoints relies on kinase cascades and regulation of transcription is one of the functions of these checkpoints. In this study, we have used tiling arrays to investigate the transcriptional role of protein kinases (PKs) and phosphatases (PPs) during damage response elicited by the alkylating agent methyl methanesulfonate (MMS). Mec1, Tel1, Rad53 (PKs) and Oca1 (PP) appeared to have primary roles in transcriptional regulation. We have integrated our results with phosphorylation, protein-protein interaction and transcription factor regulation data in order to build a comprehensive view of the MMS-induced DDR network from which regulatory circuits could be inferred. Moreover, MMS sensitivity data were obtained and integrated with the transcriptional response in order to find genes whose expression levels were correlated with relative growth rates upon MMS treatment. We have also investigated the function of non protein-coding RNA during damage response. In summary, this study has adopted a systems biology approach to provide new insights into the response to MMS-induced damage in the yeast *S. cerevisiae*.

Introduction

In response to DNA damage, the yeast *Saccharomyces cerevisiae* activates a plethora of mechanisms in order to ensure cell viability and genome stability. These mechanisms depend on specific cell cycle checkpoints, activated by pathways in which protein kinases and phosphatases play critical roles (Harrison & Haber, 2006; Heideker *et al.*, 2007; Kolodner *et al.*, 2002; Nyberg *et al.*, 2002; Zhou & Elledge, 2000).

Regulation of transcription is one of the cellular responses to DNA damage. The identification and characterization of genes involved in *S. cerevisiae* DNA damage response was initially carried out with single gene studies and early genome-wide approaches, that led to the discovery of the so-called damage inducible (*DIN*) and DNA damage response (*DDR*) genes (Gailit, 1990; McClanahan & McEntee, 1984; Ruby & Szostak, 1985; Ruby *et al.*, 1983). With the advent of the DNA microarray technology, more detailed and comprehensive studies have become possible.

The first genome-wide transcriptional study about *S. cerevisiae* DDR was performed using methyl methanesulfonate (MMS) as damaging agent (Jelinsky & Samson, 1999). This alkylating agent causes the activation of the S-phase checkpoint (Branzei & Foiani, 2009; Putnam *et al.*, 2009; Segurado & Tercero, 2009). It was found that more than 5% of the transcripts were regulated by MMS and more than 100 genes had higher induction than *MAG1*, a gene with a well-known transcriptional regulation upon DNA damage (Chen & Samson, 1991). The main finding of this study was that not only DDR genes were regulated (e.g., *MAG1*, *RAD7*, *RNR3*, *NTG1*), but also genes involved in protein metabolism, suggesting yeasts activate a response to degrade and replace damaged proteins (Burgis & Samson, 2007). As a follow-up study, the transcriptional response of several different DNA damaging agents was evaluated. Every agent gave a distinct signature response and the number of regulated genes was not a predictor of toxicity (Jelinsky *et al.*, 2000). This study confirmed that many genes with significantly changed expression were involved in protein and RNA metabolism, while fewer were involved in DNA repair, DNA replication and cell cycle. Promoter analysis suggested that the regulation of *MAG1* may be a function of the transcription factor Rpn4, already known to regulate proteasome subunit genes. Thus, this dual role of Rpn4 may explain the integration of signals from damaged DNA and proteins. Moreover, responses to MMS were found to overlap with genes regulated during stationary phase and amino acid starvation response (Jelinsky *et al.*, 2000; Natarajan *et al.*, 2001).

The effects of *MEC1*, *DUN1* and *RFX1* (*CRT1*) deletions were investigated in another study (Gasch *et al.*, 2001). Although the transcriptional response to DNA damaging agents (MMS and IR) partly overlapped with the general yeast environmental stress response (ESR) (Gasch *et al.*, 2000), the authors were able to identify a set of genes specifically responding to DNA damage and whose expression was dependent on functional Mec1 (*RNR2*, *RNR4*, *RAD51*, *RAD54*, *DUN1*, *PLM2*, *DIN7*, *YER004W*, *YBR070C*). Importantly, the induction of the ESR genes was Mec1-dependent, implying that checkpoint activation plays a role in other cellular responses. With respect to transcriptional response to MMS, several other microarray studies have been conducted

(Benton *et al.*, 2006; Caba *et al.*, 2005; Fu *et al.*, 2008; Hughes *et al.*, 2000; Oshiro *et al.*, 2002; van Attikum *et al.*, 2004; Workman *et al.*, 2006). In summary, the transcriptional response to DNA damage provides evidence that macromolecules other than DNA are damaged and suggests a link between DNA repair and proteasome activity. Moreover, different DNA damaging agents produce distinct responses, which partly overlap with the yeast ESR.

Several studies have merged the transcriptional response with phenotypic screening under damaging conditions, revealing that transcriptional responsive genes do not necessarily impair growth, in that particular condition, when deleted. In other words, there is a lack of correlation between transcriptional responsiveness and phenotypic sensitivity and this may be due to the complexity of the DDR pathways (Begley *et al.*, 2002; Birrell *et al.*, 2002; Haugen *et al.*, 2004; Hughes *et al.*, 2000).

Recent gene expression studies based on genome-wide transcription measurement tools such as tiling arrays (David *et al.*, 2006; Davis & Ares, 2006; Granovskaia *et al.*, 2010; Samanta *et al.*, 2006) have shown evidences of transcription within intergenic as well as protein-coding regions (e.g. antisense transcripts). Although the roles of these transcript regions are not very clear, some of them are evolutionarily conserved across several yeast species and have been shown to have secondary structures by Steigele *et al.* (2007). In the present study, we have used high-resolution tiling arrays to study the transcriptional response to the alkylating agent MMS in *S. cerevisiae* deletion mutants lacking protein kinases and phosphatases involved in the DDR pathway. In particular, both central players (*MEC1*, *TEL1*, *RAD53*, *CHK1*, *DUN1*, *PTC3*, *PPH3*) known to be involved in response to MMS and genes with a less understood role in DDR (*ALK1* and *OCA1*) have been taken into consideration. Moreover, the sensitivity of these strains to different MMS concentrations has been investigated. By integrating data from different cellular levels, a comprehensive network of *S. cerevisiae* response to MMS has been built in order to unravel new regulatory mechanism and extend the view proposed in an earlier study (Workman *et al.*, 2006).

Materials and methods

Yeast strains and cultures *S. cerevisiae* strains used in this study are listed in **Table 5.1**. Strains were grown in Synthetic Defined (SD) media, having the following composition (amount per liter): 1.7 g Yeast Nitrogen Base (YNB) without amino acids and ammonium sulfate (Sigma, Cat. No. Y1251), 5 g ammonium sulfate (Fluka, Cat. No. 09982), 100 mL 10X-stock of amino acids, 20 g D(+)-glucose monohydrate (Fluka, Cat. No. 49159). The 10X-stock of amino acids contained (amount per liter): 0.3 g isoleucine, 1.5 g valine, 0.2 g arginine, 0.2 g histidine, 1 g leucine, 0.3 g lysine, 0.2 g methionine, 0.5 g phenylalanine, 2 g threonine, 0.4 g tryptophane, 0.3 g tyrosine, 0.2 g uracil, 1 g glutamic acid, 1 g aspartic acid and, in addition, 0.4 g adenine. We used a SD media in order to avoid effects depending on poor media chemical definition (e.g., YPD), considering that it has been shown that media can affect MMS response (Kitanovic *et al.*, 2009). Precultures (10 mL) were grown over-night in test tubes at

Table 5.1: Yeast strains used in this study. BY4741 and W1588-4C are the reference strains (WTs).

Strain	Genotype
BY4741	<i>MATa his3-Δ1 leu2-Δ0 met15-Δ0 ura3-Δ0</i>
W1588-4C	<i>MATa ade2-1 can1-100 leu2-3,112 trp1-1 ura3-1 his3-11,15</i>
<i>alk1Δ</i>	BY4741 <i>alk1::KAN</i>
<i>alk2Δ*</i>	BY4741 <i>alk2::KAN</i>
<i>atg1Δ*</i>	BY4741 <i>atg1::KAN</i>
<i>chk1Δ</i>	BY4741 <i>chk1::KAN</i>
<i>dbf2Δ*</i>	BY4741 <i>dbf2::KAN</i>
<i>dun1Δ</i>	BY4741 <i>dun1::KAN</i>
<i>mec1Δ</i>	W1588-4C <i>mec1::TRP1 sml1::HIS3</i>
<i>oca1Δ</i>	BY4741 <i>oca1::KAN</i>
<i>pph3Δ</i>	BY4741 <i>pph3::KAN</i>
<i>prk1Δ*</i>	BY4741 <i>prk1::KAN</i>
<i>ptc3Δ</i>	BY4741 <i>ptc3::KAN</i>
<i>rad53Δ</i>	W1588-4C <i>rad53-1 sml1::HIS3</i>
<i>tel1Δ</i>	BY4741 <i>tel1::KAN</i>

* for these strains, only the phenotypic response to MMS was investigated.

30°C with shaking (150 rpm). Cultures were then inoculated (total volume 120 mL) and grown in 500 mL-shake flasks with baffles (Schott Duran) at 30°C with shaking (120 rpm). When cells reached mid-exponential phase ($OD_{600} \sim 0.8$), cultures were split into two: freshly-prepared methyl methanesulfonate (MMS; Sigma, Cat. No. M4016) was added directly to one culture (MMS final concentration 0.025% v/v), while the other culture was left untreated. Cultures were grown for an additional hour and then pelleted by centrifugation (3 min, 5,000 rpm, room temperature). Pellets were snap frozen instantaneously in liquid nitrogen and then stored at -80°C.

RNA extraction and microarray Total RNA was extracted by using the TRIzol reagent (Invitrogen, Cat. No. 15596-026) and the TissueLyser II (Qiagen). Pellets were resuspended in 800 μ L of TRIzol reagent and transferred in 1.5 mL-screw cap tubes containing 600 μ L acid-washed beads (425-600 μ m; Sigma, Cat. No. G8772). Tubes were placed in the TissueLyser II blocks (kept at -20°C) and the instrument was operated at 50 Hz for 5 min. The tubes containing the disrupted and homogenized cell extracts were immediately centrifuged (15,000 g, 20 min, 4°C) to separate the cell debris. Top phases were transferred into new screw-cap tubes and let stand for 5 min before adding 200 μ L 99% chloroform; tubes were vortexed for 15 s, let stand for 3 min and centrifuged (15,000 g, 15 min, 4°C). Top phases were again transferred to new tubes and another chloroform extraction performed. After centrifugation, upper phases were transferred to new tubes and 500 μ L of 80% cold ethanol were added. Samples

were mixed by inversion and then placed at -20°C for 2 hours. Tubes were centrifuged (15,000 g, 30 min, 4°C) and the precipitated RNA was washed once with $500\ \mu\text{L}$ of 80% cold ethanol. After centrifugation (15,000 g, 5 min, 4°C), supernatant was removed and pellet air-dried for 10-20 minutes. RNA was resuspended in RNase-free water and treated with DNA-free (Ambion, Cat. No. AM1906) in order to eliminate DNA contamination. RNA concentration was determined by using the NanoDrop 1000 spectrophotometer (Fisher Scientific). RNA sample integrity and quality was assessed prior to hybridization with an Agilent 2100 Bioanalyzer and RNA 6000 Nano LabChip kit (Agilent). Using $1\ \mu\text{g}$ of total RNA as starting material, synthesis, fragmentation and labeling of cDNA were performed using Affymetrix molecular biology kits and reagents (Cat. No. 900811-900812-900371-900301-900720) following the procedure described in the Affymetrix GeneChip Whole Transcript Double Stranded Target Assay Manual (P/N 702179 Rev. 3, protocol with amplification). $5\ \mu\text{g}$ of fragmented and labeled cDNA were hybridized onto Affymetrix *S. cerevisiae* Tiling 1.0R Arrays. Hybridization was performed in the Hybridization Oven 645; washing and staining of arrays were performed using the GeneChip Fluidics Station 450 and scanning with the Affymetrix GeneArray Scanner 3000 7G. Affymetrix Microarray Suite v5.0 was used to generate CEL files of the scanned DNA microarrays.

Microarray data analysis The GeneChip *S. cerevisiae* tiling 1.0R array provides high-density 5 base pairs probe resolution wherein each probe is a 25mer. Each array contains about 2.6 million perfect match (PM) and 2.6 million mismatch probes (MM), designed to measure transcription of the yeast genome. Since the array design was based on October 2003 *Saccharomyces* Genome Database (SGD), we re-mapped the probe-sequences to the latest genomic DNA sequences. The probe re-mapping was performed using local installation of MegaBlast (version 2.2.8) (Zhang *et al.*, 2000). No gaps or mismatches in the mapping were allowed. Probes that mapped to more than one genomic location were filtered out to avoid the cases of non-specific hybridization. This step left us with 2.5 million probes that were considered for the later analyses.

Preprocessing and normalization. Statistical analysis was performed using the R software package and BioConductor package *limma* (Gentleman *et al.*, 2004; Smyth, 2004). Non-negative signal intensities were obtained by applying NormExp background correction method (Ritchie *et al.*, 2007). These background corrected intensities were quantile-normalized by following the approach of Bolstad *et al.* (2003). The quantile normalizations were performed on each set of arrays for a strain-type because of the differences in the intensity distributions resulting from different biochemical conditions and strain variations. To be able to compare response of each strain to MMS treatment, the normalized intensities were further scaled as per median absolute deviation (MAD) values for the corresponding strain.

Differentially expressed genes. Genomic coordinates of chromosomal features were obtained from SGD (March 2009). Probes encompassing each chromosomal feature reported in SGD were collected. A feature-wise statistic was generated based on the means of differences between corresponding probes in all pair-wise combinations of

treatment and control arrays, as defined in **Equation 5.1**.

$$y_{j,k} = \frac{1}{n} \sum_{p \subset 1..n} (\log_2(PM_{jp}) - \log_2(PM_{kp})) \quad (5.1)$$

Limma was applied to the average pair-wise differences for each genomic feature using all j,k treatment-control pairs to measure differential expression due to treatment. Benjamini-Hochberg correction method was applied on P -values within *limma* to correct for multiple testing (Benjamini & Hochberg, 1995).

Detection of differentially transcribed regions containing structured RNAs. We followed the same method as above to detect non protein-coding regions showing differential transcription due to MMS treatment. These regions were reported as being transcribed by various studies (David *et al.*, 2006; Davis & Ares, 2006; Granovskaia *et al.*, 2010; Samanta *et al.*, 2006). Steigele *et al.* (2007) further assessed for enrichment of RNA structure as well as sequence conservation in multiple sequence alignments across seven yeast species.

MMS sensitivity assay Strains were grown in liquid cultures in 48-well plates by using a high-throughput cultivation system called BioLector (DASGIP AG). At mid-exponential phase, cells were treated with different concentrations of MMS: 0 (control), 0.025, 0.05 and 0.1%. The experimental parameters were: working volume 1 mL, temperature 30°C, orbital shaking 1000 rpm and humidity $\geq 95\%$. The growth detection is based on the optical determination of light-scattering. The generated light scattering values have been shown to be correlated with cell dry weight (Kensy *et al.*, 2009). Analysis of growth time-series and growth rates was performed using an R package developed by the authors specifically for the analysis of BioLector data.

Results

We have investigated the transcriptional and phenotypic responses to the DNA damaging agent MMS in *Saccharomyces cerevisiae* wild type and PK/PP knockout (KO) cells (**Table 5.1**). To measure the transcriptional response, Affymetrix tiling arrays were used to be able to investigate coding (mRNA) and non-coding RNA (ncRNA) levels. According to the genomic annotation on SGD (October 2010), roughly 5% of the yeast genome consists of non protein-coding genes. This 5% of genetic material is covered by 300 transfer RNAs (tRNAs), 77 small nucleolar RNAs (snoRNAs), 27 ribosomal RNAs (rRNAs), 6 small nuclear RNAs (snRNAs) and 15 other non-coding RNAs. Some of the non-coding RNAs are directly or indirectly involved in the transcriptional regulation of nearby protein-coding genes (e.g., *PWR1*) and others are involved in rRNA or tRNA processing. With respect to the phenotypic analysis, we have used a high-throughput cultivation system to test the sensitivity of the strains to different MMS concentrations. An integrative approach has been adopted in order to relate data produced in this study with extant data, with the aim of providing a global view of the coordinated

Table 5.2: Previous transcriptional studies about *S. cerevisiae* response to MMS.

Study	Strain	MMS conc. (%)	Treatment time (min)	Microarray platform
Jelinsky & Samson (1999)	DBY747	0.1	60	Affymetrix Ye6100
Jelinsky <i>et al.</i> (2000)	DBY747	0.05, 0.1, 0.2	10, 30, 60	Affymetrix Ye6100
Gasch <i>et al.</i> (2001)	W303-derived	0.02	5, 15, 30, 45, 60, 90, 120	Spotted array
van Attikum <i>et al.</i> (2004)	BY4733	0.1	60	Affymetrix YeastS98
Caba <i>et al.</i> (2005)	BY4730	0.12	120	Affymetrix YeastS98
Workman <i>et al.</i> (2006)	BY4741	0.03	60	Spotted array
Benton <i>et al.</i> (2006)	W303-derived	0.001, 0.01, 0.1	60	Nimblegen (custom)
Fu <i>et al.</i> (2008)	DBY747	0.1	48	Yeast 6.4K

responses of *S. cerevisiae* to MMS.

Firstly, we have analyzed the transcriptional data in order to investigate the effect of MMS within each strain (*limma* approach, see Material and methods) and then compared the results with previous reports. As can be seen in **Table 5.2**, the experimental factors (strain, MMS concentration and treatment time), as well as the microarray platform, vary among the studies. The agreement between the sets of genes implicated as MMS responsive varies considerably from study to study. When considering gene sets from the selected existing studies, only the plasma membrane sulfite pump *SSU1* was common to all. Surprisingly, we have noticed different MMS transcriptional responses of the two strain backgrounds used in this study, with W1588-4C displaying a stronger response ($P\text{-value} \leq 0.01$ and $|\log\text{FC}| \geq 1$). For example, we have found significant expression changes of *DDI* genes only in the W1588-4C background but not in the BY4741. Regarding the ribonucleotide reductase (*RNR*) genes, known to be up-regulated upon DNA damage (Elledge & Davis, 1989), two of them (*RNR1*, *RNR3*) are significantly up-regulated in BY4741, while the other two (*RNR2*, *RNR4*) show over-expression in both backgrounds. If we consider the genes common to 50% of the published expression studies (**Table 5.2**) as representative of MMS response, we see that they are enriched within the common genes between BY4741 and W1588-4C (30% of the intersection). These genes are: *AAD6*, *GTT2*, *GRE2*, *ISU2*, *HSP31*, *RNR2*, *RNR4*, *SSU1*, *YHB1* and *YNL134C*. Therefore, although the two strain backgrounds are closely related (Schacherer *et al.*, 2007, 2009), it appears they respond quite differently to 0.025% MMS at the transcriptional level. We have also analysed the expression levels of the genes known to peak during the cell cycle (Gauthier *et al.*, 2008) and observed that MMS treatment causes the up-regulation of genes at the G₁/S transition in all of the strains (data not shown).

The strain-wise microarray data analysis does not provide specific knowledge about the role of PKs and PPs during response to MMS. In order to determine which MMS responsive genes were dependent on the studied kinases and phosphatases, we ap-

plied a ‘deletion-buffering’ analysis to identify dysregulated genes, i.e. genes that are significantly differentially expressed in WT, but not in a deletion mutant (Workman *et al.*, 2006). In total, we have identified 523 genes that were shown to be dysregulated in one or more of the 9 deletion mutants. The *rad53* Δ and *ptc3* Δ strains displayed the strongest and weakest dysregulation respectively (190 and 29 genes; **Figure 5.1A**). When analyzing the gene ontology enrichment (SGD GO Slim Mapper, www.yeastgenome.org/cgi-bin/GO/goSlimMapper.pl) of the deletion buffered genes, the most over-represented biological processes were transport (13.6%), RNA metabolic process (12%), protein modification process (9.2%) and response to stress (8.8%). Analysis of macromolecular complex enrichment showed that buffered genes were mainly associated with the ribosomes, nucleosomes, proteasomes, chromatin remodeling and Rnr complexes.

In order to better understand the regulation of these buffered genes, we reconstructed a regulatory network using the deletion buffering relationships from PP/PK (this study) and TF knockout strains (Workman *et al.*, 2006) and looked for significant modules based on the strain-wise *limma* *P*-values (Cline *et al.*, 2007; Ideker *et al.*, 2002) (**Figure 5.1B**). The top-scoring module provides an integrated overview of the role of the PKs/PPs during the transcriptional response to MMS. The genes included in this module are involved in diverse processes, such as protein modification, transport, amino acid and derivative metabolism, response to stress and to chemical stimulus and other metabolic processes (e.g., lipid, cofactor, carbohydrate metabolism). Moreover, some protein complexes were enriched, for example, ribonucleoproteins, proteasomal proteins, chromatin remodeling and ribonucleotide reductase complexes. When comparing the lists of buffered genes, we have found small overlaps among them (**Figure 5.2**). For example, there is no overlap between genes buffered by the two sensors of DNA damage (Mec1 and Tel1) (**Figure 5.2A**). *rad53* Δ buffered genes overlap significantly with *mec1* Δ buffered genes, but very small overlap is observed with *dun1* Δ and *chk1* Δ buffered genes (**Figure 5.2B**). *alk1* Δ buffered genes also show small overlap with the other gene lists. With respect to PPs, only few genes are in common between *oca1* Δ and *pph3* Δ /*ptc3* Δ buffered genes (**Figure 5.2C**).

The subsequent step was to integrate the buffering network with phosphorylation events, physical interactions and TF regulation present in published studies and databases. In particular, we have considered protein-protein interactions and phosphorylation events described in the BioGRID and PhosphoGRID databases (Breitkreutz *et al.*, 2008; Stark *et al.*, 2010), as well as MMS-specific protein-protein interactions (Ho *et al.*, 2002), phosphorylation from mass spectrometry (MS) based studies (Chen *et al.*, 2010; Smolka *et al.*, 2007) and TF regulation according to the Yeast Proteome Database (Hodges *et al.*, 1999). By integrating all of these pieces of evidence, we have built a comprehensive MMS-responsive network of the central DDR pathway (**Figure 5.3**). This network includes the PKs and PPs under investigation, as well as key players of the DDR pathway. In particular we have included proteins displaying ‘multi-level’ regulation (Exo1, Hhf1, Hsp26, Plm2 and Rnr3) that appear to be both deletion-buffered and phosphorylated upon MMS treatment. Moreover, MMS causes

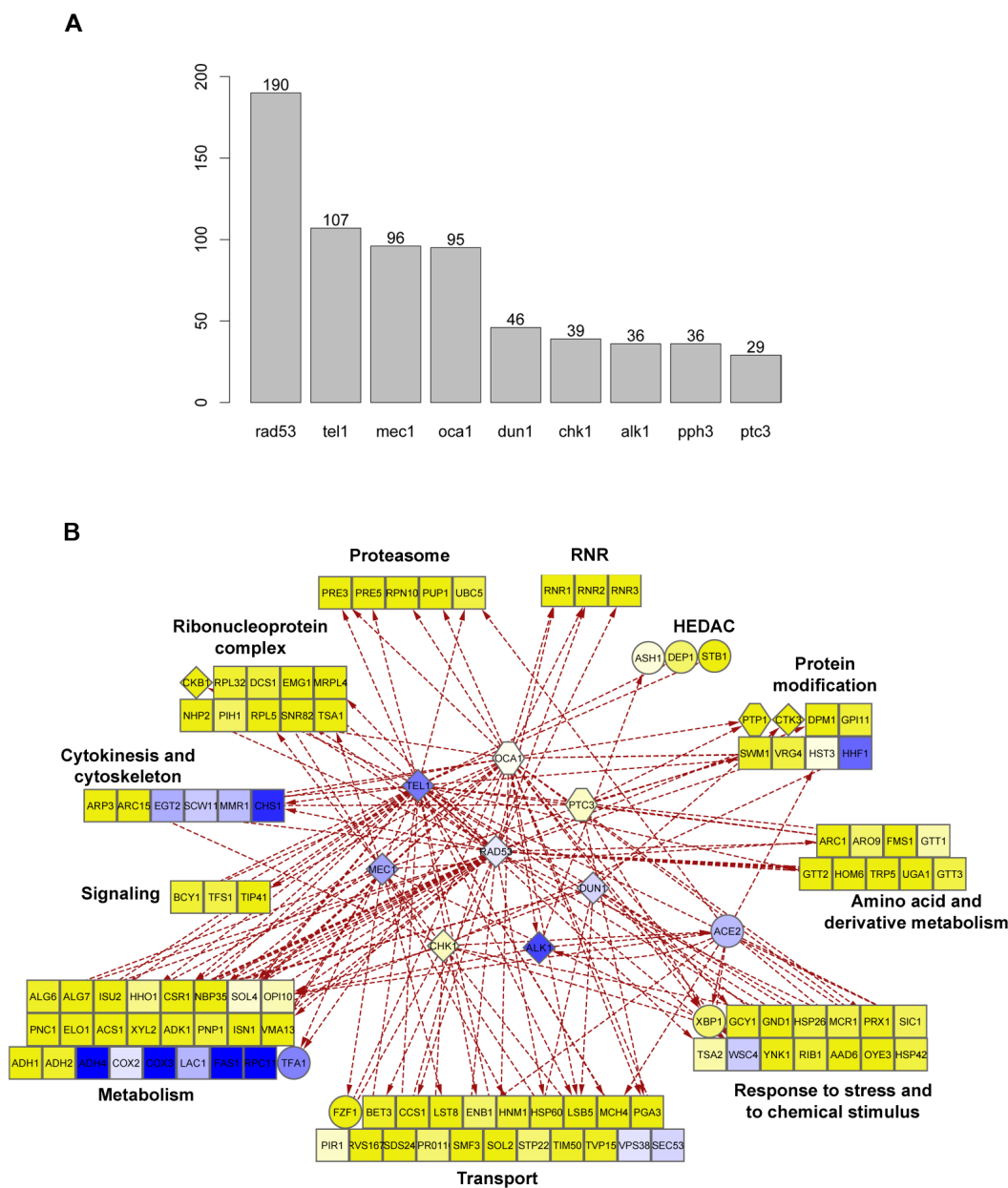


Figure 5.1: Genes buffered by PK/PP deletions during MMS response in *S. cerevisiae* - (A) Number of deletion buffered genes in the knockout strains. (B) This network represents the most significant module created by using the Cytoscape plug-in jActive module (Cline *et al.*, 2007; Ideker *et al.*, 2002); the score of the module is based on the *limma* *P*-values of BY4741 and W1588-4C. Node shape indicates PKs (diamond), PPs (hexagon), TFs (ellipse) and genes/proteins (rectangle); node color indicates gene expression levels upon MMS treatment (yellow is up-regulation, while cyan is down-regulation). Edges indicate deletion buffering relationships. The presented view shows gene expression levels relative to W1588-4C. Abbreviations: RNR (ribonucleotide reductase); HEDAC (histone deacetylase).

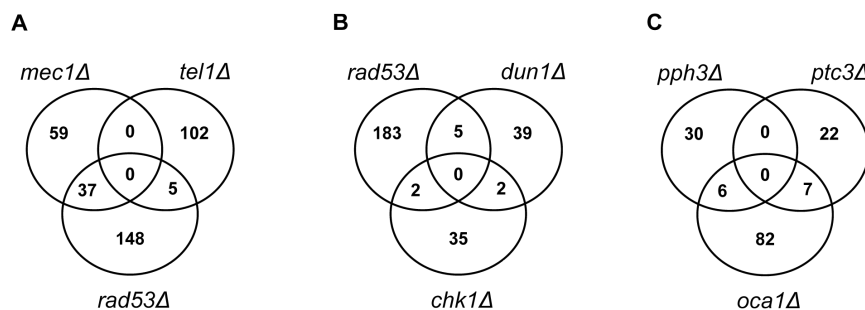


Figure 5.2: Overlap among deletion buffered gene lists -

specific physical interaction to occur, such as the interaction between Hhf1 and Lcd1, the latter known to form a complex with Mec1 (Zou & Elledge, 2003). Hug1, whose protein levels increases about 50-fold upon MMS treatment (Lee *et al.*, 2007), has been also included. Although this network is informative of the DDR pathway architecture and interactions, it needs to be interpreted in order to capture regulatory circuits that are important in response to DNA damage. Therefore, we have manually identified modules regulating the transcription of key players in the network. One of them is the *RNR* module, where the transcription of the *RNR* genes appears to be highly-regulated (**Figure 5.3**). Among the deletion mutants, *rad53Δ* and *oca1Δ* strains are the ones that present the strongest dysregulation of the *RNR* genes after MMS treatment (data not shown), while deletion of *MEC1*, surprisingly, did not compromise *RNR* induction levels in our study. Other significant modules are shown in **Figure 5.4**. Overall, the architecture of these modules describes how converging signals of different nature (gene regulation and phosphorylation) could fine-tune the activity of specific proteins.

Aim of this study was also to identify evolutionarily conserved and structured non protein-coding regions as well as SGD annotated ncRNAs showing changes in their expression profiles in response to MMS treatment. Among the SGD annotated non-coding RNAs, *PWR1* was up-regulated in response to MMS in *pph3Δ* and down-regulated in *chk1Δ* and *ptc3Δ* cells (**Figure 5.6**, page 83). *PWR1*, together with *ICR1*, is known to regulate the protein-coding ORF *FLO11* (Bumgarner *et al.*, 2009). *RNA170*, an RNA polymerase III-transcribed RNA having unknown function, was found to be significantly up-regulated in *chk1Δ* and significant down-regulated in *dun1Δ* cells in response to MMS. The differential expression extends beyond the annotated boundaries of the feature (**Figure 5.7**, page 84). Among annotated rRNAs, 15S-rRNA and 21S-rRNA showed significant down-regulation in *mec1Δ* cells (**Figure 5.8**, page 85). We also observed that the MMS response of *ptc3Δ* showed significant up-regulation for 33 of the currently known snoRNAs with respect to the control, while the other strains expressed mild or no response to MMS in terms of snoRNA expression.

Apart from the non-coding RNAs that are reported in SGD, we also investigated putative structured RNA containing regions (Steigele *et al.*, 2007) for differential expression. These regions encompass not only intergenic regions, but also coding and

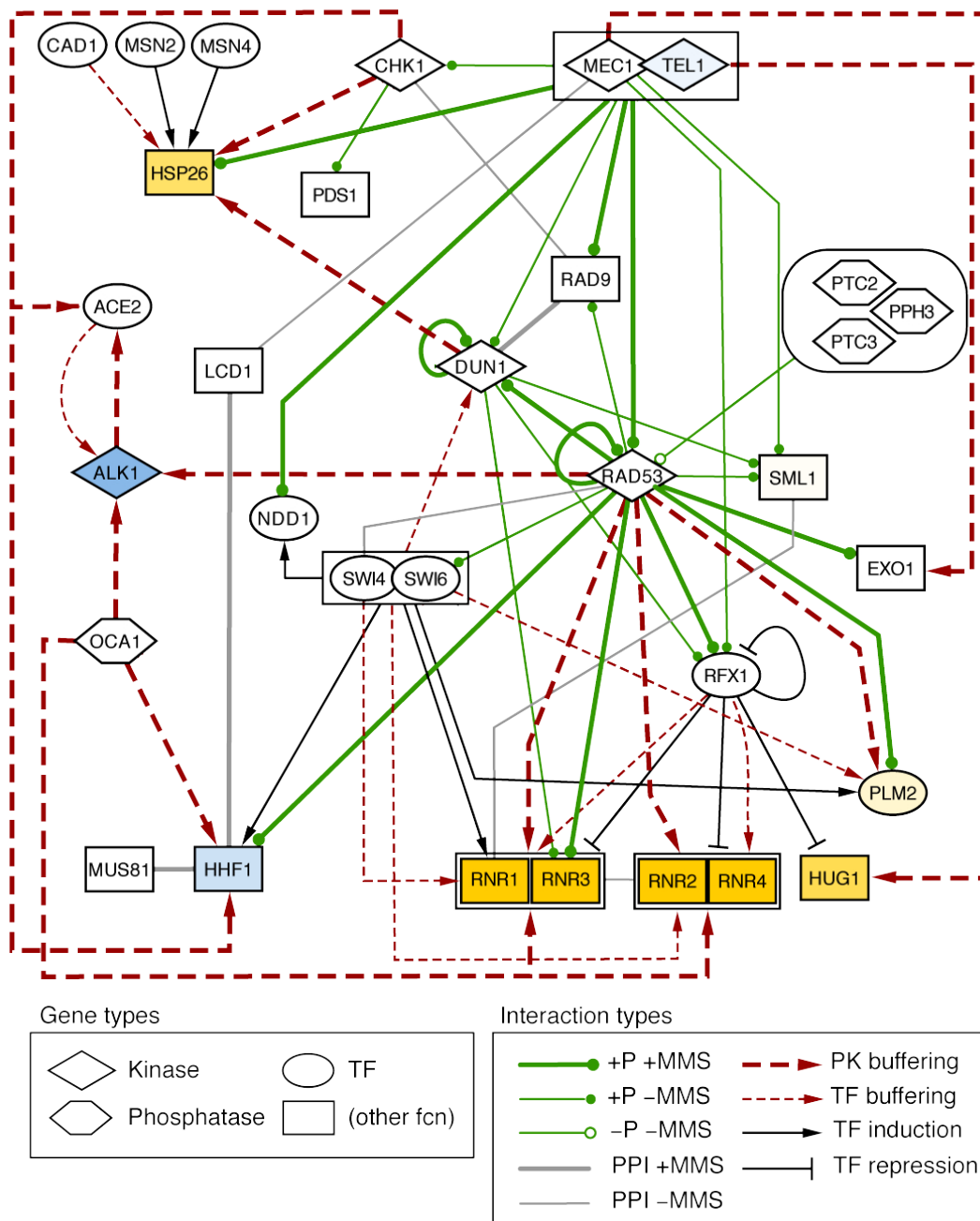


Figure 5.3: Central DNA damage pathway in *S. cerevisiae* - The network has been created by integrating diverse experimental pieces of evidence (see text). Node color indicates gene expression levels upon MMS treatment (yellow is up-regulation, while cyan is down-regulation). The presented view shows gene expression levels relative to W1588-4C. Boxes have been used to group Swi4-Swi6 (that constitute the SBF complex), Pph3-Ptc2-Ptc3 (that are the PPs known to dephosphorylate Rad53), Rnr1/Rnr3 and Rnr2/Rnr4 (that form the ribonucleotide reductase complex). Moreover, Mec1 and Tel1 have been grouped together because some of the phosphorylation data have been obtained by using a double *mec1* Δ *tel1* Δ mutant (Chen *et al.*, 2010; Smolka *et al.*, 2007).

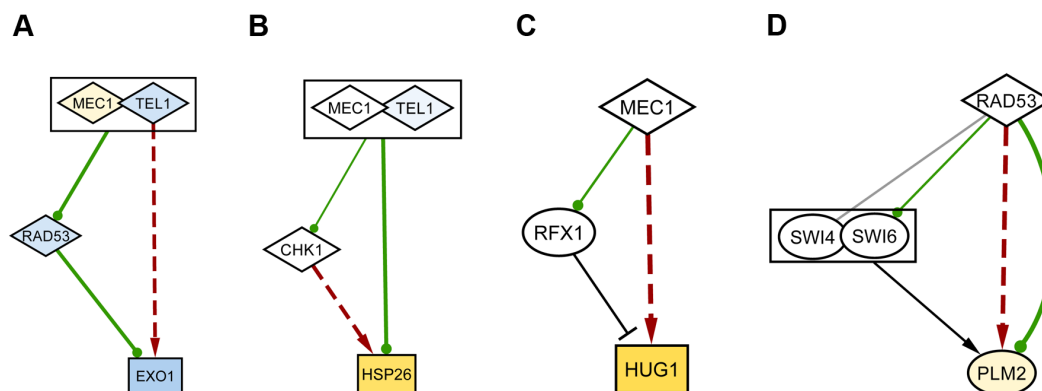


Figure 5.4: The regulation of *EXO1*, *HSP26*, *HUG1* and *PLM2* - These networks are subsets of the one presented in Figure 5.3 (the node/edge notation is the same). Gene expression values relative to BY4741 (for *EXO1* and *HSP26*) and to W1588-4C (for *HUG1* and *PLM2*) are shown.

untranslated regions of the protein-coding genes and had been previously shown to be transcribed (David *et al.*, 2006; Davis & Ares, 2006; Samanta *et al.*, 2006). We investigated predicted snoRNA regions for differential expression in response to MMS treatment. Small nucleolar RNA are molecules responsible to guide chemical modifications in their target RNAs, particularly rRNAs, tRNAs and snoRNAs. Based on the conserved sequence motifs in snoRNAs, there are two major classes: C/D box snoRNAs, involved in methylation of their targets, and H/ACA box snoRNAs, that guide pseudouridylation of rRNAs. Steiglele *et al.* reported 41 putative H/ACA box and 5 C/D box snoRNAs. Using our approach, we found that three among the five C/D box snoRNAs showed differential expression due to treatment. These three putative snoRNAs also have their target sites targeted by other known snoRNAs (snR62, snR52 and snR50). Among the 41 putative H/ACA box snoRNAs, 32 were observed to be MMS responsive in different mutant strains. Once again, these snoRNAs have been reported to have more than one snoRNAs targeting their target sites. Among the strains, *pph3* Δ and *alk1* Δ cells exhibit the higher number of putative snoRNA regions that were responsive to MMS treatment. Overall, we have shown that interesting insight into the role of ncRNAs during MMS response can be obtained by using tiling arrays.

In order to determine the MMS sensitivity of the strains under investigation, we have treated liquid cultures with different concentrations of MMS (see Materials and methods). Within each strain, the growth rate of treated cells was first normalized to the value before treatment and then to the value of 0% MMS after treatment. The calculated relative growth rates are summarized in **Figure 5.5**. We have also included three strains known not to be involved in the central DDR pathway (*atg1* Δ , *dbf2* Δ , *prk1* Δ) and a KO strain lacking the *ALK1*-homolog *ALK2* (*alk2* Δ). The results indi-

cate poor correlation between phenotype and transcriptional response. For example, *ptc3Δ* appears to be as sensitive as *rad53Δ*, while, at the transcriptional level, their responses are very different (**Figure 5.1A**). Furthermore, despite evidence that the sensitivity of *oca1Δ* decreases with increasing MMS concentration, this strain shows a disrupted transcriptional response for a number of important genes.

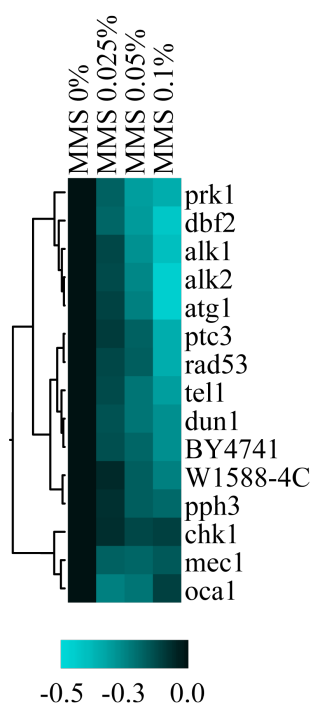


Figure 5.5: Clustering of the strain relative growth rates at different concentrations of MMS - Growth rates were calculated relatively to the 0% control concentration (values are in logarithmic scale). Growth rate profiles were clustered using a Euclidean-based distance metric.

The relationship between gene expression levels and relative growth rates at 0.025% MMS was investigated in order to discover genes whose relative transcript levels were positively or negatively correlated with relative growth rates. Based on a significance test for Pearson correlation, 24 genes were found to be significantly correlated (P -value ≤ 0.005). The 11 genes with positive correlation included two ncRNAs transcribed as part of the 35S rRNA precursor transcript (*ITS1* and *ITS2*), genes belonging to the yapsin protease family (*YPS5* and *YPS6*), genes involved in energy generation (*GPH1* and *KGD2*), and in lipid metabolism (*GPI2* and *LAP2*). Interestingly, *GPI2* is involved in the synthesis of glycosyl phosphatidylinositol (GPI) anchors and *YPS5*/*YPS6* are believed to interact with these anchors located in the cell membrane (Krysan *et al.*, 2005). Moreover, three genes with unknown biological process display positive correlation: *YPR160C-A*, which overlaps with *GPH1*, *YER076W-A* and *YLR177W*. The

13 genes with negative correlation are involved in diverse processes, among which are: ribosome biogenesis (*FAF1*, *NOP6*, *REH1*, *MTG1*), RNA metabolic process (*FAF1*, *MSS1*, *PHO80*), response to stress (*GRX7*, *PHO80*, *RVS161*) and mitochondrion organization (*ARC19*, *MTG1*).

Discussion

The transcriptional response to MMS of the wild type strains used in this study (BY4741 and W1588-4C) appears to be quite different and this could explain the poor overlap among MMS studies (**Table 5.2**). Supporting this finding, differences between backgrounds were also observed by Travesa *et al.* when investigating recovery from MMS damage (Travesa *et al.*, 2008). Moreover, the complexity of the relationship between genotype and phenotype has been recently investigated by comparing the set of essential genes in two closely related yeast backgrounds (S288c and Σ 1278b). The surprising extent of difference in essential genes likely arises from the influence of more complex, multi-gene dependencies, such as genetic interactions (Dowell *et al.*, 2010). Therefore, it seems that, even within the same species, two related strains (Schacherer *et al.*, 2007) can exhibit different responses to environmental stimuli such as MMS.

The phosphorylation cascade governing the activation of the DNA damage checkpoint could suggest that the transcriptional response elicited by the deletion of a downstream kinase largely overlaps with the response caused by the deletion of an upstream kinase. Our results do not support this view. Indeed, it appears that the deletion mutants used in this study have a specific response at the transcriptional level. The two sensors of the pathway (Tel1 and Mec1) regulate different sets of genes, in agreement with the different roles they exert: Tel1 is more important during response to double strand breaks (DSBs) (Nakada *et al.*, 2003), while Mec1 plays a critical role during replication fork stalling and S-phase checkpoint, that occur when yeast cells are treated with MMS (Branzei & Foiani, 2009; Segurado & Tercero, 2009). For this reason, the transcriptional response of *mec1* Δ cells partly overlaps with the one of *rad53* Δ cells, Rad53 being the main effector of the damage signal elicited by Mec1. However, Mec1 has been shown to have Rad53-independent functions (Friedel *et al.*, 2009) and we also show that Mec1 has a Rad53-independent transcriptional response. The strong response observed in *tel1* Δ cells (107 buffered genes) and the absence of overlap between genes buffered by *tel1* Δ and *rad53* Δ , could suggest that Tel1 has an important role during damage response that does not use Rad53 as its effector. The fact that the genes buffered by *tel1* Δ significantly overlap with the genes buffered by the deletion of three transcription factors (Ace2, Cad1, Dig1; hypergeometric test with P -value \leq 0.01) (Workman *et al.*, 2006) could suggest that this PK determines its transcriptional effect through these TFs.

With respect to the effector of the pathway (Rad53, Dun1 and Chk1), our findings implicate a poor overlap in the transcriptional responses, implying that every branch of the pathway regulates the expression of different sets of genes. While the roles of Rad53 and Dun1 during S-phase checkpoint have been widely investigated (Branzei &

Foiani, 2006; Zhou & Elledge, 1993), the role of Chk1 has been mainly associated with mitotic arrest upon DNA damage (Sanchez *et al.*, 1999). Recently, a role for Chk1 in replication fork stabilization has been proposed (Segurado & Diffley, 2008) and our results support a role of this PK during MMS-induced checkpoint response. We have also taken into consideration another protein kinase (Alk1), previously reported to peak in M phase and to be hyperphosphorylated upon MMS treatment (Nespoli *et al.*, 2006). We have shown for the first time its transcriptional role in MMS-induced damage response (36 buffered genes by *alk1Δ*, of which 25% have unknown biological process). Alk1 has a homolog (Alk2) and, in order to elucidate the role of these PKs in details, the transcriptional response of the double mutant (*alk1Δalk2Δ*) should be investigated. With respect to the protein phosphatases, our results have proposed a role for Oca1 during MMS response. Oca1 is a putative tyrosine phosphatase and little is known about this protein and its two homologs (Oca2 and Siw14) (Wishart & Dixon, 1998). Nevertheless, Oca1 appears to be required for G₁ arrest in response to oxidative stress (Alic *et al.*, 2001) and was included in this study for its possible role in DNA damage response. Our data show that *oca1Δ* has an important impact on gene expression (**Figure 5.1A**) and, together with *rad53Δ*, is involved in the regulation of *RNR* genes (**Figure 5.3**). Therefore, our findings suggest that Oca1 has an important role during S-phase checkpoint activation or de-activation although further experiments are necessary to elucidate its interactions (genetic and physical) with the other players of the pathway. In contrast, the protein phosphatases Pph3 and Ptc3 displayed only a minor role in the transcriptional response to MMS. This is probably due to the fact that these two PPs are known to have a primary role during checkpoint recovery once the damage has been repaired, rather than during checkpoint activation (Heideker *et al.*, 2007).

In summary, we have dissected the role of PKs and PPs in the transcriptional response of *S. cerevisiae* to MMS. In particular, Mec1, Tel1, Rad53 and Oca1 appear to have primary roles (**Figure 5.1A**) and be required for aspects of the global response that includes protein modification, transport, response to stress and RNA metabolic processes (**Figure 5.1B**), in agreement with previous reports (Fry *et al.*, 2005; Gasch *et al.*, 2001; Jelinsky & Samson, 1999; Jelinsky *et al.*, 2000). Our results for *mec1Δ* do not agree with previous results from Gasch *et al.* (2001). In contrast to their findings, our data do not show the loss of *RNR* induction in the *mec1Δ* strain and we do not observe that the transcriptional response in *dun1Δ* cells is largely the same as in *mec1Δ* cells. These inconsistencies could arise from the different strain backgrounds.

With the aim of further elucidating the regulatory roles of protein kinases and phosphatases in the response to MMS, we have created a network representing the central DDR pathway by integrating diverse sources of data (**Figure 5.3**). This network has allowed us to focus on specific regulatory circuits and propose possible mechanisms of regulation exerted by these PKs and PPs, for example, the regulation of the Rnr complex. The Rnr complex, which consists of two large (Rnr1/Rnr3) and two small (Rnr2/Rnr4) subunits, catalyzes the rate-limiting step for the synthesis of dNTPs; the *RNR* gene expression levels are normally up-regulated during S-phase and shows further

increase upon DNA damage (Chabes *et al.*, 2003; Elledge & Davis, 1990). According to the network in **Figure 5.3**, the Rnr complex is under the control of many proteins considered in this study including Rad53, Dun1, and Oca1 through both transcription mechanisms (via Rfx1 and Swi4/Swi6) and post-translationally via the Sml1 repressor. Upon DNA damage, *RNR* gene expression increases because of release of transcriptional repression by Rfx1, degradation of *RNR* inhibitor Sml1 and re-localization of Rnr subunits to the cytoplasm (Huang *et al.*, 1998; Yao *et al.*, 2003; Zhao & Rothstein, 2002). We found that *RAD53* deletion buffers *RNR* induction, reinforcing the central role it plays in *RNR* regulation by phosphorylating Dun1, Rfx1, Sml1, Swi6 and Rnr3 itself. Moreover, *RNR* genes are buffered by *oca1* Δ and *rfx1* Δ (Workman *et al.*, 2006). Therefore, our data show the expected control over *RNR* genes by Rad53 and show for the first time the involvement of the protein phosphatase Oca1 in this process.

Our integrated network has also allowed us to explain the regulation of other proteins involved in MMS response, such as Exo1, Hsp26, Hug1 and Plm2. During S-phase checkpoint response, one of the key function of Rad53 is to ensure replisome stability by preventing Exo1-dependent replication fork breakdown (Cotta-Ramusino *et al.*, 2005; Segurado & Diffley, 2008; Segurado & Tercero, 2009). Exo1 was found to be phosphorylated by Rad53 upon MMS treatment (Smolka *et al.*, 2007) and buffered by *tel1* Δ in our study (**Figure 5.4A**). Therefore, it could be speculated that Exo1 activity is suppressed by Rad53-dependent phosphorylation and transcriptionally repressed by Tel1-dependent activity. The small heat shock protein Hsp26 has been recently identified in a MS-based study as target of Mec1/Tel1-dependent phosphorylation in response to MMS damage (Chen *et al.*, 2010). Hsp26 interacts with members of the RSC family of chromatin remodelers, that are known to be recruited at site of damage (Chai *et al.*, 2005) and are themselves phosphorylated after MMS treatment (Chen *et al.*, 2010). Our results suggest that Hsp26 could be part of a regulatory circuit where its gene expression levels are controlled by Chk1, which is in turn phosphorylated by Mec1/Tel1 (**Figure 5.4B**). Hug1 protein levels increase about 50 times upon MMS treatment (Lee *et al.*, 2007). According to our model, the high protein levels of Hug1 can be explained by the activity of Mec1 on Rfx1 either directly or indirectly through Rad53 or Dun1, causing a de-repression of the *HUG1* gene (**Figure 5.4C**). Finally, Plm2 is an FHA-domain containing protein and putative TF and paralog of Tos4. It is a target of the SBF complex and is believed to regulate genes involved in DNA synthesis/repair, chromosome segregation and nuclear division (Horak *et al.*, 2002). *PLM2* is up-regulated in response to DNA damage and telomerase deletion (Gasch *et al.*, 2001; Nautiyal *et al.*, 2002). Our data suggest that Plm2 activity could be regulated by Rad53-mediated phosphorylation and transcriptional regulation via SBF; once activated, Plm2 could promote the activation of other genes involved in MMS response (**Figure 5.4D**).

With respect to the non-coding RNA analysis, we have obtained some promising results, in particular regarding the MMS-induced snoRNA regulation in *ptc3* Δ , *pph3* Δ and *alk1* Δ cells. Although only mild changes were detected, this study provides a first insight into the role of non protein-coding RNA during DNA damage and more specific experimental approaches (e.g., Northern blot and qPCR) are necessary to complement

the results obtained with the tiling arrays. Interestingly, the role of DNA damage-induced small RNAs in the filamentous fungus *Neurospora crassa* has been recently described (Lee *et al.*, 2009). Therefore, our data are useful in the perspective of future studies on the involvement of ncRNAs in DNA damage response in *S. cerevisiae*.

Our MMS sensitivity data shows that there is poor correlation between phenotypic and transcriptional responsiveness within each strain, in agreement with a previous report (Birrell *et al.*, 2002). However, Workman *et al.* (2006) showed that the extent of buffering by TF deletion was correlated with relative MMS sensitivity according to Begley *et al.* (2002). Our results do not confirm this correlation because we found that the strains showing the strongest transcriptional dysregulation (*rad53* Δ , *mec1* Δ , *tel1* Δ , *oca1* Δ) do not seem to be more MMS sensitive than the other strains. Therefore, with respect to PKs and PPs, MMS sensitivity is not a predictor of the transcriptional role of these enzymes within the pathway; this may be due to the fact that PKs/PPs do not directly regulate gene transcription as TFs. Moreover, there could be functional redundancy. For example, Chk1 is important in replication fork stabilization when Rad53 is missing (Segurado & Diffley, 2008).

When investigating the correlation between phenotype and expression at the level of single genes, we have found significant correlations between the transcriptional response of certain genes and the growth rates of the strains after treatment with 0.025% MMS. These genes could be interpreted as important for defining the growth response of cells upon MMS treatment. A large proportion (36%) of the positively correlated genes are involved in membrane-related processes, possibly because membrane integrity or repair of alkylation damage to membranes is important for growth after MMS treatment. Supporting this, it has been reported that some of the most MMS sensitive mutant strains were those missing constituents important for the fluidity and permeability of membranes (Begley *et al.*, 2002; Mallory *et al.*, 2005). Therefore, faster growing cells may need to protect the permeability of membranes in order to prevent excessive damage caused by MMS. The roles of the genes displaying negative correlation, instead, suggest that, at increasing relative growth rates, yeast cells down-regulate ribosome biogenesis and RNA metabolism in order to save energy during the repair phase after MMS treatment. In fact, cells need to spend a high amount of energy to respond to MMS-induced damage (Fry *et al.*, 2005) and it may be important to down-regulate ribosome synthesis as it is one of the most energy-expensive cellular processes (Warner, 1999). Moreover, some genes involved in response to stress are up-regulated at low growth rates, as observed in a previous work (Fazio *et al.*, 2008) (see Chapter 3). This may be due to the fact that slow-growth and MMS treatment represent a non-favourable environment for yeast cells. Thus, the integration of gene expression and phenotypic data can give valuable information about cellular responses that are modulated as a function of growth rate.

In summary, we have dissected the role of PKs and PPs in the transcriptional response to the alkylating agent MMS and proposed a comprehensive view of the central DDR pathway by employing a systems biology approach. Moreover, the relationship between gene expression levels and relative growth rates have been investigated. Fur-

ther work will be needed to elucidate in more details the role of the DDR players; in particular, a deeper investigation of Oca1 may lead to understand its relevant transcriptional role. Finally, our data about non protein-coding RNAs can be useful for future studies addressing the role of these RNAs during DNA damage response.

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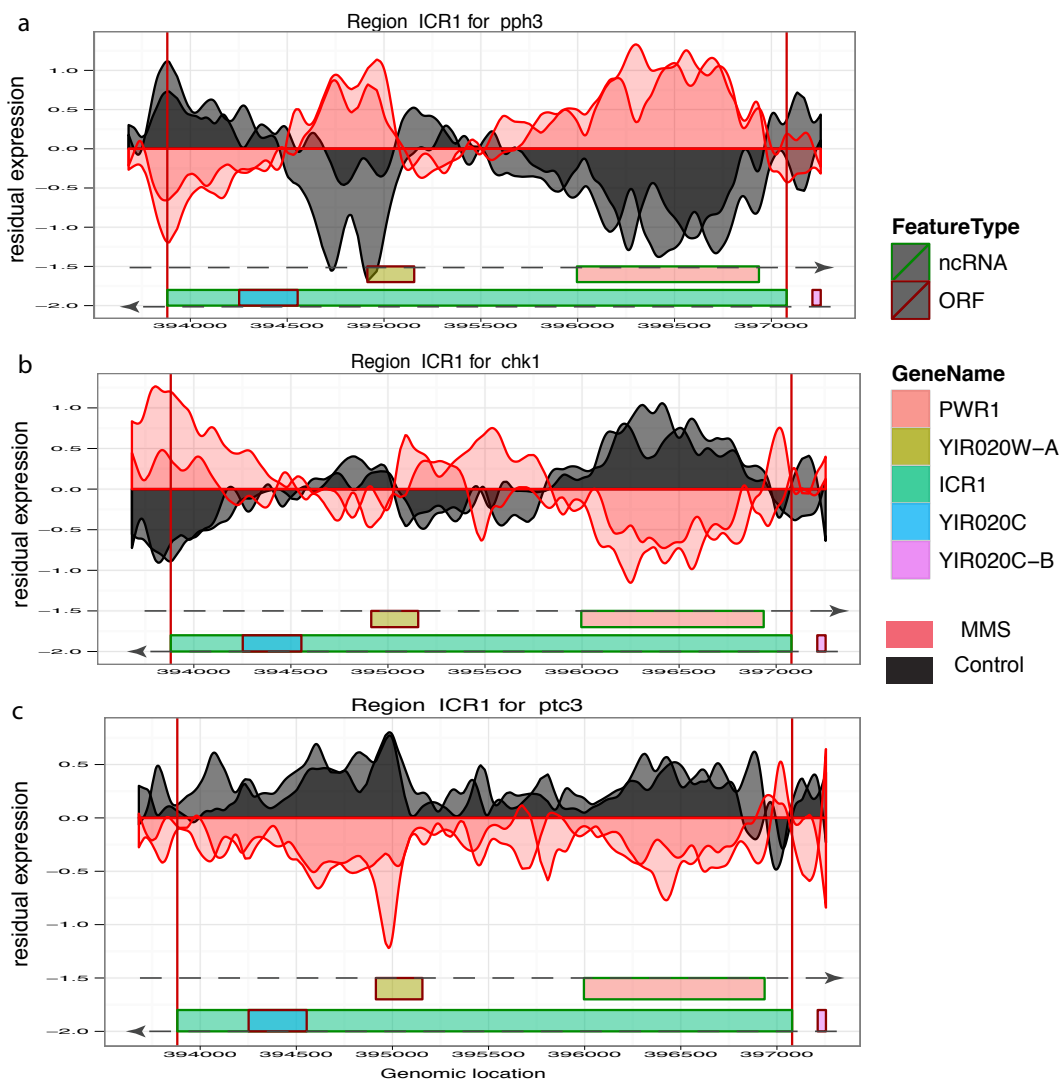


Figure 5.6: Differential transcription of *PWR1* in response to MMS - *PWR1* is found within the *ICR1* region. Y-axis shows log₂-residuals expression of treatment and control samples in (a) *pph3*Δ, (b) *chk1*Δ and (c) *ptc3*Δ strains plotted along the corresponding genomic coordinates (X-axis). The region contains overlapping transcripts of ORFs *YIR020C*, *YIR020C-B* and *YIR020W-A* as well as ncRNAs *PWR1* and *ICR1* marked as filled rectangles. The dashed arrows indicate the direction of transcription.

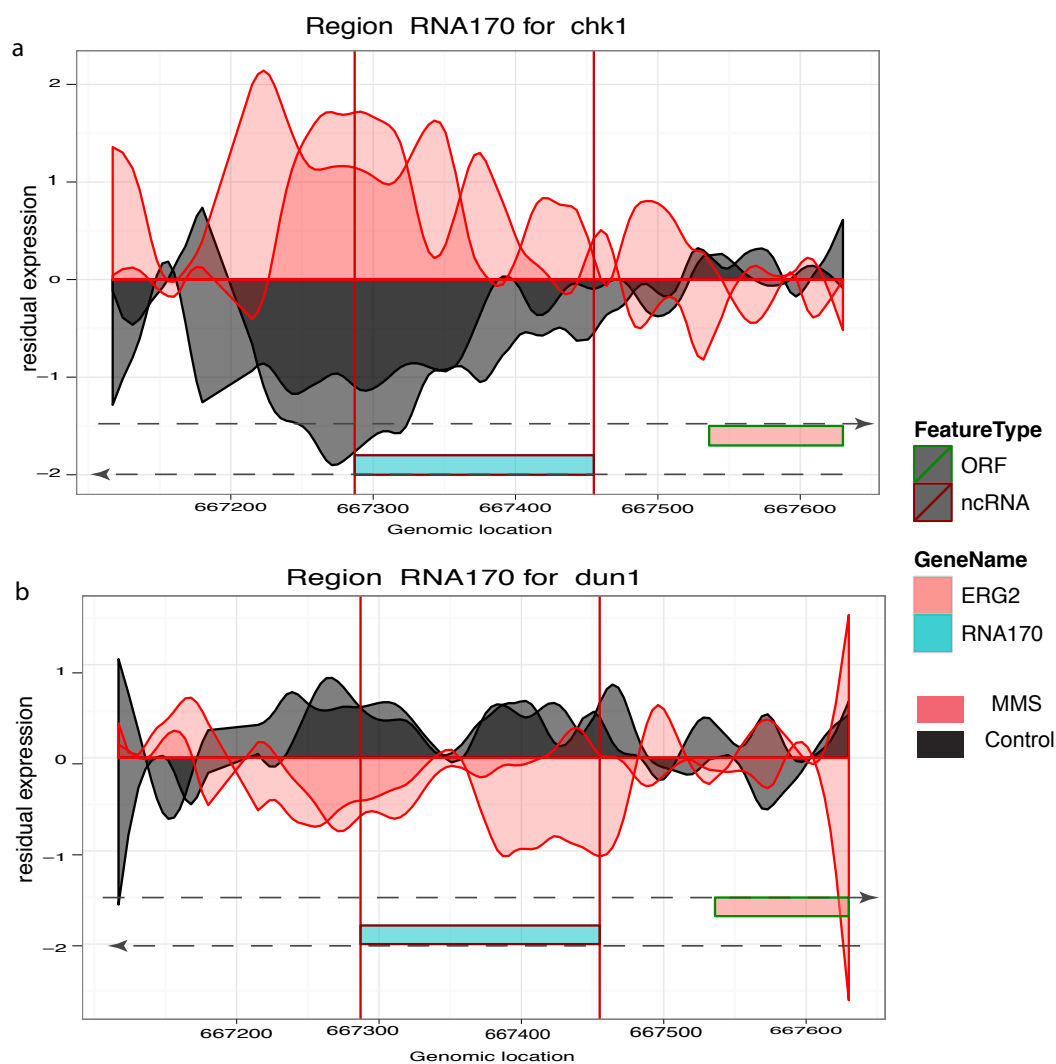


Figure 5.7: Differential transcription of *RNA170* in response to MMS - Log₂-residues of expression in (a) *chk1*Δ and (b) *dun1*Δ strains are plotted along the genomic coordinates (X-axis). The dashed arrows indicate the direction of transcription.

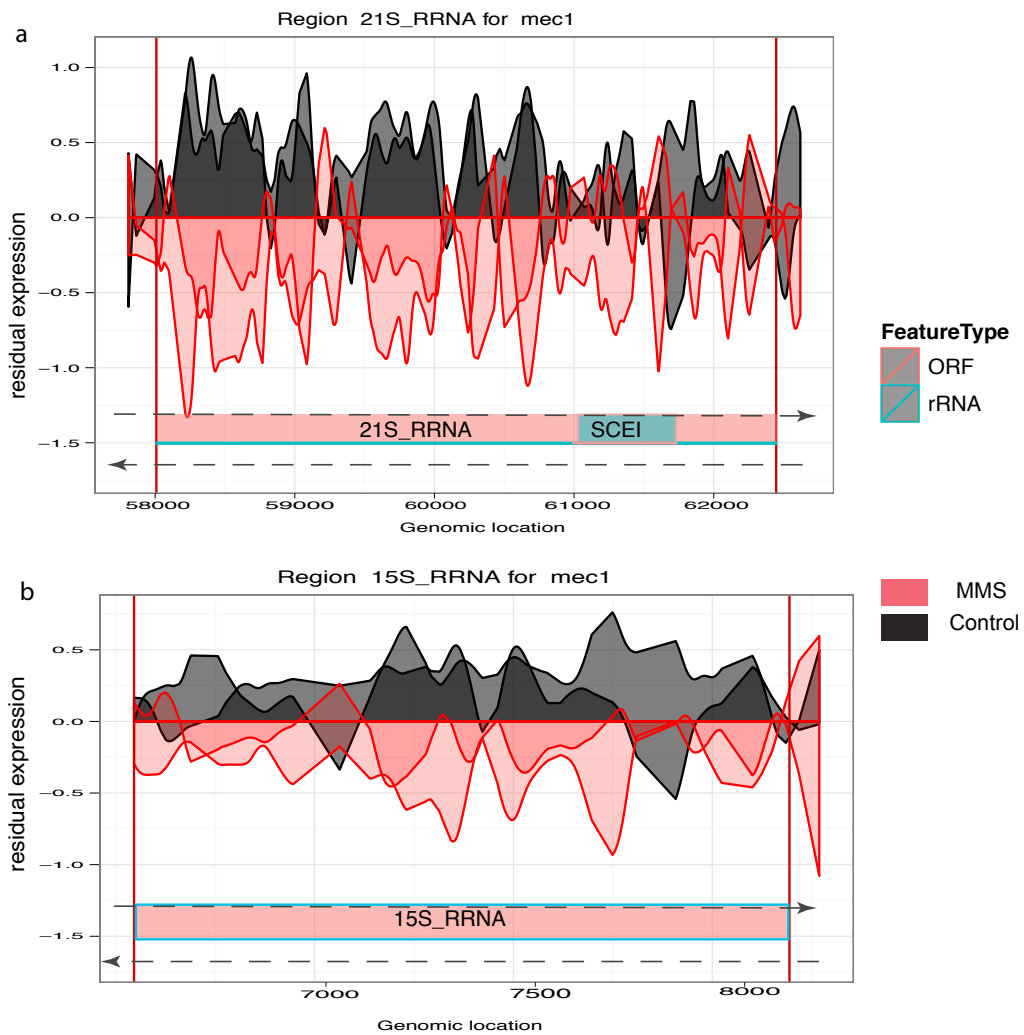


Figure 5.8: Differential transcription of 15S-rRNA and 21S-rRNA in response to MMS in *mec1*Δ cells - Y-axis shows log₂-residuals expression of treatment and control samples within the context of (a) 21S-rRNA and (b) 15S-rRNA genes respectively. Moreover, *SCEI* (an intron-encoded protein-coding gene) overlaps with the 21S-rRNA transcript. The dashed arrows indicate the direction of transcription.

Chapter 6

The role of protein phosphatases and Rad53 domains during S-phase checkpoint recovery

Alessandro Fazio¹, Nicolas C. Hoch², Jörg Heierhorst² and Christopher T. Workman^{1*}

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¹*Center for Biological Sequence Analysis, Department of Systems Biology, Technical University of Denmark, DK-2800 Kgs. Lyngby, Denmark*

²*St. Vincent's Institute of Medical Research, The University of Melbourne, 9 Princes Street, Fitzroy, VIC 3065, Australia*

* *Corresponding author: Assoc. Prof. Christopher T. Workman (workman@cbs.dtu.dk)*

Abstract

Saccharomyces cerevisiae responds to DNA damage by activating the so-called cell cycle checkpoints, which modulate DNA repair and promote viability and genome stability. Activation of the checkpoint response depends on a signaling pathway involving several protein kinases, among which Rad53 plays a key role. Indeed Rad53 stabilizes replication forks when they stall as a consequence of lesions causing cell cycle arrest in S-phase. After repair of the damage, cell cycle has to restart and this is achieved through inactivating dephosphorylation of Rad53 by protein phosphatases, whose action depends on the type of lesion and on the interaction with different Rad53 domains. In this work, we investigate the role of protein phosphatases (Ptc2, Ptc3 and Pph3) and Rad53 domains (SCD1 and FHA1) during damage recovery upon treatment with an alkylating agent (methyl methanesulfonate, MMS). We propose a role for the threonine 8 of Rad53-SCD1 and its Ptc2/3-mediated dephosphorylation.

Introduction

Protein kinase Rad53 is the main effector of the S-phase checkpoint since it regulates late origin firing and replication fork stabilization (Branzei & Foiani, 2006; Segurado & Tercero, 2009). Rad53 has two SQ/TQ cluster domains (SCD1 and SCD2) that precede two FHA domains (FHA1 and FHA2), and a kinase domain (KD) located between FHA1 and SCD2. Rad53 is the only member of the Chk2 kinase family having a second FHA domain at the carboxy-terminal, while human Chk2 only has one SCD and one FHA domain (Bartek *et al.*, 2001). During checkpoint activation, Rad53-SCD domains are phosphorylated by upstream kinase Mec1 with the intervention of mediators binding to the FHA domains (Smolka *et al.*, 2006). Phosphorylated Rad53 is bound by another Rad53 monomer and this event leads to *trans* auto-phosphorylation and kinase activation (Pelliccioli & Foiani, 2005). Up to 65 Ser and Thr sites of Rad53 are modified (Smolka *et al.*, 2005). The mediator protein Mrc1 and Rad9 are believed to facilitate Rad53 phosphorylation by Mec1 and to increase the local concentration of Rad53 at sites of damage (Alcasabas *et al.*, 2001; Osborn & Elledge, 2003; Sun *et al.*, 1998; Sweeney *et al.*, 2005; Vialard *et al.*, 1998). Once activated, Rad53 phosphorylates and regulates a wide range of substrates (Chen *et al.*, 2010; Smolka *et al.*, 2007).

Rad53 phosphorylation status is believed to correlate with its kinase activity, as shown by *in situ* auto-phosphorylation assay (ISA) (Pelliccioli *et al.*, 1999). Rad53 presents phosphorylated residues (either Ser or Thr) in the absence of DNA damage and additional sites get phosphorylated in response to MMS (Smolka *et al.*, 2005) or the UV-mimetic compound 4-nitroquinoline oxide (4-NQO) (Sweeney *et al.*, 2005); interestingly, the phosphorylation pattern seems to depend on the type of damaging agent. Moreover, the disappearance of Rad53 phosphorylation parallels recovery or adaptation (Pelliccioli *et al.*, 2001). Since new protein synthesis is not required for the appearance of unphosphorylated Rad53, it is likely that Rad53 is dephosphorylated by protein phosphatases (PPs) and not degraded during checkpoint inactivation (Tercero *et al.*, 2003).

Currently, at least three Ser/Thr phosphatases (Mustelin, 2007) are believed to act on Rad53: Ptc2, Ptc3 and Pph3. Ptc2 and Ptc3 share 62% identity at the amino acid level and belong to the PPM (protein phosphatase magnesium-dependent) family, which comprises seven isoforms (Ptc1-7) in yeast. Pph3 is a member of the PPP (phosphoprotein phosphatase) family, which relies on regulatory subunits for substrate specificity (Psy2 and Psy4).

The screening for genes able to suppress a hyperactive Rad53 form gave the first evidence of the involvement of a phosphatase (Ptc2) in the yeast checkpoint response (Marsolier *et al.*, 2000). In earlier studies, Ptc2 was found to have a role in the unfolded-protein response and in Cdc28 dephosphorylation (Cheng *et al.*, 1999; Welihinda *et al.*, 1998). Subsequently, Ptc2/3 were shown to promote Rad53 inactivation during HO-induced DSBs (Leroy *et al.*, 2003). Interestingly, Ptc2-Thr376 is phosphorylated by casein kinase 2 (CK2) and interacts constitutively with Rad53-FHA1 domain (Guillemain *et al.*, 2007; Ho *et al.*, 2002; Smolka *et al.*, 2006). This interaction is also maintained upon treatment with MMS (Smolka *et al.*, 2006). Pph3 forms a complex with Psy2 to

dephosphorylate Rad53 upon MMS treatment, while it relies also on the other regulatory subunit Psy4 during dephosphorylation of histone γ -H2A (Keogh *et al.*, 2006). Interestingly, in *pph3* Δ cells recovering from MMS, replication forks are stably stalled and not restarted, while firing of late origins is not inhibited and genome replication is eventually completed, with delay, despite of hyperphosphorylated Rad53 (O'Neill *et al.*, 2007). While Ptc2 binds to Rad53-FHA1, it has been shown that Pph3-Psy2 interacts with Rad53-KD (O'Neill *et al.*, 2007).

The generation of double and triple PP mutants led to new insights into Rad53 inactivation. In fact, when deleting *PTC2* in *pph3* Δ cells, Szyjka *et al.* observed an increased sensitivity to MMS, showing that these two PPs were not redundant. However, the authors proved a primary role for Pph3 in Rad53 dephosphorylation and fork restart during recovery from MMS (Szyjka *et al.*, 2008). Another study showed that Rad53 remains hyperphosphorylated after MMS removal in the triple mutant *ptc2* Δ *ptc3* Δ *pph3* Δ , while in HU treated cells Rad53 is eventually dephosphorylated and its kinase activity turned off, suggesting the involvement of a yet undiscovered PP (Travesa *et al.*, 2008). In a recent study, it has been described the role of protein phosphatase Glc7 during recovery from HU; however, Glc7 is dispensable during recovery from MMS (Bazzi *et al.*, 2010).

Summarizing, Ptc2/3 and Pph3 seem to be required for Rad53 inactivation during recovery from MMS, but they do not share complete redundancy. For example, after MMS damage, *pph3* Δ cells display phosphorylated Rad53 despite the presence of functional Ptc2/3 (O'Neill *et al.*, 2007; Szyjka *et al.*, 2008; Travesa *et al.*, 2008) and *ptc2* Δ *ptc3* Δ cells are not able to recover or adapt after an HO-induced DSB, but they are not hypersensitive to either UV or HU (Marsolier *et al.*, 2000). Since genotoxic agents produce different Rad53 phosphorylation patterns (Smolka *et al.*, 2005; Sweeney *et al.*, 2005), it is reasonable to speculate that different PPs may act on Rad53 according to the specific type of damage. Interestingly, the process of adaptation appears to be regulated by two kinases: CK2, which also determines Ptc2 association with Rad53 during recovery, and Cdc5 (Toczyski *et al.*, 1997; Vidanes *et al.*, 2010).

Rad53-SCD1 domain phosphorylation appears to have a primary role in Rad53 activation and functions (Chen *et al.*, 2007; Lee *et al.*, 2008a, 2003). SCD1 contains four phosphorylatable threonines (T5, T8, T12 and T15) that negatively affect Rad53 activation when substituted by alanines (*rad53-4AQ*), while adding back any of the threonine restores Rad53 activation, but not its interaction with Dun1. Indeed, the specific concomitant phosphorylation of T5 and T8 is required for the interaction with Dun1-FHA domain (Bashkirov *et al.*, 2003; Lee *et al.*, 2008a, 2003). Amongst the threonines, T8 seems to be the most important because *rad53-T8-3AQ* suppresses almost all *rad53-4AQ* phenotypes better than the other 3 single threonine mutations (Lee *et al.*, 2008a). It should be noted that *in vivo* phosphorylation of the SCD1 was missed in the two original MS studies (Smolka *et al.*, 2005; Sweeney *et al.*, 2005) most likely because the Rad53 peptides were N-acetylated *in vivo* resulting in mass change (Lee *et al.*, 2008a).

The aim of this study is to explore the phenotype and Rad53 phosphorylation

Table 6.1: Yeast strains used in this study

Strain	Genotype	Source
Y53	<i>MATa ade2-1 can1-100 leu2-3,112 trp1-1 ura3-1 sml1::HIS3 RAD5</i>	Zhao <i>et al.</i> (1998)
Y57	Y53 <i>rad53-R70A</i>	Pike <i>et al.</i> (2001)
Y1015	Y53 <i>rad53-4AQ</i>	Lee <i>et al.</i> (2008a)
Y1017	Y53 <i>rad53-T8-3AQ</i>	Lee <i>et al.</i> (2008a)
Y1461	Y53 <i>ptc2::KAN ptc3::NAT</i>	This study
Y1473	Y53 <i>pph3::TRP1</i>	This study
Y1474	Y53 <i>ptc2::KAN ptc3::NAT pph3::TRP1</i>	This study
Y1509	Y53 <i>ptc2::KAN ptc3::NAT rad53-R70A</i>	This study
Y1510	Y53 <i>pph3::TRP1 rad53-R70A</i>	This study
Y1511	Y53 <i>ptc2::KAN ptc3::NAT pph3::TRP1 rad53-R70A</i>	This study
Y1514	Y53 <i>ptc2::KAN ptc3::NAT rad53-T8-3AQ</i>	This study
Y1515	Y53 <i>pph3::TRP1 rad53-T8-3AQ</i>	This study
Y1516	Y53 <i>ptc2::KAN ptc3::NAT pph3::TRP1 rad53-T8-3AQ</i>	This study
Y1519	Y53 <i>ptc2::KAN ptc3::NAT rad53-4AQ</i>	This study
Y1520	Y53 <i>pph3::TRP1 rad53-4AQ</i>	This study
Y1521	Y53 <i>ptc2::KAN ptc3::NAT pph3::TRP1 rad53-4AQ</i>	This study

status of MMS-recovering cells harboring different combinations of mutations in *PP* and *RAD53* genes. Specifically, deletion mutants of the three protein phosphatases Ptc2, Ptc3 and Pph3 will be coupled to specific point mutations of Rad53-SCD1 (*rad53-4AQ* and *rad53-T8-3AQ*) and Rad53-FHA1 phospho-peptide binding capacity (*rad53-R70A*). We suggest a role for the interaction between the threonine 8 of Rad53 and Ptc2/3 during recovery from MMS, although further biochemical pieces of evidence would be needed in order to validate this hypothesis.

Materials and methods

Yeast strains and cultures All mutant strains were based on the W303-1a background with corrected *RAD5* and deleted *SML1* (*sml1::HIS3*), in order to suppress viability defects of *rad53* mutants (Zhao *et al.*, 1998). Constructions of *rad53-4AQ*, *rad53-T8-3AQ* and *rad53-R70A* mutants were carried out by site-directed mutagenesis of the *RAD53* locus (Lee *et al.*, 2008a; Pike *et al.*, 2001). All the other mutant strains were generated according to standard PCR-based allele replacements with auxotrophic selection or antibiotic-resistant markers (**Table 6.1**; Y53 is the wild type strain). Cells were grown in orbital shakers at 30°C in YPD (1% yeast extract, 2% peptone and 2% glucose). To prepare YPD-agar plates, 2% agar was added to the solution.

MMS recovery assay Overnight cultures were diluted to $OD_{600} = 0.2$ and grown for additional 2 hours. Aliquots were taken immediately before MMS addition and plated on YPD-agar (untreated controls). The rest of the cultures was split into three tubes

and treated with different concentrations of MMS (0.02, 0.03 and 0.04%; final volume 2 mL). After 3-hour growth, aliquots were taken and plated on YPD-agar. Appropriate dilutions were used in order to get a countable number of cells. Plates were incubated for 3 days at 30°C and visible colonies were counted. Survival was expressed as the percentage of colony forming units (CFU) relative to the untreated controls.

Western blots Overnight cultures were diluted to $OD_{600} = 0.2$ and after 3 hours a 5 mL biomass sample was taken (negative untreated control). Cells were treated with MMS (final concentration 0.05%) and another sample taken after 1 hour (treated control). MMS was removed by washing with YPD + 5% sodium thiosulfate (3 times) and YPD (2 times) (Travesa *et al.*, 2008); cells were then resuspended and grown in fresh YPD for 5 hours, while taking samples every hour. Protein extraction and Western blots were performed as described previously (Pike *et al.*, 2001). To resolve the different forms of Rad53, protein extracts were run on 8% SDS-polyacrylamide gels and probed with polyclonal anti-Rad53-FHA1, developed in our lab (Pike *et al.*, 2003). Moreover the monoclonal F9A1 antibody, which is believed to specifically detect the active conformation of Rad53, was used (Fiorani *et al.*, 2008). Horseradish peroxidase-conjugated secondary antibodies and ECL reagents (Amersham Pharmacia Biotech) were used for detection. With respect to the *rad53* mutants, Rad53 folding and expression levels were shown to be comparable to WT and its DNA-damage induced electrophoretic shift comparable to plasmid-based complementation assays (Lee *et al.*, 2008a, 2003; Pike *et al.*, 2004).

Cell cycle analyses Overnight cultures were diluted to $OD_{600} = 0.2$ and, after 2-hour growth, α -factor (15 $\mu\text{g}/\text{mL}$) was added to induce G₁-phase synchronization (~2 hours). After release into S-phase, cells were treated with MMS 0.05% for 1 hour and the damaging agent was eliminated as described before. Cells were resuspended in fresh YPD and grown for 5 hours. Aliquots (300 μL) were taken throughout the growth: after synchronization, after 1-hour MMS treatment and during recovery. These aliquots were immediately fixed in 700 μL of 100% ethanol. Cells were then washed with PBS (phosphate buffered saline) and incubated overnight with 0.25 mg/mL RNase A. Cells were treated with propidium iodide (final concentration 10 $\mu\text{g}/\text{mL}$), sonicated and analyzed using a FACScalibur machine and FlowJo software.

Results and Discussion

Previous works have reported that the triple phosphatase mutant, *ptc2 Δ ptc3 Δ pph3 Δ* (*3PPs Δ*), presents hyperphosphorylated Rad53 during recovery from MMS (O'Neill *et al.*, 2007; Szyjka *et al.*, 2008; Travesa *et al.*, 2008) and that SCD1 phosphorylation sites have a key role in Rad53 regulation (Chen *et al.*, 2007; Lee *et al.*, 2008a, 2003). Therefore, we decided to investigate the role of these sites in the phenotype of the phosphatase deletion mutants. In addition, we have also investigated the role of the

Rad53-FHA1 domain, since it is involved in binding to Ptc2/3 (Guillemain *et al.*, 2007; Ho *et al.*, 2002; Leroy *et al.*, 2003).

Our data show that, during recovery from MMS, wild type cells display modest activation of Rad53 up to 2 hours after release (**Figure 6.1A**). The deletion of PPs affects this recovery dynamic. In fact, in *ptc2Δptc3Δ* cells the increase of 'active' Rad53 is stronger, but, similarly to WT, it starts decreasing after 2 hours. In *pph3Δ* cells, instead, the strong Rad53 activation is partly sustained after 2 hours, while, in *ptc2Δptc3Δpph3Δ* cells, we noticed unmitigated hyperactivation over the entire time-course (**Figure 6.1A**). These results are in agreement with a previous study (Travesa *et al.*, 2008). With respect to the sensitivity to MMS, we observed survival defects for these mutant strains, with *ptc2Δptc3Δpph3Δ* being the most affected. In fact, this mutant shows much higher sensitivity than WT (~100-fold) (**Figure 6.2C**).

Mutations in Rad53-SCD1 domain affect the MMS recovery and survival of the PPs mutant stains. Cells harboring the *rad53-4AQ* mutation present stronger Rad53 activation than WT during recovery (**Figure 6.1B**). When coupled with the PPs deletions, *rad53-4AQ* attenuates Rad53 hyperactivation during early recovery in *pph3Δ* cells, while, during late recovery, it is similar to *pph3Δ* cells carrying a functional copy of *RAD53*. In *ptc2Δptc3Δ* cells, *rad53-4AQ* causes a subtle increase of Rad53 activity, while no major effect is observed in *ptc2Δptc3Δpph3Δ* cells (**Figure 6.1B**). However, despite negligible effects on Rad53 inactivation, *rad53-4AQ* dramatically increases the survival of *ptc2Δptc3Δpph3Δ* cells (**Figure 6.2C**); consistent with this, *ptc2Δptc3Δpph3Δ* cells irreversibly stop in S-phase, as revealed by FACS analysis, but, when combined with *rad53-4AQ*, DNA synthesis is restored after 2-hour release in MMS (**Figure 6.3B-C**). This could suggest that SCD1 domain is a key target for phosphatase action, being responsible for the recovery defect of *ptc2Δptc3Δpph3Δ* cells. One would expect the beneficial effect of *rad53-4AQ* on the survival of the other PP mutants. Instead, we have observed paradoxical results with respect to *pph3Δrad53-4AQ* cells: indeed, *rad53-4AQ* mutation makes *pph3Δ* cells the most damage-sensitive among our experiments. This could be due to reduced Rad53 activity during early recovery respect to *pph3ΔRAD53* cells, but it is unlikely because the activity does not seem to be lower than WT (**Figure 6.1A-B**).

In the *rad53-T8-3AQ* mutant, the Western blot results show similar behavior to WT and only a slight improvement in Rad53 inactivation is seen in *pph3Δrad53-T8-3AQ* cells, while no major effects are evident in the other mutants (**Figure 6.1C**). Despite these minor effects, *rad53-T8-3AQ* suppresses the survival defects of *ptc2Δptc3Δ* and *ptc2Δptc3Δpph3Δ* cells, but not as much as *rad53-4AQ* in the case of the triple mutant (**Figure 6.2**). This is consistent with a preferential role for T8 in Rad53 functions (unpublished data from the lab). Moreover, *rad53-T8-3AQ* improves resumption of DNA synthesis in *3PPsΔ* cells, even though not to the same extent as *rad53-4AQ* (**Figure 6.3B-C**). Interestingly, Rad53-T8 rescues the strong survival defect of *pph3Δrad53-4AQ* cells, accompanied by the disappearance of Rad53 'active' form during late recovery (compare **Figure 6.1B** and **C** with respect to *pph3Δ*). Thus, it seems like T8 provides a link between Rad53 inactivation and damage recovery, at least

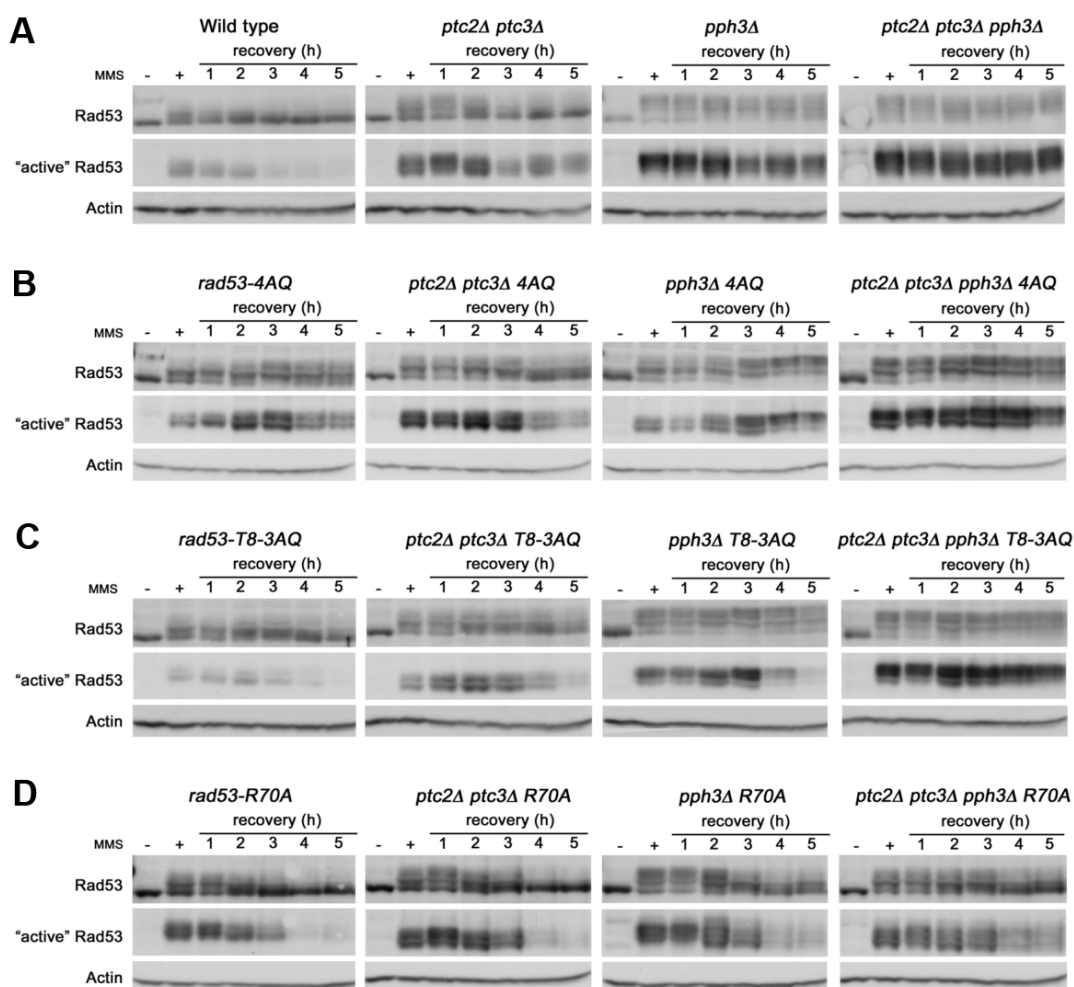


Figure 6.1: Rad53 inactivation during recovery from MMS - Western blots were obtained by using both anti-Rad53-FHA1 and F9A1 ('active' Rad53; see Materials and methods) antibodies. Samples were taken in untreated (-) and 0.05% MMS-treated (+) conditions, and during recovery (1 to 5 hours). Actin detection was used as loading control. *rad53-4AQ*, *rad53-T8-3AQ* and *rad53-R70A* are abbreviated as *R70A*, *4AQ* and *T8-3AQ* for clarity.

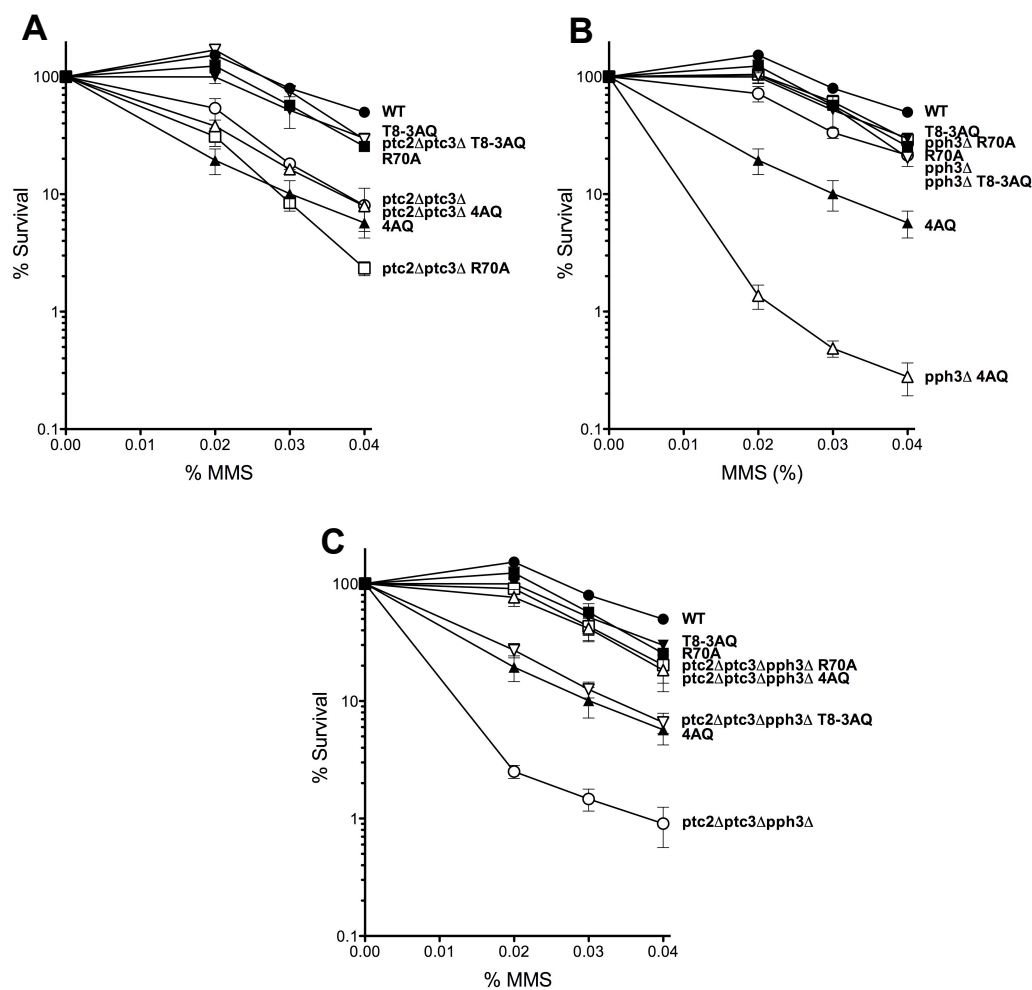


Figure 6.2: Sensitivity assays during recovery from MMS (0.02-0.03-0.04%) - Cell survival of (A) *PPsΔrad53-R70A*, (B) *PPsΔrad53-T8-3AQ* and (C) *PPsΔrad53-4AQ* mutant strains (mean \pm standard error of three independent experiments). WT indicates the wild type, while *rad53-R70A*, *rad53-4AQ* and *rad53-T8-3AQ* are abbreviated as *R70A*, *4AQ* and *T8-3AQ* for clarity.

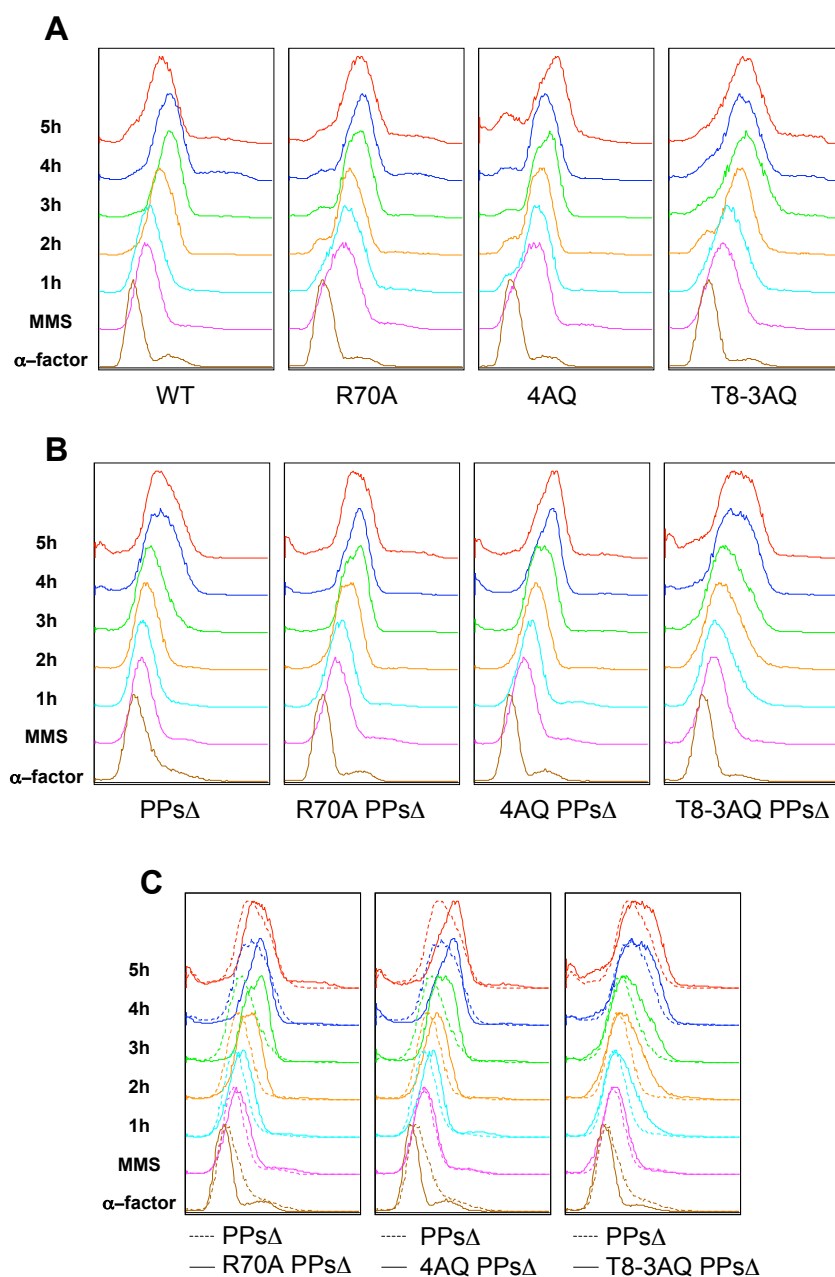


Figure 6.3: Fluorescent-activated cell sorting (FACS) analysis of DNA content
 - (A-B) Cells were synchronized in G₁ with α -factor, released in MMS 0.05% for 1 hour and let recover for 5 hours. (C) Superimposition of FACS profiles as described in the legend. WT indicates the wild type, while *ptc2* Δ *ptc3* Δ *pph3* Δ , *rad53-R70A*, *rad53-4AQ* and *rad53-T8-3AQ* are abbreviated as *PPs* Δ , *R70A*, *4AQ* and *T8-3AQ* for clarity.

in *pph3Δ* cells. Considering these findings, we speculate that Ptc2/3 may participate (directly or indirectly) in T8 dephosphorylation, probably to regulate late origin firing, since it is known that Ptc2/3 regulate this function and constitutively bind to Rad53-FHA1 (Guillemain *et al.*, 2007; Leroy *et al.*, 2003; Pike *et al.*, 2004; Smolka *et al.*, 2006). Since Pph3 is essential for replication fork restart (O'Neill *et al.*, 2007; Szyjka *et al.*, 2008), the *pph3Δrad53-4AQ* cell growth defect could be explained by the concomitant inability in replication fork restart and late origin firing.

With respect to the FHA1 domain effect during recovery, our data show that *rad53-R70A* cells have hyperphosphorylated Rad53 compared to the WT (**Figure 6.1D**), supporting previous work (Pike *et al.*, 2004). This shift is slightly increased in the PP mutants, but, with the exception of *ptc2Δptc3Δrad53-R70A*, Rad53 inactivation is faster than in the corresponding *RAD53* strains. In agreement with this, *rad53-R70A* suppresses the *3PPsΔ* survival defect and promotes efficient DNA synthesis resumption (**Figure 6.2C** and **Figure 6.3B-C**). However, *rad53-R70A* worsens the survival of *ptc2Δptc3Δ* cells (**Figure 6.2A**). This might suggest that Pph3 access to Rad53 also involves FHA1, as proposed for Ptc2/3 (Guillemain *et al.*, 2007; Leroy *et al.*, 2003) and that FHA1 and Ptc2/3 have independent roles during recovery.

In summary, our results suggest a possible role for Rad53-SCD1 in checkpoint inactivation, in particular for the threonine 8 of this domain. On the other hand, some pieces of evidences are contradictory. For example, as described before, *rad53-4AQ* renders the *3PPsΔ* mutant less DNA damage-sensitive, but worsens the recovery of *pph3Δ* cells. Probably, this illustrates the complexity of the action of the PPs: imbalance of PPs activity and accessibility of some substrates but not others (as in *pph3Δrad53-4AQ* cells) might cause misregulation of signaling pathways and detrimental effects (i.e., hypersensitivity to MMS). Rad53 is known to exert its functions via multiple interaction and phosphorylation of a wide range of substrates (Chen *et al.*, 2010; Smolka *et al.*, 2006, 2007). Therefore we cannot definitely conclude that these PPs directly dephosphorylate SCD1 and/or FHA1 domains. Phosphoproteome data would be of great value in investigating Rad53 phosphorylation patterns during damage recovery. Nevertheless, the two known studies about Rad53 phosphorylation patterns were performed in cells with functional copies of PP genes (Smolka *et al.*, 2005; Sweeney *et al.*, 2005), therefore it is not known whether PPs had dephosphorylated Rad53 when samples were taken. The ideal experimental set-up should involve the determination of Rad53 phosphorylation patterns in PPs deletion mutants treated with different DNA damaging agents in order to truly determine which sites are phosphorylated. Furthermore, it would be possible to determine whether checkpoint inactivation depends on Rad53 selective phosphorylation patterns brought about by different DNA damage agents, or selective dephosphorylation by protein phosphatases recognizing the same phosphorylated form of Rad53 (Heideker *et al.*, 2007).

It is important to make another consideration about Rad53 activation. A distinction has to be made between Rad53 phosphorylation status and the activation of its kinase activity. Although these two aspects have been correlated (Pelliccioli *et al.*, 1999), it has recently been shown that bulky Rad53 dephosphorylation can be separated from

the inactivation of Rad53 auto-phosphorylation activity (Travesa *et al.*, 2008) and our results about the ‘active’ Rad53 form partly reinforce this vision (see *pph3Δrad53-T8-3AQ* in **Figure 6.1C**). This model implies that protein phosphatases act specifically on Rad53 residues and fine-tune its functions, rather than through a simple on or off switch (Freeman *et al.*, 2010). Therefore Rad53 could have different levels of activity, carefully regulated by multiple phosphatases. However, a more accurate detection of Rad53 activity is needed, because the *in situ* kinase assay does not provide kinetically favorable conditions.

Lastly, our results support the model that proposes the separation of Rad53 functions during recovery (Heideker *et al.*, 2007; O’Neill *et al.*, 2007; Szyjka *et al.*, 2008). The fact that replication fork stabilization and late origin firing could be separable functions was already observed in a *mec1* mutant (*mec1-100*) (Paciotti *et al.*, 2001), in which Rad53 activation was delayed and reduced in response to MMS or HU. *mec1-100* cells were proficient for the stabilization of forks but defective for late-origin control, while both functions were impaired in *mec1Δ* cells (Tercero *et al.*, 2003).

Conclusion

The present study provides a first exploratory view of the interaction between *rad53* and *PPsΔ* mutations, suggesting a role for Rad53-T8 during recovery from MMS. Phosphorylation of T8 is important for the prevention of spontaneous damage during S-phase (Hoch *et al.* 2010, in preparation) and, in combination with T5 phosphorylation, is required for Dun1 activation (Lee *et al.*, 2008a). It may be that T8 cooperates with other SCD1 residues (e.g., T5, T12 or T15) to properly exert its role during checkpoint recovery. Moreover, results from a more quantitative kinase assay will be valuable to understand which residues is determinant for inactivation. It has been shown that in absence of damage, additional mutation of another phosphatase (Glc7) in *ptc2Δptc3Δpph3Δ* cells enhance growth defects (Bazzi *et al.*, 2010). Therefore multiple protein phosphatases act redundantly in unperturbed conditions in order to provide cells with increased control over the environment, while specific subsets of those PPs could be responsible for the selective response to damaging agents. A systems biology approach could be beneficial for the investigation of the interaction and (de)phosphorylation events underpinning the complex interplay between Rad53 and protein phosphatases during DNA damage recovery.

Part III

Epilogue

Chapter 7

Concluding remarks

In the work presented in this thesis, we have generated high-quality omics datasets and used computational tools in order to investigate principles governing cellular growth and response to DNA damage of the yeast *Saccharomyces cerevisiae*. Besides the conclusions presented in the previous chapters, further considerations can be made.

Which strain background should be used?

There is a complex relationship between genotype and phenotype (Dowell *et al.*, 2010). We have observed relevant differences in protein metabolism between CEN.PK (van Dijken *et al.*, 2000) and YSBN2 (S288c-derived, Mortimer & Johnston (1986)) and differences in MMS-induced damage response between W1588-4C (W303-derived, Thomas & Rothstein (1989)) and BY4741 (S288c-derived) (Brachmann *et al.*, 1998), despite the fact these strains are closely related (Schacherer *et al.*, 2007). Another example is the recently discovered difference in salt tolerance between BY4741 and W303 (Petrezse-lyova *et al.*, 2010). This poses issues about the comparability of studies employing different background strains, in particular when the same gene mutations are generated in different strains (Dowell *et al.*, 2010). The yeast community should agree on the background to be used. Ideally, a yeast scientist should be able to access the complete genome sequence of the strain used in a study, and not just the relevant genotype. In this way, one could try to understand and investigate the variability among yeast strains. Moreover, this would be useful to check whether the strains have undergone spontaneous mutations. However, full genotyping by high-throughput sequencing is still expensive and not available to every laboratory.

Phosphatases join kinases in DDR

Only recently light has been shed on the role of PPs during DNA damage response (Bakkenist & Kastan, 2004; Heideker *et al.*, 2007). Our results in Chapter 5 and 6 show the complexity and the specificity of their actions. We were not able to dissect in details the relationship between Rad53 and PPs (Ptc2/3 and Pph3) and further efforts will be invested in this direction. Indeed, a phosphoproteome study will be conducted

using our strains (**Table 6.1**, page 90), in collaboration with the University of Melbourne and the National Taiwan University. In this way, we will be able to assess whether, upon DNA damage, Rad53 phosphorylation status is determined by selective phosphorylation or selective dephosphorylation or both, and to study the dynamics of these events. With respect to another PP (Oca1), we have found evidence of its important role in the MMS-elicited transcriptional response. Little is known about Oca1 and data about its interactions with other DDR players will be valuable to understand its regulatory influence. Therefore, it appears that PPs may have a more prominent role during checkpoint response than previously thought. With respect to the role of PKs, future perspectives include the analysis of the transcriptional response of double mutant strains, lacking different combinations of PKs, PPs and TFs. Moreover, our MMS-induced network (**Figure 5.3**) would benefit from large-scale studies identifying targets of Mec1, Tel1, Chk1 and Oca1.

Systems biology next step: technology and stress

Transcriptomics is certainly the most advanced omics and the advent of new technologies, such as the transcriptome sequencing (RNA-Seq), will bring transcriptional studies to the next level (Wang *et al.*, 2009). This technology outperforms microarrays because it is quantitative and it does not require *a priori* sequence information to detect transcripts (Marioni *et al.*, 2008). However, RNA-Seq is expensive and not mature yet. Also metabolomics and proteomics have been developing remarkably in recent years. Thanks to these technological advances, we might be able to generate more and more accurate measurements, while improving the reproducibility of results among different laboratories. Nanofluidics and robotic liquid handling will likely participate in this process.

Systems biology still suffers from the lack of studies performed in stress conditions. Indeed, the majority of large-scale studies of genetic and protein interactions, that are used as scaffold in cellular network analysis, have been carried out in basal conditions. An external stimulus (e.g., MMS) can cause the rearrangement of these interactions, but, yet, we do not have a global picture of these events. Therefore, efforts in this direction will allow the investigation of global responses to specific environmental conditions.

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