The role of Not4 in cellular homeostasis during proliferation and differentiation in *Saccharomyces cerevisiae*

Dissertation zur Erlangung des akademischen Grades eines Doktors der Naturwissenschaften (Dr.rer.nat.)

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### 1. Abbreviations

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<tr>
<td>aa</td>
<td>amino acid(s)</td>
</tr>
<tr>
<td>ABC</td>
<td>ATP-binding cassette</td>
</tr>
<tr>
<td>ADP</td>
<td>adenosine diphosphate</td>
</tr>
<tr>
<td>Amp</td>
<td>ampicillin</td>
</tr>
<tr>
<td>AMP</td>
<td>adenosine monophosphate</td>
</tr>
<tr>
<td>AP</td>
<td>alkaline phosphatase</td>
</tr>
<tr>
<td>APC</td>
<td>anaphase-promoting complex</td>
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<td>APS</td>
<td>ammonium persulfate</td>
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<td>adenosine triphosphate</td>
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<tr>
<td>Avid</td>
<td>age-associated vulval integrity defect</td>
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<tr>
<td>b-isox</td>
<td>biotinylated isoxazole</td>
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<td>BCA</td>
<td>bicinchoninic acid</td>
</tr>
<tr>
<td>BF</td>
<td>bright field</td>
</tr>
<tr>
<td>bis-tris</td>
<td>bis-(2-hydroxy-ethyl)-amino-tris(hydroxymethyl)-methane</td>
</tr>
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<td>BP</td>
<td>biological process</td>
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<tr>
<td>bp</td>
<td>base pair(s)</td>
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<td>BSA</td>
<td>bovine serum albumin</td>
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<td>C. elegans/C.e.</td>
<td>Caenorhabditis elegans</td>
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<tr>
<td>CAF/Caf</td>
<td>CCR4 associated factor</td>
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<td>cAMP</td>
<td>cyclic adenosine monophosphate</td>
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<tr>
<td>CBD</td>
<td>calmodulin-binding domain</td>
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<tr>
<td>CBF</td>
<td>CCAAT-binding factor</td>
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<td>CC</td>
<td>cellular component</td>
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<td>CCR/Ccr</td>
<td>carbon catabolite repression</td>
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<td>CDC/Cdc</td>
<td>cell division cycle</td>
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<td>cDNA</td>
<td>complementary DNA</td>
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<td>CDS</td>
<td>coding sequence</td>
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<td>ChIP</td>
<td>chromatin immunoprecipitation</td>
</tr>
<tr>
<td>CHX</td>
<td>cycloheximide</td>
</tr>
<tr>
<td>CKI</td>
<td>cyclin-dependent kinase inhibitor</td>
</tr>
<tr>
<td>clonNAT</td>
<td>nourseothricin</td>
</tr>
<tr>
<td>CM</td>
<td>complete minimal</td>
</tr>
<tr>
<td>Co-IP</td>
<td>co-immunoprecipitation</td>
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<td>COMPASS</td>
<td>complex proteins associated with Set1</td>
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<td>CPF</td>
<td>cleavage and polyadenylation factor</td>
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<td>CSRE</td>
<td>carbon source-response element</td>
</tr>
<tr>
<td>Da</td>
<td>Dalton(s)</td>
</tr>
<tr>
<td>DE</td>
<td>denatured eluate</td>
</tr>
<tr>
<td>DMSO</td>
<td>dimethyl sulfoxide</td>
</tr>
<tr>
<td>DNA</td>
<td>desoxyribonucleic acid</td>
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<td>DNase</td>
<td>desoxyribonuclease</td>
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<td>dNTP</td>
<td>desoxynucleotide</td>
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<td>DTT</td>
<td>dithiothreitol</td>
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<td>DUB</td>
<td>deubiquitinase</td>
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<tr>
<td>(E. coli)</td>
<td>Escherichia coli</td>
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<tr>
<td>ECL</td>
<td>enhanced chemiluminescence</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>EGTA</td>
<td>ethylene glycol-bis((\beta)-aminoethyl ether)-(N,N,N',N')-tetraacetic acid</td>
</tr>
<tr>
<td>EM</td>
<td>electron microscopy</td>
</tr>
<tr>
<td>ER</td>
<td>endoplasmic reticulum</td>
</tr>
<tr>
<td>ESR</td>
<td>environmental stress response</td>
</tr>
<tr>
<td>ev</td>
<td>empty vector</td>
</tr>
<tr>
<td>FC</td>
<td>fold change</td>
</tr>
<tr>
<td>FG</td>
<td>filamentous growth</td>
</tr>
<tr>
<td>FRE</td>
<td>filamentous and invasion-responsive element</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>G6PDH</td>
<td>glucose-6-phosphate dehydrogenase</td>
</tr>
<tr>
<td>gDNA</td>
<td>genomic DNA</td>
</tr>
<tr>
<td>GDP</td>
<td>Guanosine diphosphate</td>
</tr>
<tr>
<td>GEF</td>
<td>guanine nucleotide exchange factor</td>
</tr>
<tr>
<td>GFP</td>
<td>green fluorescent protein</td>
</tr>
<tr>
<td>glc</td>
<td>glucose</td>
</tr>
<tr>
<td>GO</td>
<td>gene ontology</td>
</tr>
<tr>
<td>GPCR</td>
<td>G protein-coupled receptor</td>
</tr>
<tr>
<td>GPD</td>
<td>glyceraldehyde-3-phosphate dehydrogenase</td>
</tr>
<tr>
<td>GTP</td>
<td>guanosine triphosphate</td>
</tr>
<tr>
<td>HA</td>
<td>hemagglutinin</td>
</tr>
<tr>
<td>HAT</td>
<td>histone acetyltransferase</td>
</tr>
<tr>
<td>HCl</td>
<td>hydrogen chloride</td>
</tr>
<tr>
<td>HDAC</td>
<td>histone deacetylase</td>
</tr>
<tr>
<td>HEAT</td>
<td>huntingtin, elongation factor 3, protein phosphatase 2A, TOR1</td>
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<tr>
<td>HEPES</td>
<td>4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid</td>
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<tr>
<td>HG</td>
<td>high growth</td>
</tr>
<tr>
<td>HML</td>
<td>hidden MAT left</td>
</tr>
<tr>
<td>HMM</td>
<td>hidden Markov model</td>
</tr>
<tr>
<td>HMR</td>
<td>hidden MAT right</td>
</tr>
<tr>
<td>HRT</td>
<td>homologous repair template</td>
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<tr>
<td>HSE</td>
<td>heat shock element</td>
</tr>
<tr>
<td>Hsp</td>
<td>heat shock protein</td>
</tr>
<tr>
<td>HU</td>
<td>hydroxyurea</td>
</tr>
<tr>
<td>IAA</td>
<td>indole-3-acetic acid</td>
</tr>
<tr>
<td>IG</td>
<td>invasive growth</td>
</tr>
<tr>
<td>IP</td>
<td>immunoprecipitation</td>
</tr>
<tr>
<td>IPTG</td>
<td>isopropyl β-D-1-thiogalactopyranoside</td>
</tr>
<tr>
<td>kd</td>
<td>knockdown</td>
</tr>
<tr>
<td>kDa</td>
<td>kilo Dalton(s)</td>
</tr>
<tr>
<td>krpm</td>
<td>thousand revolutions per minute</td>
</tr>
<tr>
<td>LB</td>
<td>lysogeny broth</td>
</tr>
<tr>
<td>LRR</td>
<td>leucine-rich repeat</td>
</tr>
<tr>
<td>MAT</td>
<td>mating type locus</td>
</tr>
<tr>
<td>MF</td>
<td>molecular function</td>
</tr>
<tr>
<td>MFS</td>
<td>major facilitators super family</td>
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<tr>
<td>MIF4G</td>
<td>middle domain of initiation factor 4G</td>
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<td>MOPS</td>
<td>3-morpholinopropene-1-sulfonic acid</td>
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<tr>
<td>mRNA</td>
<td>messenger ribonucleic acid</td>
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<tr>
<td>mRNP</td>
<td>messenger ribonucleoprotein</td>
</tr>
<tr>
<td>NAC</td>
<td>nascent polypeptide-associated complex</td>
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<td>NaOH</td>
<td>sodium hydroxide</td>
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<tr>
<td>NCR</td>
<td>nitrogen catabolite repression</td>
</tr>
<tr>
<td>NE</td>
<td>native eluate</td>
</tr>
<tr>
<td>NEM</td>
<td>N-ethylmaleimide</td>
</tr>
<tr>
<td>NG</td>
<td>normal growth</td>
</tr>
<tr>
<td>NOT/Not</td>
<td>negative on TATA</td>
</tr>
<tr>
<td>nt</td>
<td>nucleotide(s)</td>
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<td>Ntl</td>
<td>NOT-like</td>
</tr>
<tr>
<td>o.n.</td>
<td>overnight</td>
</tr>
<tr>
<td>OD</td>
<td>optical density</td>
</tr>
<tr>
<td>ORF</td>
<td>open reading frame</td>
</tr>
<tr>
<td>P-bodies</td>
<td>processing bodies</td>
</tr>
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<td>polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>PAK</td>
<td>p21-activated protein kinase</td>
</tr>
<tr>
<td>PAN</td>
<td>poly(A) nuclease</td>
</tr>
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<td>PAPA</td>
<td>prion aggregation prediction algorithm</td>
</tr>
<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
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</table>
Abbreviations

PDS  post-diauxic shift
PGK  phosphoglycerate kinase
PKA  protein kinase A
PLAAC prion-like amino acid composition
PMSF phenylmethylsulfonyl fluoride
Poly(A) poly-adenine
PrD prion-like domain
PRE pheromone-response element
Pvl protruding vulva
rDNA ribosomal DNA
RE restriction endonuclease
RING "Really Interesting New Gene" domain
RNA ribonucleic acid
RNAi ribonucleic acid interference
RNase ribonuclease
RP ribosomal protein
RPKM reads per kilobase per million
rpm revolutions per minute
RRM RNA recognition motif
rRNA ribosomal ribonucleic acid
RT room temperature
RTG retrograde regulation
RUB/Rub related to ubiquitin
S. cerevisiae/S.c. Saccharomyces cerevisiae
SC synthetic complete
SDS sodium dodecyl sulfate
SGD Saccharomyces cerevisiae genome database
sHsp small heat shock protein
SNF sucrose non-fermenting
SPT suppressor of Ty
STRE stress-response element
TA transcriptional activator
TAE tris/acetic acid/EDTA
TAP tandem affinity purification
TBE tris/borate/EDTA
TBP TATA-binding protein
TE tris/EDTA
TEMED tetramethylethylenediamine
Tet tetracycline
TEV Tobacco Etch Virus
TF transcription factor
TOR target of rapamycin
tRNA transfer ribonucleic acid
Ub ubiquitin
UBC/Ubc ubiquitin-conjugating
UBL ubiquitin-like modifier
w/ with
w/o without
WB Western blot
wt wild type, wild-type
X-link(ing) crosslink(ing)
Y2H yeast two-hybrid
YPD yeast extract peptone dextrose
YPG yeast extract peptone glycerol
β-ME β-mercaptoethanol
2. Summary

2.1 Summary (English version)
The eukaryotic Ccr4-NOT complex constitutes a versatile regulatory platform - by making extensive contacts to a multitude of components of the protein biosynthesis machinery - and impinges on the spatiotemporal and context-specific control of the expression of genes. It accompanies mRNAs from their transcriptional synthesis, over translation, and up to their degradation. It is highly involved in the interplay between environmental stimuli and gene expression-dependent responses. The Ccr4-NOT complex comprises nine core subunits, which are arranged around the central scaffolding protein Not1. The complex can be subdivided functionally into two major modules: the deadenylase and NOT module. While the deadenylase module, comprising Caf1 and Ccr4, constitutes the major deadenylase activity, triggering mRNA degradation, the NOT module, including Not2 through Not5, is mainly involved in transcriptional repression. In addition, the NOT module component Not4 is an E3 ubiquitin ligase, which interacts with the E2 enzymes Ubc4/5, and mediates substrate-specific ubiquitination reactions. The complex is highly conserved with regard to its structural organization, as well as its immanent abilities including molecular functions and biological processes.

For more than 25 years, the Ccr4-NOT complex has been subjected to intensive studies and revealed its potential as a central hub in the context-specific regulation of gene expression. It is involved in the fine-tuning of responses during stress and development, as well as in cellular homeostasis under favorable conditions. The characterization of components of the Ccr4-NOT complex in the budding yeast *Saccharomyces cerevisiae* is mostly carried out through deletion of the respective genes, with the exception of the only essential subunit Not1.

The work presented here is focused on the E3 ubiquitin ligase Not4 in the budding yeast and its ortholog NTL-4 in the nematode *Caenorhabditis elegans*. It embodies the following insights into the complex regulatory network within which the Ccr4-NOT conducts its activity in a Not4-specific manner:

1. Although the deletion of *NOT4* is tolerated in budding yeast, it sporadically displayed the formation of colonies capable of suppressing growth-related phenotypes. Therefore, an alternative approach to investigate the loss of Not4 function was applied. Controlled Not4 protein depletion proved suitable as an alternative strategy to examine loss of function, and most phenotypic expressions observed for *NOT4*-deleted cells can be reproduced.

2. A microarray analysis comparing total mRNAs of Not4-depleted versus non-depleted cells partially revealed qualitative and quantitative differences with respect to differentially expressed genes compared to a similar study described in the literature conducted on *NOT4*-deleted cells. This observation provided for an insightful distinction between short-term and immediate effects of loss of Not4 function in contrast to long-term and probably accumulative effects on a transcriptome-wide level. In addition, the microarray data revealed a new explanatory starting point with respect to the derepressive effect on mating-responsive genes in *NOT4*-deleted cells described in the literature with the finding of inappropriate expression of genes encoding alpha-pheromone of the opposite mating type. Consequently, this led to the suggestion that autocrine stimulation of the MAPK-mediated mating response
pathway could account for an activation of these target genes, in contrast to mere derepression in dependency of Not4.

3. The function of Not4 is not essential to the reprogramming of the transcriptome and translational repression in response to amino acid withdrawal, but globally impinges on the efficiency, which can turn out critical for survival. Interestingly, polysome profiling, as a measure of global translation, revealed greater differences under amino acid-rich conditions in Not4-depleted cells than under amino acid-scarce conditions. In this context, Not4 was required to promote translation under favorable conditions.

4. During the evaluation of phenotypes characteristic for NOT4-deleted cells, a previously undescribed phenotype was discovered in Not4-depleted cells: invasive growth. This phenotype was also confirmed in NOT4-deleted cells, and surprisingly appeared in a mating type-dependent manner, as only MATa cells depleted of Not4 displayed this behavior. However, this phenotype most likely constitutes a side effect of the putative autocrine stimulation of the mating response observed for Not4-depleted cells.

5. Preliminary results obtained from the investigation of the Not4-ortholog ntl-4 in C. elegans provided insights into the localization of the protein within adult worms. The localization of NTL-4 was most apparent within gonads, thus hinting at an involvement within oogenesis, which matched the general impression of a regulatory participation during development and differentiation processes of the Ccr4-NOT complex. Within cells, NTL-4 was unambiguously detected within the cytoplasm, where it occurs evenly distributed and concentrated within distinct foci. An RNA interference-mediated ntl-4-specific knockdown demonstrated that NTL-4 had no significant effect on the survival of adult worms, but rather affected development indicated by the high occurrence of the protruding vulva phenotype, caused by compromised structural integrity of this sexual organ. The frequency with which the protruding vulva phenotype occurred could be reduced to control levels, if RNA-interference was applied post-developmentally, and thus emphasized an involvement of NTL-4 during development.

6. The glutamine/asparagine-rich C terminus of NTL-4 was predicted to resemble the defined sequence features characteristic for prion-like domains. Furthermore, it was determined that the protein can be precipitated in a biotinylated isoxazole-dependent manner, which opens up new possibilities for follow-up studies on NTL-4. Likewise, Not4 was also precipitated even though the prediction for prion-like domains yielded the yeast ortholog improbable to apply to a positive prediction. However, the precipitation behavior of Not4 was significantly affected by truncation of the N or C terminus, respectively.
Zusammenfassung (deutsche Fassung)


3. Die Funktion von Not4 ist nicht essenziell für die Umprogrammierung des Transkriptoms und der translationalen Repression aufgrund eines Aminosäureentzugs, jedoch beeinträchtigt sein Verlust global die Effizienz, mit welcher diese Prozesse ablaufen. Dies kann sich kritisch auf das Überleben der Zellen auswirken. Interessanterweise konnte durch Polysomenprofile, welche ein Maß für die globale Translation darstellen, gezeigt werden, dass Not4-dezimierte Zellen unter aminosäurearmen Bedingungen größere Unterschiede zu Kontrollzellen aufwiesen als unter aminosäurereichen Bedingungen. In diesem Zusammenhang ergab sich, dass die Funktion von Not4 unter vorteilhafte Bedingungen für die Förderung der Translation erforderlich ist.


3. Introduction

The life of the budding yeast *Saccharomyces cerevisiae* can be described from different perspectives. *Figure 1* gives a brief overview on four relevant perspectives, each of which can be further dichotomized. The following sections will give insights into each perspective in more detail, which are relevant for this work. This means, the life of yeast is highly dependent on the internal (i.e. diploid versus haploid) and external (i.e. availability of nutrients) contexts, and the diversity of different forms of existence allow for adaptation to an ever changing environment, which constitutes a prerequisite of survival. Of course, the subdivision into different perspectives and related processes are often purely artificial, but serves greater clarity. Phenotypic plasticity is often established by fluent transitions from one state into another, and is marked by intensive crosstalk between distinct and overlapping signaling networks, as well as the interdependency of relevant processes. The budding yeast is a powerful workhorse for fundamental research and many conserved processes and functions, relevant for the basic understanding of human health and disease, have initially been studied in this organism.

![Diagram](image)

*Figure 1*: Overview of the different states within the life of *Saccharomyces cerevisiae*, including cell types, cellular proliferation, cellular differentiation, and metabolic states. Colored arrows indicate the availability of the respective compounds.

A detailed description of the most relevant signaling pathways concerning proliferation and differentiation in yeast can be found in the chapter 11.1 Appendix introduction.
3.1 The Ccr4-NOT complex in regulation of proliferation and differentiation programs

The Ccr4-NOT complex is a versatile platform and accompanies mRNA transcripts from synthesis to decay. It is highly conserved in eukaryotes, and is subject of studies in all commonly used model organisms. Nevertheless, some species-dependent differences exist, which will be addressed later. Before the whole complex was recognized as such, many of the genes encoding one of the nine subunits of the Ccr4-NOT core complex had been isolated in genetic screens. A more detailed description about the structural features of the complex will be given in a separate section.

Beginning in 1980, NOT1 and NOT2 were isolated as so-called START mutants that mimic a specific cell cycle arrest at START in G1, characteristic for cells treated with pheromone (Reed, 1980). Accordingly, the original names given to those genes were CDC39 and CDC36, respectively, where cdc stands for cell division cycle. A decade later, it was discovered that G1-arrest at START in mutants of CDC39 and CDC36 most likely originated from the activation of the mating response pathway (Neiman, Chang, Komachi, & Herskowitz, 1990). The discovery of the CCR4 (carbon catabolite repression) and POP2 (PGK promoter directed overproduction) genes, encoding the deadenylases Ccr4 and Caf1, came from glucose repression and derepression studies (Denis, 1984; Sakai, Chibazakura, Shimizu, & Hishinuma, 1992). A mutant ccr4 inhibited the expression of glucose-repressible, nonfermentative genes like ADH2 and restored glucose repression on the ADH2 locus in spt10/spt6-mutant cells (Denis, 1984), and a mutant pop2 allele was isolated from a screen aiming at genes that would exhibit a glucose-derepression resistant and sucrose-non-fermentor (snf) phenotype (Sakai et al., 1992).

The transition from fermentative growth on glucose (glucose repression) to respiratory growth (glucose derepression) via the post-diauxic shift requires intensive reprogramming of the transcriptome, and is accompanied by physiological and metabolic changes. Interestingly, the post-diauxic shift can be interpreted as a specific stress response. As many stress-responsive genes, summarized as constituents of the environmental stress response (ESR), are transcriptionally activated or repressed under diverse stresses similar to post-diauxic shift (Gasch et al., 2000). Therefore, the early findings around the Ccr4-NOT complex subunits Not1, Not2, Ccr4, and Caf1 all share their involvement in biological processes which require massive cellular changes and precise spatiotemporal coordination of gene expression.

The NOT4 gene was described independently by four groups in 1994 (Cade & Errede, 1994; Collart & Struhl, 1994; Irie, Yamaguchi, Kawase, & Matsumoto, 1994; Leberer, Dignard, Harcus, Whiteway, & Thomas, 1994). It was described under three different names, as SIG1 (suppressor of inhibitory G-protein) (Leberer et al., 1994), MOT2 (modulator of transcription) (Cade & Errede, 1994; Irie et al., 1994), and finally NOT4 (negative on TATA) (Collart & Struhl, 1994). Three of the studies have performed screens for suppressors that restored mating competence to cells either carrying different temperature-sensitive mutants of STE4 or STE11, or overexpressing a dominant-negative mutant of STE4. Additionally, all three studies reported a slow growth phenotype at optimal temperatures (22°C/25°C/30°C), and inviability at 37°C. And finally, microscopy revealed an abnormal morphology, as cells were bigger in size and irregularly shaped (Leberer et al., 1994).

The fourth study, published by Collart and Struhl, identified three genes (NOT2, NOT3, NOT4) in addition to a previous report in which CDC39 was described as a negative regulator of transcription in an assay that distinguishes basal from activated transcription on the HIS3 locus (Collart &
Accordingly, these genes were described as negative regulators of transcription.

The carbon source-dependence of the Ccr4-NOT complex is further reflected by the presence and stoichiometry of its constituents. The highest protein levels of the complex partners were detected under conditions of fermentative growth on glucose. Whereas strongly reduced protein levels were especially obvious for Not1, Not3, Not4, and Not5 upon growth on non-fermentative medium containing ethanol and glycerol as a carbon source, or after cells reached stationary phase (Norbeck, 2008). In other words, under conditions where proliferation is promoted and stress or developmental responses are inhibited, the highest protein levels of the Ccr4-NOT complex subunits were detected. This fits perfectly to the observations that in the absence of any one of the previously named complex partners, developmental responses (i.e. the mating response) are being derepressed under conditions where proliferation should be favored. There exists more direct, molecular evidence for a nutrient-dependent function of the Ccr4-NOT complex, especially with regard to the two deadenylases Caf1 (Pop2) and Ccr4. Caf1 is a target of the Yak1 kinase in the nucleus under conditions of limited glucose, Yak1 itself is negatively regulated by the Ras-cAMP-PKA pathway by cytosolic retention (Moriya et al., 2001).

In addition, studies of the phospho-proteome in dependence on rapamycin revealed rapamycin-induced hypophosphorylation of Ccr4, conversely this could indicate that Ccr4 is a direct or indirect target of TORC1 signaling, and thus displays altered activity/substrate specificity in response to nutrients (Soulard et al., 2010). The functional link between the Ccr4-NOT complex and TOR signaling was further confirmed by a study concerning the regulation of rRNA synthesis by RNA polymerase I in a Ccr4-NOT complex-dependent manner (Laribee, Hosni-Ahmed, Workman, & Chen, 2015). This study provided biochemical proof of the previously found hypophosphorylation of Ccr4 upon rapamycin treatment. Furthermore, cells deleted of CCR4, CAF1, NOT4, or NOT5 were inviable on plates supplemented with rapamycin, in contrast to other tested complex partners (CAF40, CAF130, NOT3).

Taken together, the Ccr4-NOT complex and its subunits were connected to all major signaling pathways regarding proliferation and differentiation, including the MAPK-mediated mating response, as well as the nutrient-responsive Ras-cAMP-PKA pathway, the SNF network, and TOR signaling. In sum, the macroscopic activity of the Ccr4-NOT complex can be described as pro-proliferation and anti-differentiation. The following sections will give an overview of the structure of the complex, as well as insights into the molecular mechanisms underlying the manifold Ccr4-NOT complex functions.

### 3.2 The architecture of the Ccr4-NOT complex

The first visualization of the complete Ccr4-NOT complex was obtained by electron microscopy and revealed the general shape of the complex isolated from yeast cells (Figure 2 A) (Nasertorabi, Batisse, Diepholz, Suck, & Böttcher, 2011). The composition of the complex after affinity and glycerol gradient purification was verified by SDS-PAGE and Coomassie staining. The result proved the presence of all nine core complex members in a high degree of purity, namely Caf1, Caf130, Caf40, Ccr4, Not1-5. The EM results revealed the overall L-shape of the core complex, with a shorter arm of 180 Å and a longer arm of 190 Å.
Within the EM structure, the largest subunit and scaffolding protein Not1 is oriented with its N terminus matching the long arm, and the C terminus matching the short arm. According to preceding biochemical interaction studies that shed light on the direct interactions between the complex partners, the authors included the putative positions of complex partners into their result (Figure 2 A). For example, co-immunoprecipitations via Ccr4 from different Ccr4-NOT single mutants revealed the dependency of Not protein binding (Not2-Not5) on the presence of Not1’s C terminus, by the mutant NOT1 allele not1-2, which produces a C-terminally truncated Not1 protein (Not11,1315) (Bai et al., 1999). The surface of the complex displayed an accessible cavity formed between both arms, which the authors suggest could provide for substrate (RNA) interaction and/or binding of additional proteins (Nasertorabi et al., 2011). The EM-derived structural overview of the Ccr4-NOT complex was further refined by subsequent crystal structure analyses of different subunits. One study focused on the architecture of the nuclease module of the yeast Ccr4-NOT complex (Basquin et al., 2012). The deadenylase module was reconstituted from recombinantly expressed proteins (Not1754-1000, Ccr4110-837, Caf1146-453).

The stepwise analysis took the already available structural information of Caf1 into account (Thore, Mauxion, Séraphin, & Suck, 2003), and combined it with the results obtained from individually analyzed Not1754-1000. In the last step, a model of the ternary Not1754-1000-Caf1-Ccr4 complex was built based on the available information (Figure 2 B). In addition to the Not1 domain concerning the binding of the nuclease module, the authors also resolved the crystal structure of another N-terminal portion of Not1 (Not1154-753). Both obtained partial Not1 structures displayed organization in HEAT repeats. Each HEAT repeat is characterized by two α-helices (helix A-turn-helix B), packed against each other and organized in an antiparallel fashion. These repeats usually unfold their significance in extensive protein-protein interactions (Andrade, Petosa, O’Donoghue, Müller, & Bork, 2001). Not1154-753 displayed 13 HEAT repeats, mostly arranged in a parallel manner, with the exception that HEAT repeats 1-9 were rotated by about 90° toward HEAT repeats 10-13 (Figure 3 E left). The crystal structure obtained from Not1754-1000 displayed five HEAT repeats that resembled the HEAT-repeat fold of the middle domain of initiation factor 4G (MIF4G) (Figure 3 E middle). In their model the nuclease domain of Caf1 bridges the interaction of Ccr4 via its leucine-rich repeat (LRR) domain, to the MIF4G domain of Not1 (Figure 2 B). This is in accordance with biochemical data that emphasized the significant role of Caf1 in the stabilization of Ccr4 at the Ccr4-Not complex (Azzouz et al., 2009; H.-Y. Liu et al., 1998).

Structural resolution of the way Not2 and Not5 bind to the C terminus of Not1 revealed the concomitant binding of Not2 and Not5, supported by previous findings from biochemical studies (Azzouz et al., 2009; Bai et al., 1999; Bhaskar et al., 2013) (Figure 2 C). The crystal structure obtained from the C-terminal portion of Not1, relevant for the investigation of Not2/Not5-binding (Not11541-2093), displayed ten HEAT repeats of regular architecture. These HEAT repeats can be further divided into two units, one comprising HEAT 1-6 and the other HEAT 7-10, as both units displayed a perpendicular orientation toward each other (Figure 2 C). One special structural feature was found in the second unit (HEAT repeats 7-10), as they resembled four of the five HEAT repeats characteristically associated with the MIF4G domain, and therefore was considered as MIF4G-like (Figure 3 E right). Both, Not2 and Not5 carried a globular domain, which contained their C-terminal NOT boxes. Both NOT boxes were found to be similar in their structure, consisting roughly of three α-helices and a β-sheet (consisting of four antiparallel β-strands) (Figure 2 C). Not2 and Not5 interacted with each other via their NOT boxes, which were arranged in an antiparallel fashion, α1 contacting α3 and vice versa. Whereas the globular C termini mediate the interac-
tion between Not2 and Not5, the N-terminal extensions of both proteins each make multiple contacts with the C terminus of Not1. Not2 binding to Not1 concerns HEAT repeats 3-6 and 9-10 of Not1, whereas Not5 wraps around the HEAT repeats 1-5 (Figure 2 C). The cooperative binding of Not2 and Not5 to Not1 was likewise shown for the respective counterparts of the human Ccr4-NOT complex (namely CNOT2 and CNOT3 to CNOT1) (Figure 2 D).

The intimate relationship between Not2 and Not5 with regard to Not1 binding, was further expanded by a study focusing on the interaction between Not4 and Ubc4, and Not4 and Not1 (Bhaskar, Basquin, & Conti, 2015) (Figure 3 A). The obtained crystal structure of Not4_{418-589} bound to Not1_{1348-2093} showed how the C-terminal residues 426-439 of Not4 fold into an α-helix and contact Not1.
between HEAT repeat 2 and 3. Further contacts are made by Not4 residues 442-452 by connecting HEAT repeat 1 to 2 and HEAT repeat 2 to 3. And lastly, Not4 binds to Not1 via residues 462-469 by insertion between the A and B α-helices of HEAT repeat 1 of Not1. Superposition of the structures obtained from the interaction between Not1’s C terminus with Not2 and Not5, and with Not4 showed that even though all three Not proteins (Not2, Not4, Not5) make contacts to HEAT repeats 1-6 from the C-terminal Not1 structure, they are arranged in a way that allows for simultaneous interaction (Figure 3 A, B, D). In addition, the superposition revealed two helical structures from Not4 and Not5 in close proximity that suggest an interaction between Not5 and Not4 with respect to Not1 binding. This is in accordance with results from co-purification studies that showed Not5-dependent binding of Not4 to the Ccr4-NOT complex, either by complete absence of Not4 from purified fractions or significantly decreased levels of Not4 when purified from not5Δ cells (Bai et al., 1999; Gupta et al., 2016). This suggests that the interaction of Not4 to the C terminus of Not1 is further stabilized by interactions with Not5.

Besides the resolution of the Not4-Not1 interaction, the crystal structure of the interaction between the RING domain of Not4 and one of its interaction partners, the E2 enzyme Ubc4, was additionally resolved based on recombinantly expressed fusion protein comprising Not430-83-Ubc4 (Figure 3 C). The RING domain of Not4 comprises two α-helices of different length (short α1 and long α2), a total of eight cysteine residues coordinate two zinc ions as protrusions from helix α2 and the three loop regions (L1-L3). Broadly speaking, helix α2 and loops L1-L3 of Not4 interact with loops L4 and L5 of Ubc4. The binding interface of Not4 mostly concerns residues Leu35, Ile56, Cys60, Asn63, Leu70, and Pro75, which form mostly hydrophobic interactions with residues Pro62, Phe63, and Pro96 of Ubc4. Additionally, Not4Ile37 provides for a hydrophobic interaction with Ubc4 helix α1. A hydrogen bond is formed by Not4Arg78 and Ubc4Gln93, and salt bridges are formed by Not4Glu38/Ubc4Lys5 and Not4Glu69/Ubc4Lys64 (Figure 3 C). The significant role of Not4’s residues Leu35 and Ile37 with respect to binding of Ubc4, as well as the highly similar Ubc5, was previously determined in a yeast two-hybrid screen, where substitutions of the respective amino acids to alanine deter the interaction with Ubc4/5 (Mulder, Inagaki, et al., 2007).
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Figure 3: Crystal structures concerning Not4 interactions with Not1, Not2, Not5, and Ubc4. (A) Cartoon representation of the interaction between the C-termini of Not4 (blue) and Not1 (yellow). (B) Same as in A, but including the available information about Not2 (purple) and Not5 (green) interaction with the C terminus of Not1 (yellow). (C) Cartoon representation of the interaction between the RING domain of Not4 (blue) and Ubc4 (purple). The relevant interaction sites at the level of single amino acids is displayed as a subsection on the right. (D) Schematic representation of the interactions between the C terminus of Not1 with Not2, Not4, and Not5. (E) Summary of the HEAT repeats identified by X-ray crystallography. Cartoon representations of the respective HEAT repeats are displayed below.
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3.3 Transcriptional regulation through the Ccr4-NOT complex

The adequate regulation of transcription is essential for proliferation, differentiation and response to stress, and involves a highly dynamic, context-specific interplay between a variety of proteins and protein complexes (reviewed by Weake & Workman, 2010). The role of the Ccr4-NOT complex in the regulation of transcription was demonstrated on many different levels of regulation. These include the regulation and integrity of histone modifying complexes, transcriptional co-activator complexes, transcription factors, RNA polymerases I and II, as well as interactions with the nuclear RNA surveillance network.

The modification of histones through acetylation, methylation, phosphorylation, and ubiquitination is highly context-specific regarding the respective amino acid residue to be modified, or the histone protein on which a modification occurs. Accordingly, histone modifications are strictly regulated in a spatiotemporal manner. The significance of these post-translational modifications (PTMs) was well investigated regarding the fate of diploid yeast cells, where histone modifications were demonstrated to substantially impinge on whether cells undergo meiosis and sporulation, pseudohyphal growth, or apoptosis (Jaiswal, Turniansky, & Green, 2017; Walter, Matter, & Fahrenkrog, 2014). Furthermore, the importance of these modifications combined under the term "epigenetics" is widely appreciated in the research field of human health and disease, ranging from cancer, to neurodegenerative diseases, up to mental disorders like schizophrenia (reviewed by Knight, 2012).

First evidence for a connection between transcriptional control and the Ccr4-NOT complex came from studies on the HIS3 promoter through which the NOT genes had been identified. The yeast HIS3 promoter comprises two TATA elements, TC and TR, both of which support basal transcription. TR comprises a typical TATAAA sequence, promotes transcription initiation from the +13 site, and is responsive to induced transcription for example by the transcriptional activators Gcn4 or Gal4. In contrast, TC does not comprise a conventional TATA sequence and promotes transcription initiation from the +1 site. Exploitation of the HIS3 promoter to distinguish basal from induced transcriptional processes is usually read out either by Northern blot analysis or by growth on medium supplemented with 3-amino-1,2,4-triazole (3-AT). In Northern blot analysis the two transcripts (+1 and +13) produced from both promoter elements can be distinguished and their amounts quantified. Assaying growth on medium supplemented with 3-AT requires increased levels of HIS3 expression to support growth in cells carrying the mutant transcription factor gcnc4-C163, whose transcription activation domain is only partially functional. The disruption of any of the NOT genes (NOT1-5) conferred these cells viable in the presence of 3-AT, and led to an increased HIS3 transcription with a preference for TC-supported transcription (Collart & Struhl, 1993, 1994; Oberholzer & Collart, 1998). Thus, these genes were characterized as global negative regulators of transcription and called "Negative on TATA" or NOT.

The regulation of transcriptional activation is orchestrated through the interactions of histone modifying complexes, transcriptional co-activator complexes, transcription factors, and RNA polymerases. Evidence exists for a role of the Ccr4-NOT complex at all steps of transcriptional regulation. The acetylation of histones is mediated by histone acetyltransferases (HATs) and counteracted by deacetylation through histone deacetylases (HDACs). Gcn5 constitutes the catalytic subunits of the HAT complexes ADA (transcriptional ADAptor), SAGA (Spt-Ada-Gcn5 Acetyltransferase), and SLIK (SAGA-like) (reviewed by Daniel & Grant, 2007) (Figure 4). ADA, SAGA, and SLIK constitute histone modifying and transcriptional co-activator complexes, involved in the positive and negative regulation of RNA polymerase II-dependent gene expression. The SLIK complex specifically func-
tions in the retrograde response pathway in yeast important for mitochondrial integrity (Pray-Grant et al., 2002). In addition to HAT activity, SAGA further comprises deubiquitination activity mediated by Ubp8 (Henry et al., 2003). SAGA subunits Spt3 and Spt20 were demonstrated to be required for the binding of the TATA-binding protein (TBP), Spt15 to promoters (Dudley, Rougeulle, & Winston, 1999). With regard to histone acetylation, a direct interaction between the N terminus of Not2 and the SAGA/ADA subunit Ada2 was demonstrated (Benson, Benson, Howley, & Struhl, 1998; Russell, Benson, & Denis, 2002). Furthermore, an immunofluorescence-based screen for mutants that affected cellular levels of histone acetylation yielded NOT4, NOT5, and CCR4, as mutants that led to significantly reduced levels of global histone acetylation (Peng, Togawa, Zhang, & Kurdistani, 2008).

Figure 4: Schematic representation of the Gcn5-containing SAGA and ADA complexes including described protein-protein interactions with Not2, Not4, and Not5 (indicated by left right arrows). Shared subunits between SAGA and ADA complexes are marked by a bold edge.

The strong evidence with respect to the NOT module of the Ccr4-NOT complex was further supported by a study conducted with the not1-2 allele, which produces a C-terminally truncated Not1 protein, lacking the interaction site for Not2, Not5, and Not4. This mutant displayed increased binding of TFIID and SAGA to promoters of upregulated genes (e.g. HSP12) (James, Landrieux, & Collart, 2007). And finally, a connection between the Ccr4-NOT complex and the co-translational SAGA complex assembly was established (Kassem, Villanyi, & Collart, 2017). Here, it was shown that not5Δ cells displayed compromised integrity of SAGA and reduced stability of ADA, despite equally expressed levels of the SAGA/ADA subunits. RNA immunoprecipitation (RIP) experiments performed on polysome fractions, revealed that Not1-bound mRNAs were highly enriched in ADA2 mRNA, and this association was reduced in not5Δ cells. Some enrichment was also detected for the GCN5 transcript in Not1-bound fractions. In contrast, RIP experiments revealed that Ada2 and Not4, were both present on the SPT20 transcript. Thus, the authors of this work concluded that the Ccr4-NOT complex affected SAGA integrity by binding SAGA transcripts and enabling efficient co-translational complex assembly.
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The recruitment of the SAGA complex to promoters was demonstrated to be dependent on the binding of transcriptional activators, for example Gcn4 (Qiu et al., 2005) or Gal4 (Larschan & Winston, 2001), and interaction with methylated lysine 4 on histone 3 (H3\(^{K4}\)) (Pray-Grant, Daniel, Schieltz, Yates, & Grant, 2005) (Figure 5). H3\(^{K4}\) mono-, di- and trimethylation are specifically carried out by the Set1-containing COMPASS complex (complex of proteins associated with Set1) (Boa, Coert, & Patterton, 2003), and counteracted by the demethylase Jhd2 (Ingvarsdottir et al., 2007). The recruitment of COMPASS to promoters and subsequent H3\(^{K4}\) methylation was shown to require PAF complex recruitment and Rad6-Bre1-dependent monoubiquitination of lysine 123 (H2B\(^{K123}\)) on histone H2B (Kim & Roeder, 2009; Pray-Grant et al., 2005; Wood, Schneider, Dover, Johnston, & Shilatifard, 2003, 2005). Both events were further demonstrated to be dependent on the cyclin-dependent kinase Bur1 and its cyclin Bur2 (Wood et al., 2005). First evidence about a connection between H3\(^{K4}\) methylation and the Ccr4-NOT complex was provided through the finding of reduced global trimethylation on H3\(^{K4}\) detected in various deletion strains concerning members of the Ccr4-NOT complex, namely Not1 (not1-1 and not1-2), Not2 (not2\(\Delta\)), Not4 (not4\(\Delta\)), Not5 (not5\(\Delta\)), and Caf1 (caf1\(\Delta\)) (Laribee et al., 2007; Mulder, Brenkman, Inagaki, van den Broek, & Timmers, 2007). Furthermore, synthetic lethality was observed for a deletion of BUR2 in combination with not2\(\Delta\), not4\(\Delta\), or ccr4\(\Delta\) (Mulder, Brenkman, et al., 2007). However, there was disagreement whether deletions of Ccr4-NOT complex subunits had an effect on the monoubiquitination of H2B. While one study found that in not4\(\Delta\) cells, both H2B monoubiquitination and PAF complex recruitment were reduced (Mulder, Brenkman, et al., 2007), another study was unable to observe any effect on H2B monoubiquitination in not4\(\Delta\) cells (Laribee et al., 2007). A direct link between the Ccr4-NOT complex and H3\(^{K4}\) trimethylation was revealed by the finding that the demethylase Jhd2 is an E3 ligase substrate of Not4 (Mersman, Du, Fingerman, South, & Briggs, 2009).

All these insights can be summarized as follows (Figure 5). Bur1/2-dependent recruitment of the PAF complex and activation of Rad6-Bre1 leads to the monoubiquitination of H2B on K123 (Kim &
This is then followed by COMPASS recruitment and di- and trimethylation of H3K4, immediately and constantly counteracted by the demethylase Jhd2 (Boa et al., 2003; Ingvarsdottr et al., 2007; Wood et al., 2005). However, in spatial proximity of Not4, Jhd2 activity is restricted through Not4-mediated polyubiquitination of Jhd2 and subsequent degradation in a proteasome-dependent manner (Mersman et al., 2009). Strikingly, in addition to the finding of reduced global H3K4 trimethylation, not4∆ cells additionally displayed a reduced recruitment of the proteasome to genes, demonstrated by the example of the PMA1 gene (Laribee et al., 2007). Not4-dependent facilitating of stable H3K4 trimethylation allows for the recruitment of SAGA, and SAGA-dependent acetylation of lysine 14 on histone H3 (H3K14ac) in turn negatively regulates Jhd2 demethylase activity (Maltby et al., 2012), thus further promoting H3K4 trimethylation. As mentioned before, the SAGA complex also harbors DUB activity and is responsible for the deubiquitination of H2BK123 (Henry et al., 2003). Thus, SAGA recruitment allows for the full realization of H3K4 trimethylation, which was mostly detectable in the 5' end of the coding region of a gene, and generally spoken is associated with transcriptional activation (C. L. Liu et al., 2005).

The role of the Ccr4-NOT complex regarding transcriptional activation had further been expanded from a direct and indirect requirement for proper histone modification to the regulation of transcriptional co-activator complexes, like the Mediator, TFIID, and PAF complex. The Mediator complex interacts with the C terminal domain (CTD) of the largest subunit of RNA polymerase II, and was structurally divided into four submodules, called head, middle, tail, and kinase module. First evidence of a direct interaction between members of the Ccr4-NOT complex and the Mediator came from yeast two-hybrid and co-immunoprecipitation experiments, which revealed direct interactions between three out of four members of the kinase module (Med13/Ssn2/Srb9, Ssn3/Srb10/Cdk8, Ssn8/Srb11/CycC) and Not1 or Not2 (H.-Y. Liu et al., 2001). More direct evidence came from a study on Ssn8 destruction in response to oxidative stress (Cooper et al., 2012). Ssn8 was shown to relocalize from the nucleus to the cytoplasm in a Not4-dependent manner upon oxidative stress, followed by Not4-dependent polyubiquitination of cytosolic Ssn8 and proteasome-mediated degradation (Cooper et al., 2012). Interestingly, the interaction between Not4 and Ssn8 was not restricted to a specific stress condition, but also detectable under normal, vegetative growth. However, noticeable Ssn8 destruction had so far been described during meiosis and heat shock, in addition to oxidative stress (Cooper, Mallory, Smith, & Strich, 1997; Cooper et al., 2012). The kinase module of the Mediator complex plays an important role in the regulation of RNA polymerase II-dependent transcription, and was shown to display both, activating and repressing activity in a context-dependent manner. A detailed electron microscopy-based (EM) study on the conserved kinase module in conjunction with Mediator-RNA polymerase II interaction revealed how the CTD of RNA polymerase II contacts the Mediator complex on two distinct sites, one located on the middle module, the other on the head module (Tsai et al., 2013). The former was occupied by the kinase module in Mediator preparations without RNA polymerase II, while CTD-interaction with the second binding site represents the structure of the actual holoenzyme. This study conducted on Mediator and RNA polymerase II preparations from Saccharomyces cerevisiae was in accordance with a previous EM-based study on the complexes in Schizosaccharomyces pombe, which also came to the conclusion that the kinase module prevents an interaction between the Mediator and the RNA polymerase II, and that this was independent of kinase activity of Ssn3 (Elmlund et al., 2006). In contrast to the repressive function in hampering RNA polymerase II holoenzyme formation, the kinase module was further reported to act on transcriptional activation. Phosphorylation of the CTD on serine 5 and 2 (Ser5, Ser2), hallmarks of transcriptional initiation and elongation, respectively,
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was dependent on the presence of the kinase module in *Saccharomyces cerevisiae*, as well as in human cell culture experiments (Borggrefe, Davis, Erdjument-Bromage, Tempst, & Kornberg, 2002; Furumoto et al., 2007; Liao et al., 1995).

![Diagram of the Mediator complex](image)

**Figure 6**: Schematic representation of the Mediator complex. The complex can be divided into four major submodules: head, middle, tail, and kinase module. The respective protein partners are displayed accordingly. The interaction between Mediator and RNA polymerase II is displayed on the right, including the intermediate interaction which excludes a simultaneous interaction of the kinase module with the Mediator complex.

In addition, the kinase module was shown to regulate diverse transcription factors. Gcn4, the transcriptional activator regulating genes required for amino acid biosynthesis; Msn2, a general stress-responsive transcription factor; and Ste12, the main effector carrying out the transcriptional activation of the mating response, were all shown to be regulated by the cyclin-dependent kinase Ssn3 of the Mediator kinase module (Chi et al., 2001; Lallet, Garreau, Poisier, Boy-Marcotte, & Jacquet, 2004; Nelson, Goto, Lund, Hung, & Sadowski, 2003). The Ssn3-dependent phosphorylation of these transcription factors was demonstrated to constitute a mark for proteasome-mediated degradation, and thus serves to destabilize these proteins. In case of Gcn4, Ssn3-dependent phosphorylation regulated the basal levels independent on its activating stimulus, amino acid starvation (Chi et al., 2001). Whereas the requirement for Ssn3 was especially apparent upon pheromone-stimulation or heat shock for Ste12 and Msn2, respectively (Lallet et al., 2004; Nelson et al., 2003). A microarray study conducted on cells lacking Ssn3 kinase activity revealed the derepression of genes associated with diauxic shift or diverse stress responses under conditions...
which should promote growth and actually counteract transcriptional stress responses (Holstege et al., 1998). The requirement for Ssn3 activity was further expanded with regard to transcriptional repression of filamentous, invasive growth and the expression of the spore-specific water channel AQY1 under glucose-rich conditions (Law & Ciccaglione, 2015; Law & Finger, 2017; Nelson et al., 2003). At this point, the circle between histone modifications and destabilization of transcription factors closes. First, the inhibition of filamentous growth under nutrient-rich conditions was demonstrated to require both, Jhd2 and Ssn8 (Law & Ciccaglione, 2015). Second, H3K4 trimethylation was inhibited independently by Jhd2 and Ssn8, the latter through inhibition of Set1 promoter binding (Law & Finger, 2017). Third, Ssn3-Ssn8-dependent restriction of Ste12 activity was demonstrated to inhibit filamentous growth under nutrient-rich conditions (Nelson et al., 2003). In this context, it is again noteworthy to mention that both, Ssn8 and Jhd2 are Not4 substrates (Cooper et al., 2012; Mersman et al., 2009).

Extensive physical and functional interactions were demonstrated between the Ccr4-NOT complex, TBP and the general transcription factor TFIID (Figure 7). Physical interactions were mainly investigated through yeast two-hybrid (Y2H) analyses, co-immunoprecipitations or pull-down assays. Y2H interactions were demonstrated for Taf13 and the Ccr4-NOT complex through the subunits Not2, Not3, Not5, and Ccr4 (Lemaire & Collart, 2000), as well as between Not1 and Taf1 (Deluen et al., 2002). However, Y2H assays concerning yeast proteins have to be treated with caution, as interactions can be achieved directly or indirectly through additional interaction partners. In addition to the Y2H analyses, Not1, Not2, and Not5 were specifically co-immunoprecipitated by TBP (Badarinarayana, Chiang, & Denis, 2000; Lee et al., 1998), and Taf1 was co-immunoprecipitated by Not1 (Deluen et al., 2002). Several studies shed light on the functional importance of the Ccr4-NOT complex for the proper distribution of TFIID over promoters. First, cells deleted of NOT4 or NOT5 displayed stabilized TBP-TATA box interactions (Badarinarayana et al., 2000). Conversely, this could mean that Not4 and Not5 are required to restrict this interaction and thereby influence transcriptional activity. Second, chromatin immunoprecipitation (ChIP) experiments demonstrated that not4Δ cells, treated with hydroxyurea (HU) to induce replication stress, displayed defective recruitment of TBP, RNA polymerase II, and Set1 to the RNR3 gene, which is usually induced upon this condition (Mulder, Winkler, & Timmers, 2005). And finally, altered TBP, TFIID, and SAGA distribution over inducible, especially stress-regulated genes under non-stress conditions were ob-

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**Figure 7**: Schematic representation of the TFIID complex. The TFIID complex comprises TAF1-13 (TATA binding protein-associated factor 1-13) and the TATA-binding protein (TBP). Interactions between subunits of the Ccr4-NOT complex and TFIID subunits are highlighted in red.
served for deletions of almost all subunits of the Ccr4-NOT complex, and correlated well with up-regulated transcripts detected in the respective mutants (James et al., 2007; Lenssen et al., 2005). Importantly, transcript levels of TBP, or other general transcription factors including TFIIA or TFIIB, remained unaffected in cells lacking functional Not1, Not2, Not3, Not4, or Not5 (Biswas, 2005; Collart & Struhl, 1994). In other words, the distribution of TFIID over promoters of inducible genes under non-inducing conditions is strongly restricted by the presence of the Ccr4-NOT complex, but the complex is also required for TFIID recruitment to promoters under inducing conditions.

Figure 8: Strongly simplified model of Yap1 activity in the absence and presence of oxidative stress. In the absence of oxidative stress, Yap1 is constantly exported from the nucleus by Crm1, and thus kept physically away from promoters of oxidative stress-responsive genes (OXR). Upon oxidative stress, Yap1 is oxidized and remains nuclear. It interacts with the transcription factor Skn7, and activates OXR genes. A maximal oxidative stress response is enabled by the combined activity of Yap1 and Skn7. Transcription factor activity is restricted by Not4/Ccr4-NOT and was demonstrated to be crucial for inactivation of the oxidative stress response.

In addition to the physical and functional interactions between the Ccr4-NOT complex and histone modifying proteins/complexes, transcriptional co-activators, general transcription factors, and the holoenzyme, a close relationship was further documented with regard to the transcription activators Yap1 and Skn7 (Gulshan, Thommandru, & Moye-Rowley, 2012; Lenssen, Azzouz, Michel, Landrieux, & Collart, 2007) (Figure 7). Yap1 and Skn7 both respond to oxidative stress and activate the transcription of genes to enable cells to cope specifically with this insult (reviewed by Ikner & Shiozaki, 2005). While Skn7 is constitutively localized within the nucleus and activated through phosphorylation in an oxidative stress-dependent manner (He, Mulford, & Fassler, 2009), Yap1 shuttles between nucleus and cytoplasm in a Crm1-dependent manner (Kuge, Toda, Iizuka, & Nomoto, 1998). In fact, condition-dependent subcellular localization of Yap1 is part of its regulation.
While it is constantly exported from the nucleus in the absence of oxidative stress, intramolecular disulfide bond formation under oxidative conditions prevents interaction with Crm1 and thereby leads to a nuclear localization of Yap1 (Gulshan, Rovinsky, Coleman, & Moye-Rowley, 2005; Okazaki, Tachibana, Naganuma, Mano, & Kuge, 2007). Members of the Ccr4-NOT complex were found to physically and functionally interact with both transcription factors, and mainly act in the restriction of transcriptional activation upon stress (Gulshan et al., 2012; Lenssen et al., 2007). The interaction between Not1 and Skn7 was demonstrated by Y2H and immunoprecipitation experiments. Not5 also co-immunoprecipitated with Skn7 and the interaction between Skn7 and Not5 was further shown to be dependent on Not4 (Lenssen et al., 2007).

A functional relationship between Not4, Not5 and Skn7 was further detected with regard to target gene expression (OCH1), which exhibited increased Skn7 binding accompanied by increased transcript levels in not4Δ or not5Δ cells. In addition, increased OCH1 transcript levels observed in not4Δ cells were highly dependent on the presence of Ssn3. Additionally, TAP-mediated pull-down experiments revealed a Not4-dependent interaction between Skn7 and Ssn3 (Lenssen et al., 2007). Not4 was demonstrated to be required for the proteasome-dependent degradation of Yap1 following its disulfide bond formation, nuclear localization, and target gene activation (Gulshan et al., 2012). In sum, a strong connection between the Ccr4-NOT complex (especially Not4 and Not5) and oxidative stress-inducible transcriptional control through Skn7 and Yap1 was demonstrated. Hence, the role of the Ccr4-NOT complex during the transcriptional activation of oxidative stress-responsive genes through Yap1 and Skn7 mainly unfolded in the restriction of the interaction between transcription factors and promoters, and the restriction of the availability of activated transcription factors. Mind: Not4-dependent ubiquitination and subsequent destruction was mentioned earlier for Ssn8 under the exact same stress condition (Cooper et al., 2012).

In addition to the regulatory role during transcriptional initiation, it was further revealed that the Ccr4-NOT complex acts directly on RNA polymerase II-dependent elongation, and promotes the rescue of arrested RNA polymerase II during transcriptional elongation. First evidence placing the Ccr4-NOT complex at transcriptional elongation was provided by the finding that it is targeted to the coding regions of genes similarly to RNA polymerase II in vivo (Kruk, Dutta, Fu, Gilmour, & Reese, 2011). Co-immunoprecipitation assays confirmed a physical interaction between several members of the Ccr4-NOT complex and the largest of the RNA polymerase II subunits Rpo21 (also called Rpb1). Furthermore, the interaction was dependent on the presence of Ccr4, Not4, and in part Not2 (Kruk et al., 2011). The Ccr4-NOT-dependent rescue of backtracked, arrested elongation complexes was demonstrated to be mediated through enhanced recruitment of TFIIIS by Ccr4-NOT to RNA polymerase II complexes (Dutta et al., 2015). TFIIIS is a general transcription elongation factor, encoded by DST1 in yeast, and facilitates the maintenance of transcriptional elongation activity of RNA polymerase II, as well as the rescue of stalled elongation complexes through stimulation of the intrinsic cleavage activity of RNA polymerase II toward stalled, nascent transcripts (Gómez-Herreros et al., 2012; Weilbaecher, Awrey, Edwards, & Kane, 2003).

A close connection between the Ccr4-NOT complex and the dissociable RNA polymerase II sub-module Rpb4/Rpb7 was established (Babbarwal, Fu, & Reese, 2014; Dutta et al., 2015; Villanyi et al., 2014). The Rpb4/Rpb7 heterodimer was identified as a module that directly links transcription and translation, in contrast to the previous assumption regarding the uncoupled nature of both processes (Goler-Baron et al., 2008; Harel-Sharvit et al., 2010). In fact, the nuclear association of the Rpb4/Rpb7 module with RNA polymerase II was demonstrated to be required for efficient translation in the cytoplasm. The Rpb4/Rpb7 module was shown to accompany mRNAs throughout their
lifetime by binding of the 3’ end of newly transcribed mRNAs, and to be functionally involved in the stimulation of both, translation and mRNA decay in the cytoplasm (Goler-Baron et al., 2008; Lotan, Goler-Baron, Duek, Haimovich, & Choder, 2007). The Rpb4/Rpb7 module was required for the recruitment of the Ccr4-NOT complex to RNA polymerase II elongation complexes (Babbarwal et al., 2014). Similar to the mRNA-associated shuttling behavior of Rpb4/Rpb7, it was demonstrated that newly synthesized transcripts experienced Not1-imprinting which served as a determinant for translation efficiency (Gupta et al., 2016). Taken together, the Ccr4-NOT complex was demonstrated to accompany RNA polymerase II during elongation, for which the dissociable Rpb4/Rpb7 heterodimer was required. Strikingly, the Rpb4/Rpb7 heterodimer and the Ccr4-NOT complex have many similarities. Both shuttle between the nucleus and cytoplasm. Within the latter, both entities associate with the translation machinery and can be found in processing bodies (P-bodies), where they impinge on translational efficiency and mRNA decay rates.

The detailed investigation of physical and functional interactions between the Ccr4-NOT complex and the network of factors and complexes orchestrating transcription usually focused on the effects on single or a manageable number of genes and raised the question of how the Ccr4-NOT complex affects transcription and the transcriptome globally. This was examined through a microarray-based transcriptome-wide analysis on the total content of mRNAs (Cui et al., 2008). The functional subdivision within the Ccr4-NOT complex into the deadenylase module (comprising Caf1 and Ccr4) and the Not module (comprising Not2-Not5) was also apparent by the way single deletion mutants affected the transcriptome (Cui et al., 2008). A great correlation was observed regarding differentially expressed genes in caf1∆ and ccr4Δ, as well as in not4∆ and not5∆ but not between both functionally distinct units (Cui et al., 2008).

A functional analysis on differentially expressed genes revealed that all kinds of cellular components (e.g. mitochondria, cytoplasm) and biological processes (e.g. mRNA synthesis, ribosome biogenesis, glycolysis/ gluconeogenesis) were affected in mutants of the Ccr4-NOT complex. However here, the functional separation into deadenylase and Not module was not visible (Cui et al., 2008). Even though the NOT genes were functionally described as transcriptional repressors, not4∆ and not5∆ both displayed a similar number of up- and down-regulated genes (Cui et al., 2008). In the case of not4∆, this distribution was confirmed by a second microarray-based study, but not for not5∆, which displayed almost three times more upregulated over down-regulated genes (Azzouz et al., 2009). In general, both studies yielded comparable results on one hand, but also some inconsistencies on the other. The total number of genes which were considered as differentially expressed was much higher for all Ccr4-NOT mutants in the study from Cui et al., compared to the study of Azzouz et al. Another difference emerged with regard to the distribution between the number of genes which were upregulated or down-regulated. This was especially apparent for the deletion of CAF1 or NOT5 (Figure 9).

Whether this was simply due to differences in handling, experimental procedures, and data evaluation or actually reflected naturally occurring phenotypic variability of these mutants remains unaddressed so far. Nevertheless, it was proven that the Ccr4-NOT complex can make a wide range of functional contacts with the transcription machinery, and the consequences were measurable on a global scale. Given that the Ccr4-NOT complex is involved in the regulation of general, as well as specific transcription factors and complexes, it would not be surprising if the consequences vary between experiments. Especially since it appears to mainly impinge on the context-specific efficiency of transcriptional regulation.
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Figure 9: Bar chart comparison between microarray data described in the two publications indicated. The chart shows the total number of genes which displayed at least two fold differential expression in the different null mutants of the Ccr4-NOT complex.

3.4 The Ccr4-NOT complex in translation and mRNA decay

The regulation of gene expression is not restricted to transcriptional control. Instead, it is regulated at all possible levels, including transcript processing, translation and decay. The interdependency of transcription and translation was already discussed in the previous section. The control of translational activity and mRNA degradation will be elaborated here with regard to the Ccr4-NOT complex, and especially Not4 which is the main focus of the work presented here.

Gene expression can be simplified in the following model (Hargrove & Schmidt, 1989):

\[
\begin{align*}
\text{Gene} & \xrightarrow{k_{S1}} \text{mRNA} \xrightarrow{k_{D1} R_t} \text{Protein} \xrightarrow{k_{D2} P_t} \text{Nucleotides} \xrightarrow{k_{S2} R_t} \text{Amino acids} \\
\end{align*}
\]

- \( k_{S1} \): rate constant for transcriptional synthesis
- \( R_t \): steady-state level of mRNA at time point t
- \( k_{D1} \): rate constant for transcript degradation
- \( k_{S2} \): rate constant for translational synthesis
- \( P_t \): steady-state level of protein at time point t
- \( k_{D2} \): rate constant for protein degradation

This simplified model demonstrates the interdependency of synthesis and degradation rates, steady-state pools, and the dependency of protein synthesis on mRNA levels (Hargrove & Schmidt, 1989). The simplicity presented here can never meet the complexity of a network that further comprises regulatory feedback mechanisms and the entirety of context-specific interdependencies between the transcriptome and proteome. However, it is still useful for the rough visualization of gene expression.

The Ccr4-NOT complex was not only found to affect transcription in a quantitative and qualitative manner (represented as \( k_{S1} \) in the model), but to be likewise involved in the regulation of mRNA degradation rates (represented as \( k_{D1} \) in the model). Consequently, this complex directly and indirectly affects both, the transcriptome and proteome of a cell. While the NOT module (Not2-5) was
identified as the major contributor in the Ccr4-NOT complex-dependent regulation of transcription, the deadenylase module, comprising Caf1 and Ccr4, was demonstrated to present the major cellular deadenylase activity in the cell and thereby affecting mRNA degradation directly (Tucker, Staples, Valencia-Sanchez, Muhlrad, & Parker, 2002; Tucker et al., 2001). A second complex displaying deadenylation activity is the PAN complex (poly(A) nuclease complex), and was demonstrated to be involved in the initial trimming of newly polyadenylated mRNAs from approximately 200 nt to 60-80 nt (Brown & Sachs, 1998). The PAN complex was further shown to deadenylate mRNAs to a remaining length between 20-25 nt and most likely hands over the transcript to the Ccr4-NOT complex for complete deadenylation (Tucker et al., 2001). The poly(A)-binding protein Pab1 was demonstrated to play a major role in the regulation of deadenylation and affects both, the PAN complex and Ccr4-NOT complex. While deadenylation by PAN was shown to be stimulated in the presence of Pab1, deadenylation by Ccr4-NOT was inhibited (Boeck et al., 1996; Tucker et al., 2002).

Two pathways control the general degradation of mRNAs, and both decay pathways are initiated by deadenylation (Figure 10). Besides the two general pathways, specific decay pathways have evolved as part of the cellular quality control network to deal with non-functional transcripts that escape the general degradation pathways or as part of a specific regulatory pathway, for instance during stress (Parker, 2012).

The decapping-dependent 5'->3' end decay pathway was demonstrated to take place in defined cytoplasmic foci referred to as processing bodies (P-bodies) (Sheth & Parker, 2003). The formation of cytoplasmic P-bodies was further demonstrated to depend on deadenylation, while the dissolution was dependent on decapping activity. Microscopy-based analyses showed that P-bodies comprise decapping enzymes, Dcp1 and Dcp2, decapping activators like Pat1, Lsm1, or Dhh1, as well as the 5'->3' exonuclease Xrn1. Ccr4 - although mostly distributed throughout the cytoplasm - was also detectable within distinct cytoplasmic foci (Sheth & Parker, 2003). The Not proteins, Not1-
Not4, were found to localize into P-bodies in strains lacking decapping activity (Muhlrad & Parker, 2005). P-bodies comprise non-translating mRNAs, RNA-binding proteins, and other proteins which are localized to these distinct structures through protein-protein interactions. They belong to the superior class of messenger ribonucleoprotein granules (mRNP granules) which are defined by their composition of proteins and mRNAs, and the formation of distinct, but dynamic cytoplasmic foci (Buchan, 2014; Teixeira, Sheth, Valencia-Sanchez, Brengues, & Parker, 2005). They function as sites of mRNA storage (e.g. stress granules, P-granules), mRNA degradation (e.g. P-bodies), or mRNA transport (e.g. neuronal transport granules). P-bodies play a role during stress responses in yeast, embryonic development and neuronal function in higher eukaryotes (Buchan, 2014; Buchan, Muhlrad, & Parker, 2008; Strome & Wood, 1983). While the deadenylase activity was shown to be a prerequisite for P-body formation - demonstrated through decreased P-body occurrence in a ccr4Δ mutant - the Not proteins were found to promote mRNA decapping, and thus are involved in P-body dissolution (Alhusaini & Coller, 2016; Muhlrad & Parker, 2005). P-body formation was demonstrated to increase in number and size in response to various stresses (Teixeira et al., 2005), a condition upon which translation experiences repression. Moreover, the decapping activators Dhh1 and Pat1 were demonstrated to be required for translational repression and P-body formation upon stress induced by nutrient withdrawal (glucose or amino acids) (Coller & Parker, 2005).

The DEAD-box helicase Dhh1 and the mRNA-decapping activator Pat1 were shown to associate with the core Ccr4-NOT complex (Alhusaini & Coller, 2016; Hata et al., 1998; Maillet & Collart, 2002). Dhh1 was demonstrated to physically interact with Caf1 and the N terminus of Not1 and severe synthetic growth defects were observed for several Ccr4-NOT mutants in combination with dhh1Δ (Maillet & Collart, 2002). In more detail, synthetic lethality was observed for dhh1Δcaf1Δ, dhh1Δnot2Δ, and dhh1Δnot4Δ cells, and synthetic sick phenotypes for dhh1Δnot3Δ and dhh1Δnot5Δ. The importance of the NOT module for dhh1Δ cells was further demonstrated through the combination with the mutant not1-2 allele, which is a C-terminally truncated variant lacking the interaction with the NOT module (Maillet & Collart, 2002).

In contrast to the association of Dhh1 with the N terminus of Not1 and the deadenylase module, Pat1 was demonstrated to interact with Not3 and Not5 of the NOT module, which is localized to the C terminus of Not1. Furthermore, the NOT module was demonstrated to function in the promotion of decapping (Alhusaini & Coller, 2016; Muhlrad & Parker, 2005). The interaction between Dhh1, Pat1 and the Ccr4-NOT complex occurs on mRNAs, which are subject to translation by ribosomes. Like Dhh1 and Pat1 (Drummond et al., 2011; Huch, Gommlich, Muppavarapu, Beckham, & Nissan, 2016), components of the Ccr4-NOT complex specifically interact with ribosomes during translation, as shown for Not1, Not4, Not5, Caf1, and Ccr4 (Halter, Collart, & Panasenko, 2014; Preissler et al., 2015). Accordingly, the Ccr4-NOT complex was not only found to impinge on mRNA turnover merely through deadenylation, but furthermore through promoting subsequent decapping via interaction with decapping activating proteins. In addition, Not4 was required for global translational repression upon glucose or amino acid withdrawal similar to Dhh1 (Preissler et al., 2015).
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Figure 11: Schematic representation of the interactions between the mutually exclusive processes of translational initiation (bottom) and translational repression (top). mRNA can either interact with the translational initiation complex or the translational repression network because both target the m\(^7\)Gppp-cap at the 5' end or the poly(A) tail on the 3' end. Black arrows indicate protein-RNA interaction, red and green arrows represent inhibitory or activating interactions, respectively.

In analogy to the functional distinction between eu- and heterochromatin, polysomes and P-bodies can be considered similarly functional units with regard to mRNA-associated activity, respectively. Whether a transcript is being translated or not, strongly depends on the opposing effects between the translation initiation machinery and the deadenylation-decapping machinery, which either promote initiation or repression of translation, and constitute mutually exclusive processes (Figure 11).

3.5 Structural and functional conservation of the Ccr4-NOT complex

The Ccr4-NOT complex is structurally and functionally highly conserved from yeast to humans, and subject to investigation within most model organisms or cellular systems. Although the underlying molecular mechanisms, as well as the complex composition, can turn out species-specific, the biological function is conserved.

The following section will give some insights into species-specific features of the Ccr4-NOT complex. For most of the Ccr4-NOT complex subunits identified in the budding yeast, S. cerevisiae, orthologs were found in other eukaryotes. These include the scaffolding protein Not1, the deadenylases Ccr4 and Caf1, the NOT proteins Not2, Not3/5, and Not4, the Ccr4-associated factor Caf40, and the DEAD-box helicase Dhh1. The structural and functional conservation of these subunits was demonstrated for example by complementation assays conducted in respective yeast mutants, which displayed rescue of growth phenotypes when complemented with human orthologs (Albert et al., 2002, 2000).
Table 1: Nomenclature und composition of some eukaryotic Ccr4-NOT complexes.

<table>
<thead>
<tr>
<th></th>
<th>S. cerevisiae</th>
<th>S. pombe</th>
<th>C. elegans</th>
<th>H. sapiens</th>
</tr>
</thead>
<tbody>
<tr>
<td>Not1</td>
<td>CDC39/NOT1</td>
<td>NOT1</td>
<td>let-711/ntl-1/spn-3</td>
<td>CNOT1</td>
</tr>
<tr>
<td>Not2</td>
<td>CDC36/NOT2</td>
<td>NOT2</td>
<td>ntl-2</td>
<td>CNOT2</td>
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<tr>
<td>Not3</td>
<td>NOT3</td>
<td>NOT3</td>
<td>ntl-3</td>
<td>CNOT3S</td>
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<td>Not5</td>
<td>NOT5</td>
<td></td>
<td></td>
<td>CNOT3L</td>
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<tr>
<td>Caf1</td>
<td>POP2/CAF1</td>
<td>CAF1</td>
<td></td>
<td>CNOT7</td>
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<tr>
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<td>CCR4</td>
<td>CCR4</td>
<td>ccf-1</td>
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<td>RCD1</td>
<td>ntl-9</td>
<td>CNOT6</td>
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<tr>
<td>Not4</td>
<td>SIG1/MOT2/NOT4</td>
<td>MOT2</td>
<td>ntl-4</td>
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<td>MMI1</td>
<td>ntl-11</td>
<td>CNOT7</td>
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<td>STE13</td>
<td>cgh-1</td>
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Differences exist with regard to Not3/Not5, the association of Not4 with the complex, and additional complex subunits. The N termini of the NOT proteins Not3 and Not5 were found to share 44% sequence identity, and moreover it was demonstrated that the overexpression of NOT3 could suppress mutations in NOT5, thus Not3 and Not5 appeared interchangeable (Oberholzer & Collart, 1998). In other eukaryotes, these proteins are represented by a single homolog. Across species, the N terminus of Not4, including the RING domain and RNA-recognition motif, is highly conserved, in contrast to the respective C termini, which display differences with regard to length and sequence composition. This was also reflected by the finding that the human Not4 ortholog, CNOT4, was not stably associated with the Ccr4-NOT complex in affinity purified complex preparations. Although it showed a positive interaction with CNOT1 in a Y2H assay (Lau et al., 2009). Likewise, Ccr4-NOT complex preparations from Drosophila melanogaster lacked a stable association of Not4, even though a weak interaction with Not1 was detected (Temme et al., 2010). In contrast, the Not4 ortholog in a close relative, the fission yeast Schizosaccharomyces pombe, displayed stable association of Mot2 with the complex (Stowell et al., 2016).

The Ccr4-associated factor Caf130 constitutes a specific subunit in budding yeast, and has no ortholog in other eukaryotes. Corresponding orthologs were identified in C. elegans and H. sapiens for the RNA-binding protein Mmi1, which was present in Ccr4-NOT complex preparations from S. pombe (Stowell et al., 2016). In S. pombe, Mmi1 has a role in the exosome-dependent destruction of meiosis-specific transcripts during vegetative growth, which was further demonstrated to be promoted by Mot2 (Simonetti, Candelli, Leon, Libri, & Rougemaille, 2017). Pho92 constitutes the Mmi1-homolog in S. cerevisiae and was recently identified to physically interact with Caf1, but was not considered as a Ccr4-NOT component so far. Disruption of either PHO92 or CAF1 resulted in an increased half-life for the PHO4 mRNA, encoding an important transcription factor of the phosphate signal transduction pathway. This emphasized the functional connection between Pho92 and the Ccr4-NOT complex in S. cerevisiae as well (Kang et al., 2014). So far, no orthologs could be identified for CNOT10 in fungi or in the nematode C. elegans. Its association with the Ccr4-NOT complex was demonstrated to depend on CNOT11, which contacts the N terminus of CNOT1 (F. Mauxion, Prève, & Séraphin, 2013). And finally, Ccr4-NOT complex preparations from human cell lines revealed that at least four distinct complex variants can be isolated. They differed in the composition of the deadenylase module (Lau et al., 2009). Contrary to yeast, human Ccr4 and Caf1 function was confirmed to be provided each by two orthologs, which were demonstrated to occur in...
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all possible combinations. Another major difference between budding yeast and Ccr4-NOT complexes from higher eukaryotes was the finding that in yeast, Caf1 was dispensable for in vitro deadenylation reactions, and Ccr4 constituted the catalytic subunit of the deadenylase module (Tucker et al., 2002). In contrast, both Caf1 and Ccr4 were demonstrated to be required for the catalysis of deadenylation in D. melanogaster or human cell lines (Fabienne Mauxion, Faux, & Séraphin, 2008; Temme et al., 2010).

Similar to what was observed in S. cerevisiae, the Ccr4-NOT complex was found to be functionally involved in the regulation of proliferation and differentiation in higher eukaryotes. As an example, the role of the Ccr4-NOT complex in the regulation of the JAK/STAT signaling pathway was demonstrated in D. melanogaster and human cell lines (Chapat et al., 2013; Grönholm et al., 2012). The JAK/STAT pathway constitutes an essential signaling network important for innate immunity, response to stress, cellular growth and differentiation control. The misregulation of JAK/STAT signaling is linked to numerous diseases, including cancer and autoimmune diseases (reviewed by Bousoik & Montazeri Aliabadi, 2018). In D. melanogaster, Not4 was identified as a positive regulator of the JAK/STAT pathway by means of the JAK/STAT-responsive transcription factor Stat92E. Not4 was co-immunoprecipitated by Stat92E, and flies overexpressing Not4 displayed significantly increased transcript levels of Stat92E-regulated genes (Grönholm et al., 2012). A role for the JAK/STAT-dependent transcription factor STAT1 was further demonstrated for the deadenylase subunit CNOT7 (Caf1) in human cell lines. First, knockdown of CNOT7 resulted in increased occupancy of STAT1 at target gene promoters and their expression. Second, CNOT7 was demonstrated to physically interact with STAT1 in the cytoplasm and to control the trafficking of the transcription factor under unstimulated conditions. Third, CNOT7 was shown to accelerate the degradation of some of the STAT1-induced transcripts (Chapat et al., 2013). And as a final example, CNOT4 was demonstrated to be involved in the RBM15-dependent cell-fate determination of megakaryocytes, from which thrombocytes mature (Zhang et al., 2015). Failure to establish homeostasis between proliferation and differentiation is a common and crucial feature of leukemia, where mutations within a single pluripotent stem cell can turn out life-threatening. The RNA-binding protein RBM15 was demonstrated to be restricted in its activity by PRMT1-mediated methylation and subsequent CNOT4-mediated ubiquitination, which eventually led to proteasome-dependent degradation of RBM15 (Zhang et al., 2015).

The structural and functional conservation of the Ccr4-NOT complex throughout eukaryotes and the involvement as a major conductor during gene expression in response to internal and external stimuli emphasize the significance of the complex, especially with regard to human health and disease. Furthermore, due to the high degree of conservation, studies on the Ccr4-NOT complex conducted in S. cerevisiae are useful with regard to the general role and functions of the complex because this organism lacks the extreme complexity of context-dependency found in higher eukaryotes.
4. Aims of this study

The work aimed at the characterization of NOT4 in the model organism Saccharomyces cerevisiae. NOT4 is not an essential gene for haploid yeast cells, its deletion yields a wide range of phenotypes including slow growth, transcriptional derepression, changes in morphology and inhibition of translational repression upon nutrient withdrawal. However, deletion of a gene which such far-reaching effects can be problematic, as the disruption of a single function can lead to a chain of events, which accumulate over time. This can include adaptations within the genome that suppress the original insult within the cellular homeostasis. Therefore, the described phenotypes of a NOT4 deletion were compared to an alternative approach of inducible and controlled Not4 protein depletion. The comparison of both approaches served the distinction of immediate, primary effects in contrast to long-term, accumulative effects, a loss of NOT4 function can bring forth.

Another aim of this work was to gain insights into the global effects a loss of NOT4 function had on a global scale. Transcriptome- and translatome-wide data in combination with Gene Ontology analyses yielded insights into biological processes, molecular functions, or cellular components affected by the disruption of NOT4 function.

The number of detected Not4 substrates is rather small considering the pleiotropic effects observed upon NOT4 deletion. The exploitation of new experimental approaches based on some Not4-specific properties therefore could reveal new substrate interactions. For this purpose, two methods were tested and refined in a Not4-specific manner to obtain optimal results.

Finally, little is known about the NOT4 ortholog of the nematode Caenorhabditis elegans ntl-4 and its associated functions within this multi-cellular organism. The specifics about the life of this model organism is a well-described developmental landscape and a post-mitotic adult stage which is ideally suited for aging-related investigations. Because NOT4 has a big impact on transcriptional and translational control, this model organism could prove very useful in the investigation of NOT4 functions with regard to developmental processes, as well as aging.
5. Results

5.1 Haploid not4Δ cells sporadically and spontaneously suppress not4Δ growth phenotypes

Although the deletion of NOT4 is tolerated by haploid yeast cells and produces viable progeny, it was repeatedly observed that colonies displayed a huge variation in their size when material from a single colony was transferred and spread onto a fresh agar plate. Similar observations were made for not4Δ cells grown in liquid medium. In most cases not4Δ cells behaved uniformly and displayed a more or less similar growth rate and colony size. Throughout this study it was ensured that only colonies which would meet the expectation in size were used in experiments. Nevertheless, colonies increased in their size or with increasing growth rates could be observed sporadically and spontaneously. These cultures were dismissed from experiments.

To further investigate this observation, not4Δ cells were freshly taken from a cryogenic stock and selected for colonies with the typical smaller size or atypical larger size compared to wild-type cells. The latter will be referred to as suppressors from here on. Growth was assayed for wild-type, not4Δ and not4Δ suppressor cells. Adjusted cell suspensions were diluted and transferred onto YPD, YPG, and YPD+clonNAT plates to elucidate whether the previously described impressions were reproducible.

The growth analysis confirmed the slow growth phenotype of typical not4Δ cells compared to wild-type cells at 30°C and 20°C (Cade & Errede, 1994; Collart & Struhl, 1994; Leberer et al., 1994) (Figure 12). Typical not4Δ cells displayed no growth at 37°C and on YPG plates (Cade & Errede, 1994; Collart & Struhl, 1994; Dimmer et al., 2002; Leberer et al., 1994). YPG plates contain glycerol as the sole carbon source and thus only support respiratory growth. Indeed, the analysis of the suspected not4Δ suppressor mutant showed improved growth at 30°C and 20°C. And strikingly, even minimal growth on YPG plates was observed for not4Δ* despite the fact that not4Δ was described as a respiration-deficient, petite mutant (Dimmer et al., 2002). On the other hand, not4Δ* was still unable to grow at 37°C. Growth on YPD+clonNAT served as a control for the respective genotype, because the NOT4 gene was replaced by a NatMX4 cassette in not4Δ cells, which confers resistance toward the antibiotic clonNAT. Accordingly, only not4Δ cells should be able to grow on YPD+clonNAT, and indeed that was the case.

This experiment was repeated three times. In each case, selected not4Δ* suppressors displayed enhanced growth on YPD and minimal growth on YPG. There seems to be a correlation between atypically enhanced growth rates and the ability to overcome the deficiency in respiration of these suppressor cells. Accordingly, only typical not4Δ cells were selected for reproduction to gain sufficient amounts of cell material. In the unusual and sporadic event that typical not4Δ cells would ex-
hibit suspicious growth behaviors, the cultures were dismissed and not used further into experiments.

5.2 Not4 function is dependent on the capacity to interact with E2s and the Ccr4-NOT complex

The examination of point-mutants or truncated variants of a protein can help to expand the knowledge about different functional parts or domains of a protein contributing differently to the protein-specific functions. In case of Not4, truncation variants, as well as an already published point mutation were investigated with respect to growth (Albert et al., 2002). These experiments were conducted in not4Δ cells transformed with plasmids carrying different variants of NOT4 under the control of the NOT4 promoter. Two Not4 characteristics were tested, the N-terminal RING domain, necessary for the interaction with the E2 enzymes Ubc4/5, and the C terminus, required for the interaction of Not4 with the Ccr4-NOT complex (Figure 13A).

![Figure 13: Different Not4 mutants and their growth behavior. (A) Schematic representation of the different Not4 mutants. All Not4 variants carry an HA-tag on their respective C terminus. The RING and RRM domain are marked, as well as the protein region essential for binding to the Ccr4-NOT complex. The position of the amino acid substitution on position Leu35 is marked in the respective Not4-L35A mutant. (B) Growth assay results for growth on YPD plates at the indicated temperatures and incubation periods. Empty vector (ev) controls carry the respective plasmid without any NOT4.](image)

The requirement for the RING domain was tested in two ways: the Not4ΔRING is a truncated version lacking the entire domain, whereas Not4-L35A carries a point mutation previously shown to disrupt the interaction with Ubc4/5 (Albert et al., 2002; Mulder, Inagaki, et al., 2007). Accordingly, E3 ligase substrate interactions and the interaction with the Ccr4-NOT complex should remain intact - contrary to the interaction with the E2 enzymes Ubc4/5 - and thus these mutants could reveal E3 ligase-specific functions of Not4. Not4ΔC carries an intact RING domain, but lacks the C-termi-
Results

Growth was analyzed on YPD agar plates at different temperatures (Figure 13 B). Wild-type cells transformed with an empty vector plasmid (ev) were spotted as serial dilutions, alongside not4Δ cells transformed with ev or complemented with full-length NOT4 (Not4). Wild type ev and not4Δ Not4 - although both carry a functional and complete NOT4 gene under the control of the endogenous promoter - did not display the same growth capacity. In other words, complementation of not4Δ with full-length NOT4 did not restore growth to a wild-type level. This was especially apparent for growth at 20°C (Figure 13 B). not4Δ cells without complementation (ev) displayed the well-known slow growth at 30°C and 20°C, as well as no growth at 37°C. There was no difference in growth between Not4- and Not4-L35A-complemented not4Δ cells. Both truncated variants (Not4ΔRING and Not4ΔC) displayed a growth behavior at 30°C and 20°C similar to the non-complemented not4Δ cells. However, at 37°C Not4ΔC was capable to restore a little growth in contrast to not4Δ ev and Not4ΔRING. In sum, Not4’s association with the Ccr4-NOT complex was partially dispensable at 37°C, whereas the RING domain was absolutely required under all tested temperatures.

5.3 Not4-specific substrates can be isolated by the Rubylator

The identification of E3 ligase substrates has been challenging ever since this protein class has been identified. The challenge arises from the transience of the substrate modification with ubiquitin, that, in most cases, destines substrates for degradation by the 26 S proteasome - especially in case of polyubiquitination. It needs to be noted, that ubiquitination can also destine substrates for change in their subcellular localization, regulate protein-protein interactions, or protein activity (Yau & Rape, 2016). In 2013, Zhuang and colleagues introduced an elegant new approach for the identification of E3 ligase substrates: the so-called Neddylator (Zhuang, Guan, Wang, Burlingame, & Wells, 2013).

Their *in vitro* study was based on purified recombinantly expressed E3 ligases, having their RING domain substituted with the Nedd8-specific E2 enzyme, Ubc12. Nedd8 is an ubiquitin-like modifier.
(UBL) with low abundance compared to ubiquitin. It is mainly involved in the control of cell cycle regulators and unlike the predominant polyubiquitination, it mostly occurs as monneddylation on its substrates. The Neddylation pathway is highly conserved among eukaryotes and essential for the viability of most model organisms, with the exception of S. cerevisiae (Rabut & Peter, 2008). Rub1, the Nedd8 homolog in S. cerevisiae, is a UBL with 53% sequence similarity to ubiquitin (Liakopoulos, Doenges, Matuschewski, & Jentsch, 1998). It is conjugated to substrates in a manner similar to ubiquitin, but with enzymes specific for Rub1 and its targets (Figure 14). Even though each UBL modifier comes with its own specific set of E1, E2, and E3 proteins, Nedd8/Rub1 can be a substrate of ubiquitin-specific E1s in human or yeast cells upon stress, where, in consequence, free ubiquitin levels are depleted (Leidecker, Matic, Mahata, Pion, & Xirodimas, 2012; Singh et al., 2012).

A strategy similar to the Neddylator was applied to yeast in order to identify Not4-specific substrates. For this purpose, the RING domain of plasmid-encoded NOT4 was removed and substituted with the coding sequence of UBC12, the Rub1-specific E2. The resulting construct will hereafter be referred to as UNΔ (Figure 15 A).

The coding sequence of RUB1 was also inserted into the plasmid, including a N-terminal 3XFLAG tag to allow for subsequent immunoprecipitation of conjugated proteins. Whereas UBC12-NOT4ΔRING (UNΔ) was transcriptionally controlled by the endogenous NOT4 promoter (about 600 nt upstream of the start codon), the GPD promoter sequence (TDH3) was inserted to control the expression of 3XFLAG-RUB1. An identical plasmid without the insertion of UBC12 was constructed to serve as a control, hereafter referred to as NΔ. not4Δ cells were transformed with either NΔ or UNΔ plasmid, grown to early exponential phase and prepared lysates were subjected to α-FLAG immunoprecipitations. First of all, in vivo rubylation was feasible for Not4 in yeast, as demonstrated by immunodetection of denaturing eluates after immunoprecipitation (Figure 15 B left). However, signals were also detectable in samples from control cells (NΔ), possibly presenting endogenous Rub1 substrates. Silver stained gels displayed a tremendous amount of unspecific background (Figure 15 B right). Taken together, these initial results called for a refinement of the applied protocol.

The first refinement involved the manner in which cells were harvested and lysed, by switching from more time-consuming centrifugation-based harvest to rapid vacuum suction through a filter, as well as from FastPrep-mediated to cryomill-mediated lysis (the former being represented by Figure 15 B, the latter by Figure 15 C through E). Furthermore, as Rub1 can alternatively be conjugated within the ubiquitin-conjugation pathway when free ubiquitin is scarce, four lysine residues (K6, K11, K27, K33) within Rub1 were mutated to arginine residues in order to prevent putative branching, provided that it actually occurs, hereafter indicated by the addition of K4R. The protocol was changed with respect to washing of the beads, from initially four times with low salt washing buffer, to two times with high salt washing buffer and three times with low salt washing buffer to reduce unspecific protein binding. And finally, proteins bound to α-FLAG-coupled magnetic beads were natively eluted by the addition of 3XFLAG peptide, prior to a denaturing elution. The immunodetection results obtained for NΔ and UNΔ, after the protocol has been adapted, showed stronger and more distributed signals according to the molecular weight (Figure 15 C left and middle panel). However, signals appeared stronger in the control (NΔ) compared to the Rubylator (UNΔ). On the other hand, signals detected in UNΔ concentrated narrower between 65 kDa and >140 kDa. Compared to NΔ and UNΔ, the K4R variants displayed no visible signals at first (Figure 15 C top left), but selectively changing the tonal correction between 65 kDa and 115 kDa revealed a faint signal.
for UN∆ K4R, which was more specific upon comparison to N∆ K4R (Figure 15 C bottom left). Unfortunately, this was not accompanied by an increase in specificity regarding unspecific binding to the magnetic beads (Figure 15 C right).

Figure 15: Rubylation of Not4-specific substrates. (A) Schematic representation of the Rubylation concept. (B) Immunoblot and silver staining of denatured elution samples from three independent replicates. (C) Comparative analysis of wild-type Rub1 versus Rub1K4R mutant. Immunoblot of native elution samples (left) including a section with enhanced tonal correction for Rub1K4R samples. Immunoblot of denatured elution samples (middle). Silver staining of native elution (NE) and denatured elution (DE) samples of Rub1K4R samples. (D) Immunoprecipitation results of cells subjected to amino acid-rich (+aa) or amino acid-scarce (-aa) conditions. Immunoblot (left) and silver staining (right) of native elution samples. (E) Immunoprecipitation results of cells subjected to glucose-rich (+glc) or glucose-scarce (-glc) conditions. Immunoblot of native elution samples. Rub1-conjugated proteins were immunodetected by α-FLAG. Asterisk indicates unconjugated 3xFLAG-Rub1.

Switching from α-FLAG-coupled magnetic beads to Protein G magnetic beads mixed with α-FLAG further improved specificity. As did the addition of a preclearing step which involved pre-incubation of the lysates with Protein A magnetic beads to decrease unspecific binding. These further changes were applied in combination with either amino acid or glucose withdrawal of the yeast cul-
Results

tures, to possibly increase Not4-substrate interactions upon addition of nutritional stress (Figure 15 D left and E, respectively). Immunoblot analyses confirmed the increased specificity of the K4R mutants regarding 3XFLAG-Rub1 conjugation to Not4 substrates (Figure 15 D left and E), and more importantly improved the result of silver-stained gels (Figure 15 D right). However, the resulting silver staining showed no obvious signal of a Not4-specific substrate. And only in case of glucose withdrawal (-glc) did the exposure to nutritional stress improve the amount of 3XFLAG-Rub1 conjugated proteins - although collectively, the samples of the glucose withdrawal experiment showed decreased signals in total, compared to the amino acid withdrawal experiment (compare Figure 15 D and E).

Taken together, the results showed that in vivo Rubylation works for Not4, and that the K4R mutant versions of N∆ and UN∆, Protein G magnetic beads with α-FLAG antibody, as well as the addition of a pre-clearing step, provided for cleaner and more specific results. However, the results require further improvement in order to be analyzed by a mass spectrometer to identify putative Not4-substrates. Already performed mass spectrometric measurements were inconclusive so far, mostly due to an extremely low amount of Rub1-conjugated substrates in the elution samples, which were insufficient for a conclusive identification by mass spectrometry.

5.4 Ribosome profiling reveals severe changes of the translatome in not4Δ cells

Ribosome profiling is an elegant method to obtain a global snapshot of actively translated mRNAs within cells, the translatome (Ingolia, Ghaemmaghami, Newman, & Weissman, 2009). The results not only allow for a distinction between the pool of actively translated transcripts in contrast to the total of transcripts present within cells, it allows for a position-specific resolution of ribosomes within each transcript. For the purpose of ribosome profiling, total cell lysates were subjected to RNase I digestion to generate ribosome-protected mRNA footprints of an average length of 28 nt. mRNA footprint isolation was followed by generation of a cDNA library and analysis using next generation sequencing.

Exponentially grown not4Δ cells, as well as wild-type cells, were subjected to ribosome profiling in order to gain insights into the composition of the respective translatomes in a qualitative, and - relative to wild-type cells - quantitative manner. For each strain, two biological replicates were examined. The efficacy of RNase I digestion was verified by separation of ribosomal particles using density gradient centrifugation. Furthermore, this allowed for the fractionation of the total lysate and the separation of 80 S monosomes from undigested polysomes possibly remaining (Figure 16 A-D).

Data sets were processed by removing any artificially attached sequences (e.g. linker sequence, index oligonucleotides), removing reads aligning to ribosomal RNA, and aligning the remaining reads to a list of non-dubious, low redundancy ORFs. Low redundancy per definition was achieved if less than 20% of reads mapping to a gene were matched to a second ORF. Data output as a measure of translation was managed as RPKM (reads per kilobase per million). Total reads within a gene were normalized against the length of the ORF and against the total number of reads. Finally, a filter was applied that set a threshold of at least 128 total reads per gene for both replicates to omit low-frequency reads. More than 50% of reads originated from rRNA, tRNA or intergenic regions (Table 2, see chapter 11. Appendix for the final data set).
Results

Figure 16: Evaluation of the experimental procedure and obtained data from ribosome profiling.
Continuation Figure 16: A-D: Polysome profiles obtained from RNase I-digested lysates of either wild-type (A and B) or not4Δ cells (C and D). Polysome profiling was performed by detection of the absorption at 254 nm. The 80 S fractions applied to ribosome profiling are highlighted. E-F: Scatter plots of obtained results in RPKM after filters were applied. Results from two biological replicates are displayed for wild type (E; number of ORFs included: 4883) and not4Δ samples (F; number of ORFs included: 4988) to show the correlation between duplicates including the calculated Pearson correlation coefficients (R²). G: Scatter plot of the average RPKM from reads of wild-type and not4Δ samples, including the Pearson correlation coefficient. Only ORFs with overlapping sufficient reads are included (number of ORFs: 4853). H: Bar graph showing the number of ORFs with at least 2-fold enrichment or depletion in not4Δ compared to wild-type samples.

<table>
<thead>
<tr>
<th>Data set</th>
<th>Number of ORF reads</th>
<th>Total reads*</th>
<th>ORF/ total reads [%]</th>
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<tbody>
<tr>
<td>wild type replicate 1</td>
<td>23640238</td>
<td>50150496</td>
<td>47.1</td>
</tr>
<tr>
<td>wild type replicate 2</td>
<td>25414615</td>
<td>59609793</td>
<td>42.6</td>
</tr>
<tr>
<td>not4Δ replicate 1</td>
<td>22616772</td>
<td>46650708</td>
<td>48.5</td>
</tr>
<tr>
<td>not4Δ replicate 2</td>
<td>26834484</td>
<td>61732418</td>
<td>43.5</td>
</tr>
</tbody>
</table>

Table 2: *Total reads include rRNA, tRNA, intergenic, and below threshold ORF reads

<table>
<thead>
<tr>
<th>Strain</th>
<th>Replicate Correlation Coefficient (Pearson)*</th>
<th>Number of ORFs considered</th>
<th>ORF/ background genes [%]</th>
</tr>
</thead>
<tbody>
<tr>
<td>wild type</td>
<td>0.9952</td>
<td>4883</td>
<td>68.2</td>
</tr>
<tr>
<td>not4Δ</td>
<td>0.9929</td>
<td>4988</td>
<td>69.6</td>
</tr>
</tbody>
</table>

Table 3: *Correlation coefficients were only determined for ORFs exceeding 128 total reads between two biological replicates.

Data sets obtained from two biological replicates strongly correlated for samples from both strains (Figure 16 E and F) with a Pearson correlation coefficient of 0.9952 and 0.9929 for wild type and not4Δ, respectively (Table 3). However, correlation between the average RPKM of wild-type and not4Δ samples showed differences and resulted in a Pearson correlation coefficient of 0.89957 (Figure 16 G).

16 ORFs showed a difference greater than 50% between replicates in both strains (Figure 17). Dividing the 16 ORFs according to the replicate sample in which it was increased showed that only three ORFs displayed increased read density (SNZ1, SPG4, and HSP30) in replicate 1 of wild-type cells. These ORFs have in common being associated with stationary phase growth of yeast cells. The 13 ORFs with increased read density in replicate 2 of wild-type cells can be associated to positive regulation under low glucose conditions. In conclusion, the differences observed between wild type replicates probably arose from minor differences regarding growth phases, where replicate 1 displayed advanced growth toward stationary phase over replicate 2. However, because the remaining 4867 ORFs correlated between both wild type replicates, these 16 ORFs were considered especially sensitive to growth phase progression. These observations could be interpreted as follows: the 16 ORFs indicated slight differences in growth phase progression between both replicates in wild type. In turn, this proves the robustness of all other ORFs correlating despite the difference in growth phase progression.

In case of the 16 ORFs displaying a difference greater 50% between the two replicates of not4Δ cells, replicate 1 showed an increased read density for five ORFs significantly associated to the biological process of sexual reproduction (ASG7, FIG1, FUS2, PRM1, and PRM2). Their presence suggests an activated mating response in not4Δ samples of different magnitude. The comparison to wild-type samples supports the suggestion of an activated mating response in not4Δ, as FIG1,
FUS2 and PRM2 did not produce enough reads from wild-type samples to be included in the analysis, and ASG7 and PRM1 were increased in not4Δ over wild type 1.66-fold and 2.05-fold, respectively. The remaining 11 ORFs with differential read abundance between replicates of not4Δ allowed no straightforward explanation, especially since four of them are described as "proteins of unknown function".

Calculation of the fold enrichment of the average RPKM resulted in 1275 ORFs with ≥2-fold increased reads and 131 ORFs with ≥2-fold decreased reads in not4Δ compared to wild type (Figure 16 H). Gene Ontology (GO) term finder¹ was applied to ORFs with a ≥2-fold enrichment of reads to gain more understanding about the nature of ORFs affected by the deletion of NOT4. 83.7% of ORFs with enriched reads in not4Δ could significantly be assigned to a GO term of biological processes (Figure 18). GO terms were summed under a suitable category and almost 50% of ORFs could be assigned to the category "metabolism", including both, positive and negative regulations. 58% of ORFs significantly assigned to the biological process "positive regulation of transcription from RNA polymerase II promoter in response to stress" displayed an enrichment in read density in samples from not4Δ cells.

1 https://www.yeastgenome.org/goTermFinder
Results

Figure 18: Biological process evaluation of ORFs with increased read density in *not4Δ* by ribosome profiling.
Continuation Figure 18: ORFs with at least 2-fold enrichment in not4Δ samples were subjected to GO Term Finder to identify significant (P-value ≤0.05) assignments to biological processes (BP). Biological process terms were additionally clustered into superordinate categories. The bar graph displays the \(-\log_{10}(P\text{-value})\) to each BP term.

The same was done for molecular functions, to which 27.2% of enriched ORFs were assigned. About 11% of ORFs with increased reads in not4Δ were assigned to the categories "transcription factor activity" and DNA binding activity in general (Figure 19 A and C). In total, 36 transcription factors displayed increased read density in not4Δ samples. Including MSN2 and MSN4 with 2-fold and 2.5-fold increased read density, respectively. MSN2 is constitutively expressed in yeast cells and regulated through its localization in the cytoplasm in a cAMP-PKA-dependent manner, whereas MSN4 is stress-inducible and itself under the control of Msn2/4 (Gasch et al., 2000). Further, PDR1 and its paralog PDR3, the major transcriptional regulators of the pleiotropic stress resistance, were enriched 2.4-fold and 3.3-fold. Their major targets are genes encoding various ABCs (ATP-binding cassette) like PDR5, -10, -15, SNQ2, and YOR1, as well as MFS (major facilitators super family) transporters like HXT9 and HXT11 (reviewed by Kolaczkowska & Goffeau, 1999). However, an increased read density was only confirmed for PDR15 by ribosome profiling.

Three of the four constituents of the CBF complex (CCAAT-binding complex) displayed enrichment (HAP2: 2.2-fold; HAP3: 2.3-fold; HAP4: 3.7-fold; HAP5: 1.2-fold). This complex is the global regulator of respiratory gene expression, transcriptionally induced during diauxic shift and glucose-repressed. An increase in read density for the described target genes (Mantovani, 1998) was however not observed. The transcript encoding Gis1, another transcription factor involved in the regulation of gene expression during the diauxic shift, was enriched 3.4-fold. The described target genes of Gis1 (Orzechowski Westholm et al., 2012) were not part of the filtered data set, except GND2, which did not show a significant enrichment (only 1.8-fold) - although it was slightly increased compared to wild-type samples. And finally, CAT8 and ADR1 displayed the largest enrichment among the transcription factors, with 8.8-fold and 11.2-fold enrichment. Both transcription factors control gene expression of respiratory and gluconeogenic genes during the diauxic shift. Some of their targets displayed increased read density in not4Δ cells. The Adr1 target USV1 was the transcript with the highest increase with a 46.3-fold enrichment (Hlynialuk, Schierholtz, Vernooy, & van der Merwe, 2008).

In summary, ribosome profiling revealed an enrichment in ribosome occupancy for transcripts encoding transcription factors involved in stress response, diauxic shift, and glucose limitation. Whether this increase of ribosomal footprints, indicative of an increased translational rate, is actually accompanied by an increase in transcriptional activity of said transcription factors cannot be conclusively assessed. However, target genes for which data was accessible displayed contradictory information, and hence, tend to support the suggestion that despite an increased translational rate, these transcription factors remain inactive with regard to transcriptional activation of target genes.
Figure 19: Molecular function and cellular component evaluation of ORFs with increased read density in not4Δ by ribosome profiling.
Continuation of Figure 19: A-B: ORFs with at least 2-fold enrichment in not4Δ samples were subjected to GO Term Finder to identify significant (P-value ≤0.05) assignments to molecular functions (MF; A) and cellular components (CC; B). Terms were additionally clustered into superordinate categories. Bar charts display the -log_{10}(P-value) to each term. C-D: Bar graphs of the number of ORFs assigned to each MF (C) or CC (D).

In addition to these specific transcription factors, ribosome profiling further revealed increased ribosomal footprints for transcripts encoding constituents of general transcription factors (e.g. holo TFIIH and TFIIIF), transcriptional co-activator complexes (e.g. TFIID, mediator, SAGA, NuA3), chromatin remodeling complexes (e.g. SWI/SNF, INO80, RSC), histone modifying complexes (e.g. COMPASS and Set3 complex), the SBF complex, which activates transcription during the G1/S-phase transition, and the PAF complex, which is involved in transcriptional initiation and elongation of RNA polymerase II-dependent genes (Table 4). More than 50% of the transcripts encoding constituents of the TFIID complex showed increased footprint density, but not the TATA-binding protein (TBP) encoded by SPT15, which was even 2-fold decreased. In contrast, transcripts of the RNA polymerase II core itself, or encoding histone proteins H2A (HTA1: 0.9-fold/HTA2: 1.2-fold) and H2B (HTB1:0.9-fold/HTB2: 1.1-fold) did not display increased footprints in not4Δ.

The enrichment of ORFs associated with gene expression, especially regarding transcriptional processes, was reflected by the GO term search of cellular components, with 39.8% of enriched ORFs significantly assigned to the cellular component "nucleus" (Figure 19 B and D). 4.2% of enriched ORFs were assigned to the GO term "mitochondrial ribosome" and both its daughter branches "mitochondrial large ribosomal subunit" and "mitochondrial small ribosomal subunit". These 4.2% of enriched ORFs make up 57.8% of all ORFs significantly assigned to this GO term. In other words, more than half of all ORFs significantly assigned to mitochondrial ribosomes displayed a significant enrichment in read density in samples of not4Δ. This finding was in accordance with the above described results of increased footprint density observed for transcription factors involved in the regulation of genes characteristic for diauxic shift, gluconeogenesis and respiration.

Another interesting observation was the footprint enrichment of ORFs associated with the biological process "cell cycle" (Figure 18). 41.5% of ORFs associated with this process displayed an enrichment, including the main cell cycle cyclin-dependent kinase CDC28 (3.7-fold), the G1 cyclin CLN1 (2.4-fold), the S-phase-specific B-type cyclins CLB5 and CLB6 (2.7- and 2.3-fold), and constituents of the anaphase-promoting complex (APC; CDC16: 2.3-fold; CDC20: 2.1-fold; CDC27: 2.8-fold; CDH1: 2.4-fold; MND2: 3.3-fold) (Figure 20 A and B). Taken together, transcripts involved in the regulation of mitotic key steps displayed an enrichment in footprint density. Of particular note is the magnitude of the observed enrichment oscillating around 2-fold. This could indicate that proportionally more cells were committed to cell cycle-specific processes or that these cells spent more time on cell cycle progression. Because ribosome profiling only reports on read density of a whole population of cells, not on a single-cell basis, it is not possible to draw an unambiguous conclusion from these results.

Finally, in accordance with the above described finding of an enriched read density for stress-responsive transcription factors, several heat shock proteins displayed an enrichment, too (Figure 20 D). The seven Hsp-encoding transcripts are stress-inducible, usually in a Hsf1- and Msn2/4-regulated manner. Some are specifically enriched upon glucose limitation/carbon starvation, or characteristic for growth phase transitions (diauxic shift, stationary phase).
## Table 4: Ribosome profiling results for constituents of the RNA polymerase II core complex, general transcription factor complexes, transcriptional co-activator complexes, chromatin remodeling and histone modifying complexes. All reads received after filtering were included and divided into either enriched (at least 2-fold) or not enriched.
Figure 20: Cell cycle and heat shock protein associations of ORFs with enriched read density in not4Δ cells.
Continuation Figure 20: A: Bar graph of the RPKM fold enrichment in not4∆ over wild-type samples concerning ORFs associated to cell cycle with at 2-fold enrichment. B: Pie charts showing the proportion of ORFs with at least 2-fold enrichment in not4∆ samples compared to all ORFs assigned to the biological process "cell cycle". 201 ORFs assigned to N/A produced a sufficient read density but did not meet the limit set to ≥2-fold. 16 ORFs (ISC10, NDT80, ZIP2, YOR019W, DMC1, AMA1, SSP1, FYV5, ADY3, YLL017W, SPO21, MPC54, SDC25, ADY4, SPR28, DON1) were excluded from the filtered results, and thus not part of the analysis. C: Bar chart of ORFs encoding heat shock proteins (Hsps) with ≥2-fold enrichment in not4∆. D: Scheme representing signals positively regulating gene expression of the HSP genes in C.

In conclusion, ribosome profiling revealed 1406 ORFs with at least 2-fold difference in their read density in not4∆ samples compared to wild-type samples. Of those, 91% showed an increase in the number of ribosomal footprints. However, several questions arise which require further clarification, as they can not solely be answered by the data obtained. First, does the footprint enrichment indicate an increased engagement in translation or rather reflect an increased availability of transcripts. In other words, do these results point to a role of Not4 in transcriptional or translational regulation, or both? Second, because the data constitute an average over many cells, it remains unclear whether the whole population equally displayed the observed differences or whether a sub-population experienced large differences. Therefore, consideration might be given to the proportion of cells engaged in cellular growth or cell division, which could hold an explanation for the enrichment of transcripts involved in cell cycle progression.

5.5 Auxin-inducible Not4 protein depletion works as an alternative method to study immediate loss-of-function effects

Because not4∆ cells randomly displayed suppressor mutants another system was chosen to investigate the loss-of-function of Not4. Common methods to study the loss-of-function of a gene of interest include RNA interference (RNAi)-mediated gene-silencing or inducible, degron-mediated protein depletion. However, RNAi, though conserved in eukaryotes, is not an option in the budding yeast Saccharomyces cerevisiae (Drinnenberg et al., 2009). Therefore, an auxin-inducible degron (Morawska & Ulrich, 2013; Nishimura, Fukagawa, Takisawa, Kakimoto, & Kanemaki, 2009) for Not4 was established and proved as an alternative system to study the effects of Not4 depletion (Baker, 2016; Blessing et al., manuscript in preparation, 2018).

Figure 21: Representative growth assays of serially diluted, OD600-adjusted cell cultures. Wild-type (BY4741), not4∆, AFB2, and AID*-NOT4 cells were tested. Growth was analyzed on YPD or YPD+IAA [1 mM] at the temperatures indicated. Incubation period at 30°C was two days, at 20°C three days. The respective mating-type is displayed in brackets.

Plate growth assays of serially diluted, OD600-adjusted cell suspensions confirmed that under Not4-depletion conditions (+IAA [1 mM]), growth was slowed down in cells expressing the AID*-Not4 fusion protein as sole source of Not4 (AID*-NOT4) at 30°C and 20°C (Figure 21). The growth impairment was not as pronounced as in not4∆ cells, but strong enough to unambiguously differ from the
control cells (AFB2), and wild type. And finally, AID*-NOT4 cells grew indistinguishable from control cells on YPD without the addition of auxin at 30°C and 20°C.

Preliminary work on the AID*-NOT4 strain established the optimal growth and depletion conditions for Not4 (Baker, 2016). Accordingly, Not4 depletion was usually carried out for six hours in exponentially growing cells, and a depletion efficacy of 80-90% was usually achieved during that time. Depletion efficacy was routinely determined for each experiment to ensure sufficient Not4-depletion.

5.6 The depletion of Not4 leaves the integrity of the Ccr4-NOT complex unaffected

So far, studies on the Ccr4-NOT complex components in yeast were mostly conducted through deletion mutants. This can be problematic because disruption of a single subunit can potentially have destabilizing effects on other complex partners (Azzouz et al., 2009; Bai et al., 1999; H.-Y. Liu et al., 1998). For example, the stable association of Ccr4 with the complex requires the presence of Caf1 (Azzouz et al., 2009; H.-Y. Liu et al., 1998). Several pull-down experiments emphasized how important the presence of all nine core subunits is for complex integrity. The NOT module was demonstrated to be especially sensitive toward disruption of single subunits of their kind. Their interaction and mutual stabilization was later confirmed and explained by structural analysis (Bhaskar et al., 2015). Especially Not5 seems to play an essential role in the formation of the NOT module, as three different published pull-down approaches demonstrated how Not2 and Not3 no longer associate functionally with the complex when isolated via Caf40, Ccr4, or Not1 (Azzouz et al., 2009; Bai et al., 1999; Gupta et al., 2016). Results obtained from not4Δ cells furthermore displayed dependency on whether pull-down was carried out via Caf40-TAP or Ccr4 immunoprecipitation, the latter showing no effect of NOT4 deletion on the constitution of the complex (Bai et al., 1999), whereas the former showed that Not4 was required for the association of Not2 and Not3 with the complex (Azzouz et al., 2009).

Protein depletion of a single complex subunit can affect the complex stability differently than the respective gene deletion. Potentially, other/neighboring complex proteins might also be marked for degradation due to the fact that they are in close proximity of the protein that is depleted. Therefore, to test whether the depletion of Not4 negatively affects its fellow complex partners Not1-mediated TAP pull-downs were performed (Figure 22 A).

Control (AFB2) and AID*-NOT4 cells were treated with auxin for six hours (indicated by “+” in Figure 22 BCF). Immunodetection was possible for the subunits Not1 (via α-CBD), Caf1 (via α-HA) and Not4 (via α-FLAG). Immunoblot analysis of the adjusted cell lysates serving as input for the TAP pull-down procedure showed efficient Not4 depletion upon auxin treatment (Figure 22 B). Signals were quantified and displayed relative to either the signals obtained from G6PDH (Figure 22 D and E), or CBD (Figure 22 G and H). The values obtained from the control group (AID*-NOT4 -) were normalized to a value of 1 and the respective signals from treated samples (AID*-NOT4 +) were displayed relative to it (Figure 22 E and H). Not4 depletion was efficient relative to detected Not1 signals, and signals were significantly decreased down to 24% on average (P value 0.003) (Figure 22 H). α-HA, representing Caf1, on the other hand displayed no significant difference between cells depleted of Not4 and control cells (P value 0.539).
After TAP pull-down, eluate samples were additionally analyzed via immunodetection of Not1, Caf1 and Not4 (Figure 22 C). Their quantitation and normalization revealed a similar result as for the input samples: the amount of Not4 detected was significantly different between depleted and non-depleted cells (P value 0.007). In contrast to Caf1, whose detection displayed no significant difference (P value 0.951) (Figure 22 I and J). The remaining complex subunits were identified in a silver-stained gel based on their molecular weight (Figure 22 F). A difference between non-deplet-
ed and Not4-depleted eluates was not observed with respect to the eligible areas, except for Not4 itself. These experiments qualitatively showed that Not4-depletion has no obvious effect on the association of other complex partners to Not1. And quantitatively, they showed that at least Caf1 remains unaffected by Not4-depletion with regard to total amounts in the cleared lysate (input samples) and Not1-bound amounts (eluate samples).

5.7 Not4 affects transcriptional repression of stress-inducible genes and genes involved in cellular differentiation programs

Microarray-based studies to describe transcriptome-wide changes in not4∆ revealed a large number of differentially expressed genes in not4∆ cells compared to wild type (Azzouz et al., 2009; Cui et al., 2008). Both studies reveal an approximately equal distribution between transcripts with increased and decreased expression in the deletion mutant. To investigate whether auxin-induced depletion of Not4 similarly effects the transcriptome, a microarray analysis was conducted.

Cells were grown in synthetic complete medium and depleted of Not4 for six hours (Baker, 2016). Total RNA was isolated and subjected to an Affymetrix yeast genome 2.0 gene chip. Transcripts were considered as being expressed significantly different if expression was at least two-fold increased or decreased compared to samples from non-depleted cells (see chapter 11. Appendix for the final data set). Upon depletion of Not4, 183 genes exhibited increased expression levels, 34 of which could be assigned to the biological process of reproduction (GO:0000003, Figure 23 A and D). These data confirmed the previously reported increased amounts of transcripts of the pheromone-responsive genes in not4∆ (Cade & Errede, 1994), Figure 23 B). In support of a role of Not4 in modulating the amount of reproduction-associated transcripts, FIG1, a known target of the mating response pathway, was upregulated more than 100-fold (Figure 23 B). Additionally, previously described transcriptional reporter genes for the activation of the MAPK-mediated mating response pathway, besides FIG1, were found to be significantly increased, including FUS1, FUS3, and PRM3 (C. J. Roberts et al., 2000), Figure 30 B). And finally, increased transcript levels were observed for the pheromone-inducible Ty3 retrotransposon genes, encoded by YGR109W-A, YGR109W-B and YIL082W-A (Bilanchone, Claypool, Kinsey, & Sandmeyer, 1993; Figure 23 B). Surprisingly, the transcripts MF(alpha)1 and MF(alpha)2, encoding the α-pheromone, were enriched almost five-fold and two-fold, respectively. This was surprising since the cells used for the microarray analysis were of mating-type a, and therefore should not produce the α-specific mating pheromone. Besides the clearly induced transcription of mating responsive genes, the microarray data revealed additional transcripts with significantly increased levels upon Not4-depletion, among them were stress-inducible genes, e.g. SSA4, HSP26, HSP42 or XBP1 (Figure 23 A).

In contrast, 43 transcripts were significantly reduced at least two-fold in Not4-depleted cells. Of which 17 could be assigned to the cellular component "mitochondrion" by GO: Slim Mapper (provided by https://www.yeastgenome.org/cgi-bin/GO/goSlimMapper.pl). The number of genes expressed differentially in Not4-depleted cells (226 genes) obtained in this study, greatly differed from what was previously reported for not4Δ under comparable conditions (i.e. exponential growth on glucose-containing medium) (Figure 23 C; Azzouz et al., 2009; Cui et al., 2008); whereas studies conducted on not4Δ resulted in an approximately equal distribution between increased and decreased transcripts, the data presented here not only showed an overall reduced number of differentially expressed genes, but four times more genes with increased over decreased levels.
Results

The microarray data set of differentially expressed genes obtained from samples of Not4-depleted cells was directly compared to the data set of Cui et al. and showed that only a small subset of genes was differentially expressed.

Figure 23: Microarray results of Not4-depleted cells compared to non-depleted cells. (A) Volcano plot of the obtained microarray data displayed as the log₂(fold change) against -log₁₀(adjusted P-value) to visualize the extent (fold change) and significance (adjusted P-value) of the observed changes. (B) Bar chart of log₂(fold change) values of pheromone-responsive genes with increased expression. (C) Bar chart of the assignment of >2-fold enriched genes to GO terms of biological processes. The -log₁₀(P-value) obtained from GO term finder is shown. (D) Bar chart to compare the number of differentially expressed genes between two previous studies (Cui et al. 2008, Azzouz et al. 2009) and the data obtained from the microarray in this study. (E) VENN diagram of the comparison between the Cui et al. data set and this study.

The microarray data set of differentially expressed genes obtained from samples of Not4-depleted cells was directly compared to the data set of Cui et al. and showed that only a small subset of genes was differentially expressed.
Results

genes were similarly affected in both studies. In total, 30 transcripts with increased expression, and 14 transcripts with decreased expression, overlapped between both data sets (Table 5).

Both data sets displayed a significant increase in transcripts of the small HSP-encoding genes HSP26 and HSP42. In summation, microarray analysis of Not4-depleted cells showed an increase in transcripts associated with an activated mating response (e.g. FIG1, FUS1, FUS3, YGR109W-A, YGR109W-B, and YIL082W-A). The most striking result however were increased transcript levels of the two α-pheromone-encoding genes. The data of Cui et al. also revealed a 7.9-fold increase in the MF(alpha)2 transcript, but it was excluded from their final data set of "good genes" together with other pheromone-responsive transcripts due to inconsistencies between their biological replicates of not4Δ. Furthermore, Not4-depleted cells displayed a decreased abundance of transcripts associated with the mitochondrion.

The microarray data of Not4-depleted cells were further compared to the results of ribosome profiling from not4Δ cells. Similarly to what was observed upon the comparison of the Not4-depleted microarray data set to published data obtained from not4Δ cells, the overlap of genes with at least 2-fold enrichment/depletion between ribosome profiling and microarray data was only small. About half of the transcripts with at least 2-fold enrichment in Not4-depleted cells also displayed an increased read density in the not4Δ ribosome profiling data set (88 out of 184, Figure 24 A). GO term analysis with regard to the involvement in biological processes of all overlapping genes yielded a significant association which can be summarized under "response to stimulus" (Figure 24 B).

A closer look at the results obtained for genes encoding constituents of the MAPK pathways that mediate the mating response/filamentous growth revealed an increased read density for the transcriptional repressors DIG1 and DIG2, the MAPK kinase STE11, the G-protein γ-subunit STE18, and the scaffolding protein STE5 in not4Δ by ribosome profiling (Figure 24 C). The microarray data of Not4-depleted cells displayed increased transcript levels of DIG2, the MAPK FUS3, α-pheromone-encoding MF(alpha)1 and MF(alpha)2, and almost 2-fold increased transcript levels for the α-cell-specific pheromone receptor STE3 (1.99-fold) (Figure 24 D). The direct comparison of MAPK pathway constituents between ribosome profiling data from not4Δ and microarray data from Not4-depleted cells revealed that only DIG2 was at least 2-fold enriched in both data sets (Figure 24 E). Because both data sets were obtained from different loss-of-function systems (gene deletion vs. protein depletion), and measured different aspects of gene expression (translatome vs. transcriptome), both differences could be the reason for the observed discrepancy. If the latter was the predominant reason for the differences, one explanation would be that Not4 regulates expression of certain mating response pathway constituents on a transcriptional (DIG2, FUS3, MF(alpha)1, MF(alpha)2), as well as on a translational level (DIG1, DIG2, STE11, STE18, STE5). It is also likely, that the observed effects on the mating response pathway constituents merely derived from a general regulatory role of Not4 during transcription and translation, and represent genes that are particularly sensitive toward Not4-dependent regulation of gene expression.

Taking all four "-omics" analyses into account it became apparent that the microarray data by Cui et al. and Azzouz et al. revealed a similar distribution between genes with increased and decreased abundance (Figure 24 F and G), while a similar ratio between the ribosome profiling data from not4Δ cells and the microarray data from Not4-depleted cells was observed. The major difference between microarray- and ribosome profiling-based analyses is that the former is a measure of the steady-state levels of total mRNAs, while the latter measures the engagement of mRNAs in translation.
### Table 5: Overlap of transcripts with differential expression in both microarray studies. not4Δ indicates the results from Cui et al. 2008.

<table>
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<th>systematic name</th>
<th>name</th>
<th>name description</th>
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<th>not4Δ (fold change)</th>
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Figure 24: Comparison of ribosome profiling (not4Δ) and microarray (Not4-depleted) data. (A) VENN diagram of the number of genes with at least 2-fold increase (left) or decrease (right). (B) GO term assignment to biological processes of shared ORFs. (C+D) Bar chart of the fold enrichment of ORFs associated with the mating response/filamentous growth pathway of ribosome profiling (C) and microarray (D) data. (E) Bar chart of the fold enrichment of ORFs associated with the mating response/filamentous growth pathway of which values were available in both data sets. (F+G) Comparison of the number of genes affected in the displayed data sets in total numbers (F) and in percent (G).
In other words, the presence and amount of transcripts must not necessarily be reflected in increased protein synthesis and *vice versa*, as both, transcription and translation can be independently regulated. Further, inducible protein depletion and gene deletion can have different, even opposing effects on biological processes. Experiments that exploit inducible protein depletion for loss-of-function studies will probably yield more immediate, albeit weaker effects as experiments with a corresponding deletion mutant. Deletion mutants might display stronger and/or adaptive changes over generations. The occurrence of suppressors in *not4Δ* as discussed above (see chapter 5.1) – but also other deletion strains (Ryu, Wilson, Mehta, Hwang, & Hochstrasser, 2016) - further supports this suggestion.

In conclusion, the microarray data obtained from Not4-depleted cells revealed that the immediate response to the loss of Not4 was fairly different from what was previously reported for *not4Δ* cells. Despite the differences, certain themes were constantly present, that is the activation of pheromone-responsive genes (Cade & Errede, 1994; Irie et al., 1994) and of the small HSPs, Hsp26 and Hsp42 (Cui et al., 2008). The finding of the inappropriate expression of α-pheromone-encoding genes upon Not4-depletion could provide an explanation why pheromone-inducible genes display increased expression. Assuming that α-pheromone was actually produced and secreted, it could stimulate Not4-deficient cells and thereby induce the mating response pathway in an autocrine fashion, independent of the presence of MATα cells.

### 5.8 Not4-depletion induced changes of the transcriptome are not due to proteasome impairment

The literature on the Ccr4-NOT complex provides ample evidence for a functional connection between Not4 and the proteasome (Laribee et al., 2007; Panasenko & Collart, 2011; Panasenko, David, & Collart, 2009). First, genetic and direct protein-protein interactions have been shown by epistasis and immunoprecipitation experiments between Not4 and subunits of the proteasome (Laribee et al., 2007). Second, association of the α-subunit of NAC (Egd2) with the proteasome was shown to require Not4-mediated ubiquitination of Egd2 (Panasenko et al., 2009). Third, proteasome integrity was demonstrated to be dependent on Not4, including direct interaction between Not4 and the proteasome chaperone Ecm29, as well as decreased interaction between Ecm29 and the proteasome in *not4Δ* cells (Panasenko & Collart, 2011). Fourth, microarray analysis revealed that 30% of transcripts associated with proteolysis are increased in *not4Δ* cells (Cui et al., 2008).

The proteasome does not merely function in the degradation of terminally misfolded proteins, it is also involved in the regulation of various processes through specifically targeted degradation of regulators. The 19 S regulatory and the 20 S core particle (RP and CP) not only form a functional 26S proteasome, the 19 S particle was further shown to carry out a non-proteolytic regulatory role during transcriptional elongation (Ferdous, Gonzalez, Sun, Kodadek, & Johnston, 2001). This was followed by other studies on the proteasome with regard to transcription. Proteasome inhibition induced by chemicals (MG132 or PS-341) or temperature-sensitive proteasome mutant subunits revealed significant alterations within the transcriptome (Auld, Brown, Casolari, Komili, & Silver, 2006; Dembla-Rajpal, Seipelt, Wang, & Rymond, 2004; Fleming et al., 2002). Proteasome inhibition was followed by an increase in transcripts associated with protein degradation (including genes encoding subunits of the proteasome and involved in ubiquitination), stress response, mitochondria, and carbohydrate metabolism. Also a decrease in transcripts involved in amino acid
metabolism, protein synthesis and mating. Additionally, transcripts encoding histones or ribosomal proteins were decreased in samples subjected to proteasome inhibition. One study established a direct interaction and activity of the proteasome on DNA and during transcription by ChIP experiments, directly demonstrating a role of the proteasome in the regulation of transcription (Auld et al., 2006).

Figure 25: Comparison of microarray data of published data sets from not4Δ cells or proteasome inhibited cells. (A) GO term results for the assignment to cellular components of transcripts which were 2-fold enriched in not4Δ cells (Cui et al. 2008). (B) GO term results for the assignment to cellular components of aggregated proteins identified by mass spectrometry in aggregation preparations from not4Δ cells (Preissler et al. 2015). (C) VENN diagram of transcripts displaying differential expression obtained from proteasome inhibition experiments (Dembla-Rajpal et al. 2004) and Not4-depleted cells. The overlapping genes are indicated below including the measured ratios (Ratio1: proteasome inhibition, Ratio2: Not4-depletion). (D) Scatter plot of at least 2-fold increased/decreased transcripts according to (C). Values meeting on the green diagonal line would indicate a positive correlation, while values meeting on the red diagonal line would indicate a negative correlation. The six overlapping transcripts are marked in orange, and FIG1 is marked for better orientation.
To exclude the possibility that the changes within the transcriptome observed in Not4-depleted cells were due to an altered activity of the proteasome, published data sets were compared to the microarray data of Not4-depleted cells presented in this work.

First, GO term analyses were conducted for the transcripts with 2-fold increase observed for not4Δ (Cui et al., 2008), as well as for aggregated proteins identified in not4Δ (Preissler et al., 2015) (Figure 25 A and B). Indeed, the microarray analysis conducted by Cui et al. revealed a significant enrichment in transcripts associated with the proteasome complex. About 30% of all genes associated with the cellular component "proteasome complex" were enriched. A similar result was obtained for the aggregated proteins isolated from not4Δ by Preissler et al. with more than 40% of all genes associated with this cellular component appearing in aggregates. While the proteasome core complex seemed more affected at the transcript level in not4Δ (Figure 25 A), there was a strong enrichment for constituents of the regulatory particle in aggregates of not4Δ (Figure 25 B). Taken together, GO term analyses expanded the relationship between Not4 and proteasome described above, by an increase in transcripts encoding proteasomal proteins, as well as increased aggregation of such proteins in not4Δ.

The microarray analysis results obtained from Not4-depleted cells were further compared to microarray-based data from proteasome inhibition experiments conducted by Dembla-Rajpal et al. to exclude the possibility that the observed effects were merely due to altered proteasome integrity and activity (Dembla-Rajpal et al., 2004) (Figure 25 C and D). Of all at least 2-fold differentially expressed genes, only six overlapped between both data sets (Figure 25 C). Neither a positive nor a negative correlation could be observed between the data sets (Figure 25 D). Thus, it can be assumed that the differential expression observed in Not4-depleted cells was not due to impaired proteasome function.

5.9 Not4 depletion leads to increased sensitivity toward α-pheromone and provokes agar invasion

Increased sensitivity of haploid MATα not4Δ cells toward α-pheromone has been previously demonstrated via the so-called halo assay (Leberer et al., 1994). To study whether increased pheromone sensitivity can also be observed in cells depleted of Not4, halo assays were performed on haploid MATα AID*-NOT4 cells. Indeed, halo assays confirmed increased sensitivity of Not4-depleted cells toward α-pheromone like previously reported for not4Δ cells (Leberer et al., 1994). The discontinuation of growth in close proximity to an external source of α-pheromone affected a significantly larger area in Not4-depleted as in control cells (Figure 26 A). While the edges of the formed halos by control cells (with and without IAA) and AID*-NOT4 cells (without IAA) were smooth and incisive, cells depleted of Not4 displayed frayed edges.

The final effector of the MAPK-dependent pheromone response pathway is the transcription factor Ste12. Its short-term activation by the pheromone-responsive MAPK pathway was shown to be followed by rapid inactivation and degradation. Failure to inactivate and degrade Ste12 had further been demonstrated to stimulate invasive growth (Esch, Wang, & Errede, 2006; Nelson et al., 2003; R. L. Roberts & Fink, 1994). Halo assays only provide the mating pheromone of the opposite mating type, and as a consequence, cells can not productively mate. Instead, these cells experience a constant stimulation and activation of Ste12, which eventually leads to the activation of invasive growth. Normally, this is restricted to the area in which cells react sensitively toward the pheromone provided. To investigate pheromone-induced agar invasion, cell material was gently
washed off from plates after documentation. Indeed, control cells and AID*-NOT4 cells without deple- tion revealed invasive growth underneath the area that previously displayed α-pheromone sen- sitivity indicated by discontinuation of growth (Figure 26 C). Surprisingly, cells depleted of Not4 dis- played agar invasion independent of the area affected by α-pheromone. Therefore, agar invasion was either independent of α-pheromone or triggered by very low concentrations of α-pheromone in Not4-depleted cells.

An experiment without an external source of α-pheromone showed that invasive growth was independent of α-pheromone in Not4-depleted cells (Figure 26 D). In this case, too, agar invasion was observable for AID*-NOT4 cells in the presence of auxin, but only in cells of mating-type a. Finally, agar invasion was demonstrated for MATa not4Δ, as well.

**Figure 26**: Halo assay with Not4-depleted cells. (A) Control (AFB2) and AID*-NOT4 cells were evenly spread onto YPD or YPD+IAA (1mM) plates and exposed to 20 µM α-pheromone spotted on a filter. Documentation was carried out after 3 days at 30°C. (B) Bar chart depicting the degree of α-pheromone sensitivity (by affected area/size) and invasive growth (n = 3) according to (A) and (C). One-way RM ANOVA resulted in significantly increased α-pheromone sensitivity of Not4-depleted cells (A, AID*-NOT4 bottom) compared to control cells (A, AFB2 top) (P value: 0.032). Invasive growth was significant in Not4-depleted cells (C, AID*-NOT4 bottom) compared to cells without Not4-depletion (C, AFB2 top, AFB2 bottom, and AID*-NOT4 top; P value: <0.001). (C) Same plates as in (A), but after the cell lawn has been washed off to reveal invasive growth. (D) Growth assay depicted in Figure 21 before and after cell material has been washed off. Mating types are indicated in brackets.
Results

In sum, halo assays confirmed an increased sensitivity toward α-pheromone in Not4-depleted cells, in accordance with what has been reported for not4Δ cells (Leberer et al., 1994). The investigation of agar invasion following the halo assay yielded a new phenotype for cells lacking Not4, namely α-pheromone-independent invasive growth of Not4-depleted or not4Δ MATa cells. This observation was mating type-specific in case of AID*-NOT4 cells and only occurred in MATa cells. Taking into account that the microarray results described above revealed inappropriate overexpression of the α-pheromone-encoding genes (MF(alpha)1 and MF(alpha)2) it is tempting to speculate that an autocrine stimulation occurred in cells depleted of Not4 which leads to the pheromone-independent agar invasion.

5.10 Not4 depletion and NOT4 deletion affect translation and translational repression upon nutrient withdrawal differently

The effect of a NOT4 deletion on translational repression upon nutrient withdrawal has already been investigated (Preissler et al., 2015). Therein, it was reported that translational repression is inhibited in not4Δ cells. Therefore, it was promising to compare translational repression in wild-type, not4Δ, and Not4-depleted cells with regard to translation and amino acid withdrawal-induced translational repression by polysome profiling (Figure 27).

In a polysome profile, translational repression is characterized by a relative decrease in polysomal fractions alongside an increased 80 S peak upon stress. The comparison of polysome profiles from control cells subjected to growth in presence or absence of amino acids, showed the above described characteristic changes between 80 S and polysomal fractions (Figure 27 A and B). Upon amino acid withdrawal, polysomes decreased, while the 80 S peak increased due to translational repression. In case of Not4-depleted cells (AID*-NOT4 1 mM IAA), amino acid withdrawal led to an increase in the 80 S peak, accompanied only by a small decrease in polysomal fractions (Figure 27 A and B). However, compared to control cells, polysome profiling also revealed decreased levels of 80 S and polysomes in Not4-depleted cells under amino acid-rich conditions. The Not4-depletion efficacy was controlled for by immunoblot analysis. After six hours, Not4 protein was reduced to approximately 20% (Figure 27 C). Profiles from not4Δ cells only displayed subtle changes in the 80 S peak and polysomal fractions upon amino acid withdrawal (Figure 27 A and B). Under amino acid-rich conditions, not4Δ samples displayed an 80 S peak comparable to control cells, and only a slight reduction in polysomes - in contrast to the profiles obtained under amino acid withdrawal conditions, where not4Δ behaved more similar to what was observed for Not4-depleted cells.

Of the total ribosomal particles, all three groups (control, Not4-depleted, not4Δ) displayed changes regarding the 80 S peak and polysomal peaks upon amino acid withdrawal (Figure 27 B). But while control cells displayed a decrease in polysomes of 29%, Not4-depleted and not4Δ cells showed a decrease of only 18% and 15%, respectively (Figure 27 D).

Therefore, it can be concluded that cells lacking Not4 by either protein depletion or gene deletion are still capable of translational repression, albeit not as efficient as control cells. The finding that polysome profiles of Not4-depleted cells displayed a divergent behavior already under amino acid-rich conditions, was puzzling. It very much looks like cells depleted of Not4 already experience translational repression even without the application of amino acid stress. Accordingly, one would not expect great changes following amino acid withdrawal. However, this conclusion requires further support from additional analyses.
Figure 27: Translation and translational repression analyzed by polysome profiling.
Continuation Figure 27: (A) Representative polysome profiles from wild type-like, non-depleted cells (AID*-NOT4 control), Not4-depleted cells (AID*-NOT4 1 mM IAA), and not4Δ cells. Displayed are the polysome profiles under amino acid-rich conditions (black) and without amino acids (gray), as well as the overlay of both profiles. The identity of the displayed peaks is indicated below. (B) quantitation of the relative amount of ribosomal particles according to 40 S, 60 S, 80 S, and polysomal fractions in percent. (C) quantitation of Not4 protein relative to the loading control (G6PDH) with and without depletion. Depletion was significant according to a two-tailed paired t-test under amino acid-rich (+aa, P-value 0.02) and amino acid withdrawn conditions (-aa, P-value: 0.04) (D) Quantitation of the reduction of polysomes upon amino acid withdrawal in percent.

Regarding not4Δ and control cells, the results obtained in this work contradict the findings of an earlier study (Preissler et al., 2015). The authors claimed that translational repression is inhibited in not4Δ cells. However, there are many differences between both studies, which will be addressed in the following. First, polysome profiles from cells under amino acid-rich conditions in the published work (Preissler et al., 2015) already resembled translational repression (i.e. large 80 S peak and small polysomal peaks), and there were only small changes observed upon amino acid withdrawal in comparison to the data shown here. Second, the ratio of 80 S and polysomes to the total ribosomal particles was different. In the Preissler et al. study the ratio is 30%/50% for wild-type 80 S/polysomes under amino acid-rich conditions compared to a 20%/>60% ratio in the current work. Hence, the current work shows a higher fraction of polysomes. Third, Preissler et al. used centrifugation-based cell harvest and glass bead-mediated cell lysis, in contrast to the methods used in this work, with comparatively rapid harvest by vacuum suction through a filter and cryomill-based cell lysis. In order to investigate the effect of the different methods on polysome profiling results, non-depleted and depleted AID*-NOT4 cells were harvested and lysed in accordance with the methods published (Figure 28).

Indeed, polysome profiles were more comparable to the ones published, when the same protocols for cell harvest and lysis were used. Under amino acid-rich conditions, 80 S peaks were already quite high compared to polysomes (compare Figure 27 A and Figure 28 A). Likewise, the "old" methods yielded an 80 S/polysomes ratio of 30%/50% (Figure 28 B). In contrast, amino acid withdrawal in the publication reversed the ratio in wild-type (50%/30%), and not4Δ samples revealing an approximation of the 80 S to polysomes ratio (roughly 40%/40%) (Preissler et al., 2015), similar to what was observed for Not4-depleted cells subjected to the "old" method (+IAA-aa: 44%/43%) (Figure 28 B).

Despite the methodological differences concerning the published data and data presented here, one result obtained from not4Δ cells was identical for both methods. That was the percentage decrease of polysomes upon amino acid withdrawal. In both cases, not4Δ samples displayed a decrease of 15%, compared to approximately 38% (Preissler et al., 2015) and 29% (this study) in wild type/control samples. So, the results provided here, and the comparison with the published results, support the notion that Not4-depleted or NOT4 deleted cells already experience translational repression independent of amino acid withdrawal. The findings presented here contradict the interpretation of Preissler et al. in that translational repression is inhibited in not4Δ upon amino acid withdrawal. The current work suggests that it seems more likely that the presence of Not4 is required for effective translation during optimal growth, as indicated by already reduced levels of 80 S and polysomes in Not4-depleted samples, which was confirmed regarding polysomes in not4Δ samples (Figure 27 A).
Strikingly, the polysome profiles revealed a greater total difference between control or not4Δ cells and Not4-depleted cells. This further emphasized the difference between short-term protein depletion versus gene deletion.
Not4-depletion negatively affects the adaptation of the transcriptome in response to amino acid withdrawal

Amino acid withdrawal not only affects the translational capacity of a cell in a quantitative and qualitative manner, but also provokes massive changes within the transcriptome. These changes include for example the transcriptional repression of genes involved in ribosome biogenesis (ribosomal proteins, ribosome biogenesis factors) and transcriptional activation of genes involved in amino acid metabolic processes or environmental stress response (ESR, (Gasch et al., 2000)). Therefore, total RNA was isolated from non-depleted and Not4-depleted cells – in the presence or absence of amino acids - and subjected to microarray analysis to examine transcriptome-wide changes by microarray analysis in dependency on Not4 (Figure 29).

The microarray results showed global changes of total mRNAs upon amino acid withdrawal in both data sets, in non-depleted control cells and in Not4-depleted cells (see chapter 11. Appendix for data sets). In accordance with previously published results (Gasch et al., 2000), amino acid withdrawal provoked differential expression for many transcripts. Control cells displayed roughly the same number of transcripts with increased (468) and decreased abundance (471) (Figure 29 C). In Not4-depleted cells, both numbers were reduced down to 260 with increased, and 308 with decreased abundance, respectively. The intersections between control cells and Not4-depleted cells were 226 transcripts with increased and 304 transcripts with decreased abundance upon amino acid withdrawal. Conversely, this means that 242 and 34 transcripts were exclusively upregulated in control cells and Not4-depleted cells, respectively. Gene ontology analysis of those genes yielded annotations to "catabolic process" and "carbohydrate metabolic process" for control cells, and "cellular amino acid metabolic process" for Not4-depleted cells. The intersection between both, control and Not4-depleted cells, regarding down-regulated genes was larger. Only 167 transcripts and 4 transcripts were exclusively down-regulated in control or Not4-depleted cells. Here, gene ontology analysis mainly yielded processes associated with "ribosome biogenesis" in control cells (Figure 29 compare E and F). Taking a closer look at transcripts which were assigned to the biological process of "ribosome biogenesis" and failing to reach at least a 2-fold decrease in both groups showed that even though the threshold of 2-fold was not always met, the tendency to down-regulate these transcripts was present in both data sets (Figure 29 D).

In contrast, most transcripts encoding ribosomal proteins remained below the applied threshold to count as differentially expressed, although most of these transcripts were rather underrepresented in cells subjected to amino acid withdrawal (Figure 29 compare G and H). Two of the four transcripts exclusively decreased in Not4-depleted cells encode the ribosomal proteins Rps14B and Rps28B, the other two encode proteins involved in ribosome biogenesis, Rpc40 and Nop56.
Results

Figure 29: Microarray analysis results for the differential expression upon amino acid withdrawal.
Continuation Figure 29: Microarray analysis results for non-depleted (orange) and Not4-depleted (lilac) cells upon amino acid withdrawal. Differential expression of transcripts of amino acid-withdrawn versus amino acid-rich conditions is displayed. (A+B) Volcano plots of the obtained microarray results display the log₂(fold change) against -log₁₀(adjusted P-value). The circle marks the area with the greatest difference between the two groups. (C) Bar chart of the total number of transcripts with differential expression values of at least 2-fold for non-depleted and Not4-depleted data sets. (D) Bar chart of transcripts assigned to the biological process "ribosome biogenesis" with significant decrease in either non-depleted samples or Not4-depleted samples. (E+F) Volcano plots according to (A+B), restricted to the biological process "ribosome biogenesis". (G+H) Volcano plots according to (A+B), restricted to the cellular component "ribosome". Genes encoding ribosomal proteins with significant differential expression are marked.

Transcripts associated with the biological processes "metabolic process", "DNA-templated transcription", or "cell cycle" (Figure 30), displayed differential expression upon amino acid withdrawal similarly in Not4-depleted cells as did non-depleted cells, but often with a reduced magnitude in the former. However, some transcripts displayed strikingly different results in Not4-depleted cells. For example, the DAL80 transcript, encoding a negative regulator of genes involved in multiple nitrogen degradation pathways (Cunningham & Cooper, 1991). It was almost 16-fold increased upon amino acid withdrawal in Not4-depleted cells, while it was only 1.5-fold increased in non-depleted cells experiencing amino acid withdrawal (Figure 29 A and B). DAL80 is a member of the regulatory network of the nitrogen catabolite repression pathways (NCR). The activation of these pathways was to be expected given that amino acid withdrawal shifts cells from growth on favored nitrogen sources (e.g. asparagine and glutamine) to ammonium, and thus effects derepression of the NCR pathways. Another transcriptional regulator of this network GAT1, was also increased 3.3- and 3.9-fold in non-depleted and Not4-depleted cells in response to amino acid withdrawal (Figure 31).

Other major regulators (URE2 and GLN3) and a selection of target genes (UGA1, UGA2, UGA4, DUR1,2, DAL2, DAL3, DAL7, DUR3) of the NCR were not differentially affected in dependency on Not4 (Figure 31). Therefore, the effect observed for DAL80 might not necessarily express different regulation of the whole NCR pathways, but rather be specific for the DAL80 transcript.

To sum up these observations, amino acid withdrawal provoked similar changes of the transcriptome in Not4-depleted cells and non-depleted cells. The major difference between both data sets was the magnitude to which transcripts were differentially expressed in Not4-depleted cells, which was generally smaller. In some cases, e.g. DAL80, the results were significantly different from non-depleted cells, but this scenario remained an exception within the whole transcriptome-wide data set. In conclusion, Not4 was not required for cells to respond to nutrient stress, but might impinge on the efficacy with which cells respond. These results implicate a more general role of Not4 in the regulation of gene expression.
Figure 30: Microarray analysis results for non-depleted (orange) and Not4-depleted (lilac) cells upon amino acid withdrawal. Differential expression of transcripts of amino acid-withdrawn versus amino acid-rich conditions is displayed. Data points were restricted to the biological processes "metabolic process" (A+B) and "DNA-templated transcription" (C+D) and "cell cycle" (E+F). Individual transcripts are marked for better comparison of the data.
5.12 ntl-4 knockdown provokes developmental defects in C. elegans

In yeast, deletion of NOT4 is tolerated and thus in principle amenable for loss of function studies. However, in C. elegans, deletion of the NOT4 ortholog ntl-4 is embryonically lethal (Maeda, Kohara, Yamamoto, & Sugimoto, 2001). Thus, RNAi-mediated gene knockdown is the means by which loss of function studies can be conducted with regard to ntl-4. In order to examine whether ntl-4 knockdown has an impact on the survival rate of worms, survival with or without ntl-4-specific RNAi was tracked and evaluated by the Kaplan-Meier estimator (Kaplan & Meier, 1958).

For the first survival rate experiment, worms were subjected to ntl-4 knockdown from the beginning (L1 larval stage), and hence developed under ntl-4 knockdown conditions. Only animals that died under natural circumstances were included in the analysis. The first survival experiment (marked as (1) in Table 6, Table 7 and Figure 32) was set up to compare the life spans of wild-type N2 worms either subjected to ntl-4-specific RNAi (ntl-4 kd (L1)) or not (ev). Of 120 wild-type control worms fed with empty vector bacteria, 70% were included in the data analysis, in contrast to only 40% wild-type worms fed with ntl-4 knockdown bacteria. Median survival was 20 days for control animals (ev) and 18 days for knockdown animals (ntl-4 kd) (Table 6). Survival rates were not significantly different from each other, as demonstrated by performing Peto-Mantel-Haenszel Test (Log-Rank Test) (Table 7).
Results

Table 6: Overview of the number of worms fed into life span experiments (analyzable). Including the number of censored worms, divided by the reason of censorship (bagging, protruding vulva (Pvl) and other).

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Total</th>
<th>Bagging</th>
<th>Pvl</th>
<th>Censored (other)</th>
<th>Analyzable</th>
<th>Median survival time [days]</th>
</tr>
</thead>
<tbody>
<tr>
<td>ev (1)</td>
<td>120</td>
<td>10</td>
<td>2</td>
<td>24</td>
<td>84</td>
<td>20</td>
</tr>
<tr>
<td>ntl-4 kd (L1) (1)</td>
<td>120</td>
<td>11</td>
<td>38</td>
<td>23</td>
<td>48</td>
<td>18</td>
</tr>
<tr>
<td>ev (2)</td>
<td>120</td>
<td>19</td>
<td>2</td>
<td>8</td>
<td>91</td>
<td>18</td>
</tr>
<tr>
<td>ntl-4 kd (L1) (2)</td>
<td>120</td>
<td>7</td>
<td>31</td>
<td>10</td>
<td>72</td>
<td>16</td>
</tr>
<tr>
<td>ntl-4 kd (A) (2)</td>
<td>120</td>
<td>2</td>
<td>17</td>
<td>5</td>
<td>96</td>
<td>17</td>
</tr>
</tbody>
</table>

Table 7: Results of the statistical evaluation of the Kaplan-Meier graphs obtained by Peto-Mantel-Haenszel Test (Log-Rank Test).

<table>
<thead>
<tr>
<th>Treatment</th>
<th>compared to</th>
<th>Log-Rank</th>
<th>degree of freedom</th>
<th>P-value</th>
<th>result</th>
</tr>
</thead>
<tbody>
<tr>
<td>ev (1)</td>
<td>ev (2)</td>
<td>7366.56</td>
<td>1</td>
<td>1.00</td>
<td>not significant</td>
</tr>
<tr>
<td>ntl-4 kd (L1) (1)</td>
<td>ntl-4 kd (L1) (1)</td>
<td>3005.53</td>
<td>1</td>
<td>1.00</td>
<td>not significant</td>
</tr>
<tr>
<td>ev (2)</td>
<td>ntl-4 kd (L1) (2)</td>
<td>7002.85</td>
<td>1</td>
<td>1.00</td>
<td>not significant</td>
</tr>
<tr>
<td>ntl-4 kd (A) (2)</td>
<td>ntl-4 kd (L1) (2)</td>
<td>9877.30</td>
<td>1</td>
<td>1.00</td>
<td>not significant</td>
</tr>
<tr>
<td>ntl-4 kd (L1) (1)</td>
<td>ntl-4 kd (L1) (2)</td>
<td>3741.93</td>
<td>1</td>
<td>1.00</td>
<td>not significant</td>
</tr>
</tbody>
</table>

Figure 32: Life span experiments of N2 animals subjected to ntl-4-specific RNAi, and control animals (ev). (A) Bar chart displaying the proportion of analyzable or censored worms. The total number of censored worms is marked on the side of each bar and censorship was further divided into: protruding vulva (Pvl), bagging, or other. (B) Kaplan-Meier graphs starting with the first worm dying from natural causes at day 6. The survival rate (S(t)) is an iterative method and takes censorship into account under the assumption that censoring does not change the probability of survival. (C) Pie charts of censored worms divided into the three groups in percent according to A.
Because survival rates of animals did not differ much, but the number of animals that required censorship did,ntl-4 knockdown was investigated further. Censorship was divided into three categories: bagging, protruding vulva (Pvl) and other. The bagging phenotype is usually provoked when animals encounter scarce food supply and is interpreted as a strategy to increase survival of the progeny by allowing larvae to hatch inside and devour the hermaphrodite from within. The Pvl phenotype on the other hand is described as a developmental defect, and was distinguished from age-associated vulval integrity defects (Avid) with regard to their temporal occurrence (Leiser et al., 2016). More precisely, Pvl occurs before reproduction is completed, whereas Avid is defined by a post-reproduction occurrence. Animals subjected to ntl-4 knockdown displayed the Pvl phenotype with high frequency (Figure 32 C), in contrast to bagging, which was equally frequent between ev and ntl-4 kd animals (Table 6). As the Pvl phenotype represents a developmental defect, a second survival experiment (marked as (2) in Table 6, Table 7, Figure 32) was carried out, including a third group (marked with (A)). These animals developed on RNAi plates with ev-feeding bacteria and were only subjected to ntl-4-specific RNAi upon reaching adulthood to test whether Pvl-associated censorship could be reduced. The second survival experiment confirmed the results from the first, in that survival rates did not differ significantly (Figure 32 B, Table 6, Table 7). Indeed, the number of animals that required to be censored could be reduced to levels comparable to ev control animals, if RNAi treatment was only applied in adulthood (Figure 32 A, Table 6). But even though the total number of animals that required censorship was reduced, still over 50% were censored due to Pvl phenotype regardless of the onset of RNAi (Figure 32 C).

RNAi-mediated knockdown efficacy was verified during the first life span experiment by semi-quantitative qPCR on RNA prepared from 120 animals (day 2 adulthood) treated the same way as for the determination of the survival rate. qPCR results showed that ntl-4 transcripts were knocked down to levels below 20% (Figure 33). In addition to ntl-4, qPCRs were performed on heat shock protein transcripts associated to different subcellular compartments. Transcripts for hsp-70 (C12C8.1) and hsp-17 (small Hsp) were increased at least 2-fold in worms after ntl-4 knockdown, indicating cytosolic stress. In contrast, hsp-60 and hsp-4, as representatives of mitochondrial or ER stress responses, remained mostly unaffected. In sum, ntl-4-directed knockdown was efficient and
accompanied by a more than 2-fold increase of two cytosolic heat shock protein transcripts, indicative of a cytosolic stress response.

5.13 NTL-4 protein is predominantly present within gonadal structures

FLAG-GFP::ntl-4 worms were generated by CRISPR/Cas9-mediated genomic engineering to allow for protein detection in biochemical experiments and microscopy-based protein localization studies. Confocal fluorescence microscopy of FLAG-GFP::ntl-4 alongside wild-type N2 worms revealed the strongest GFP signals within the gonad (Figure 34).

Both strains exhibited intestinal autofluorescence triggered by the excitation wavelength of 488 nm applied to detect GFP (Pincus, Mazer, & Slack, 2016). GFP signals representing NTL-4 protein varied between diffuse to granular, punctiform organization and were detectable in most cell types and tissues. Signals were especially strong within the cytoplasm of gonads and oocytes, and relatively weak in zygotes and developing embryos, where they changed from a more diffuse to a punctiform pattern (Figure 35 I, K, M, N). Nuclei displayed no GFP signal, thus NTL-4 is mainly detectable within the cytoplasm. In addition to the autofluorescence of intestinal cells, the cuticula gave an unspecific signal as well (Figure 35 D, F, H, J, L). In summary, NTL-4 was detected via GFP in the majority of tissues and cell types and either displayed a granular structure or even distribution throughout the cytoplasm. Due to the intestinal autofluorescence, no statement can be made about the localization of NTL-4 in this type of cells. Nevertheless, the microscopic analysis clearly showed the strongest GFP signals within gonads. And a differential abundance during the transition from maturing oocytes with strong, evenly distributed signals, to zygotes after fertilization through the spermatheca with low signals, to developing embryos where GFP fluorescence reappears with a regional, granular-like distribution.
**Results**

Figure 34: Confocal fluorescence microscopy of N2 and FLAG-GFP::ntl-4 worms. (A) and (B) show two different images taken of the same worms (5x objective). The three worms on top are N2, accordingly only autofluorescence is detectable with excitation at 488 nm (middle). Top: bright field images with scale bars (100 µm). Middle: fluorescence images with excitation at 488 nm for GFP. Bottom: drawing of the above images. Pharynx (red), intestine (blue), gonads (green), anterior (A), posterior (P), vulva (arrow).
Figure 35: Confocal fluorescence microscopy of N2 and FLAG-GFP::ntl-4 worms. (A) and (B) are the same as in Figure 34. Images (C)-(N) were taken with a 63x water immersion objective. Images (C), (E), (G), (I), (K), (M), and (N) are of FLAG-GFP::ntl-4 worms, whereas (D), (F), (H), (J), and (L) are of N2 worms and serve as a control. (C)-(H) show the head region, the pharynx is the explicit structure observable here. Images (I)-(N) display the reproductive system, including gonads, oocytes, spermatheca, zygotes and embryos. i = intestine, p = proximal gonad, d = distal gonad, e = embryo, o = oocyte, s = spermatheca, z = zygote, arrow head = vulva.
5.14 Biotinylated isoxazole can be exploited for the examination of Not4 and NTL-4

Not4 homologs of different eukaryotic model organisms share an extensive protein sequence homology regarding RING and RRM domains within the N terminus. Whereas, C-terminal protein sequences are different. Sequences of Not4 and NTL-4 (isoform a) were subjected to PLAAC analysis (Lancaster, Nutter-Upham, Lindquist, & King, 2014) to predict prion-like domains. The C-terminal sequence of NTL-4 comprises an enrichment of sequential glutamine (Q) stretches, a hallmark of proteins that form prions. The protein sequences were analyzed with settings that consider organism-specific background comparison as suggested by the authors. The minimum length was set to default (L_{core} = 60).

The results of the PLAAC analysis are displayed in Figure 36. For yeast Not4 only a small region spanning 10 amino acids (residues 271-280) was predicted to be PrD-like. However, the short sequence displayed only a mediocre probability based on the output (Figure 36 A). The result obtained for Not4 further showed that the most intrinsically disordered region indicated by the Fold-Index lies between the N-terminal RING and RRM domains, a sequence segment highly improbable to be prion-like as indicated by -PLAAC (Figure 36 B and C). In case of NTL-4, a highly probable prion-like domain was predicted spanning 220 amino acid residues (residues 531-750) (Figure 36 D) with a high PLAAC value coinciding with intrinsic disorder (Figure 36 E). Compared to Not4, NTL-4 additionally displayed two noticeable stretches of positive charge accompanied by intrinsic disorder between residues 300 and 400 (Figure 36 E and F).

Proteins comprising KH or RRM domains, as well as low complexity sequences, are often associated with RNA granules (Mitchell & Parker, 2014). These granules contain assemblies of RNAs and proteins, which either directly bind to RNA or facilitate protein-protein interactions. RNA granules have been observed in different eukaryotic cell types and play a crucial role in the spatial and temporal regulation of gene expression (Anderson & Kedersha, 2006). For example during development and mRNA shuttling of neuronal granules from the cell body to the synapses for localized translation. Biotinylated isoxazole (b-isox) has previously been shown to facilitate the precipitation of RNA-binding proteins (Han et al., 2012). Furthermore, b-isox-derived precipitates comprise a significant fraction of proteins associated with RNA granules in vivo. B-isox thus allows for enrichment and isolation of RNA granules from total cell lysates for further examination. Heretofore, RNA granules have mostly been examined in microscopy-based subcellular localization studies, because their highly dynamic nature complicated their biochemical isolation. As Not4 associates with polysomes and NTL-4 was detected via GFP in granular structures within the cytosol (Figure 35), it was tested whether both proteins can be precipitated by b-isox. And whether this approach can be used to identify interaction partners and putative substrates.
Figure 36: PLAAC analysis of Not4 and NTL-4.
Continuation Figure 36: PLAAC analysis of Not4 (A - C) and NTL-4 (D - F). (A) and (D) show the probability for the prion-like domain state in the Hidden Markov model (HMM) where a value of 0 equals improbable and 1 equals probable. The organism-specific background of amino acid frequencies in the HMM is also shown. (B) and (E) show the negative values of the probability for a prion-like amino acid composition (-PLAAC; +1 = probable, -1 = improbable) alongside the FoldIndex, which shows the probability of intrinsically unfolded protein segments (+1 = folded, -1 = unfolded). (C) and (F) show the distribution of charged amino acids (CHARGE, +1 = positively charged, -1 = negatively charged) and the distribution of hydrophobic amino acids (HYDRO, +1 = hydrophobic, -1 = hydrophilic). Graphs also display the position of the RING and RRM domains, as well as the essential region of Not4 for binding to the Ccr4-NOT complex. A protein region is considered “prion-like” if two predictions appear in combination: the region is predicted as unfolded and is highly probable for a prion-like amino acid composition.

Yeast and worm lysates were treated with 100 µM b-isox according to the schemes depicted in Figure 37 A and F. Samples were prepared from not4Δ yeast cells complemented with either empty vector (-), or vectors expressing wild-type Not4 (Not4), N-terminally truncated Not4 (Not4ΔRING), C-terminally truncated Not4 (Not4ΔC) or Not4 carrying a point mutation at leucine 35 (Not4-L35A). The resulting immunoblot obtained from these samples revealed that a fraction of wild-type Not4 was precipitated by addition of b-isox (Figure 37 B P1 b-isox). However, the truncated mutant Not4 proteins (Not4ΔRING and Not4ΔC) remained unaffected by b-isox addition, as proteins were only detectable in the supernatant fractions (Figure 37 B compare P2 and S2). The point-mutated Not4-L35A displayed a decreased amount in the precipitated sample compared to wild-type Not4 (Figure 37 B compare P1 b-isox and P2 b-isox). Thus, the ability of yeast Not4 to be precipitated in a b-isox-specific manner required wild type-like integrity, i.e. the presence of the RING domain, as well as the C terminus which facilitates binding to the Ccr4-NOT complex. The steady-state levels varied between Not4 truncation variants. Whereas Not4ΔRING displayed reduced amounts, Not4ΔC was increased 2-fold compared to wild-type Not4 (Figure 37 B and D). Truncations can affect the native fold of proteins and thus protein stability. In case of Not4ΔC, increased protein amounts could also hint at a complex-dependent protein turnover of Not4 which is bypassed by Not4ΔC and its inability to bind the complex.

The sequences of the different Not4 mutants were analyzed to predict the probability of disorder. This was done to exclude the possibility that the resulting differences in b-isox-mediated precipitations were caused by the respective truncation or point-mutation (Figure 38).

Comparison of the probability of disorder did not result in obvious differences based on the truncations or the point mutation, with the exception of the N terminus of Not4ΔRING. Which displayed a higher probability for disorder. This can be explained by the way this construct was designed. The codons encoding the first 80 amino acids were deleted from the NOT4-HA sequence and a start codon was reintroduced to allow for protein translation of Not4ΔRING-HA. The disorder probability for the initial methionine was similarly high in all samples, but is shifted by 80 amino acids for Not4ΔRING. The prediction of the disorder probability further revealed that the regions with the highest probability for disorder (between the RING and RRM, and RRM and C terminus) should remain present and intact in both truncations variants.

As Not4 variants with mutations affecting the RING domain were readily available (Not4ΔRING and Not4-L35A), ubiquitinated protein levels were investigated as well (Figure 37 C and E). The two replicates depicted in Figure 37 C showed opposing trends for the analyzed Not4 mutants. Wild-type Not4 showed strong signals detected by the ubiquitin-specific antibody, whereas samples prepared from not4Δ cells containing the empty vector clearly displayed reduced amounts of ubiquitinated proteins, both in the input, as well as precipitate samples (Figure 37 C and E).
Figure 37: b-isox-mediated precipitation of Not4, different Not4 mutants and NTL-4. (A) Schematic representation of the protocol applied to yeast lysates. (B) Immunoblot detection of Not4 mutants via α-HA in mock treated (DMSO) and b-isox-treated (b-isox) samples. Protein detection was carried out in precipitate (P), supernatant (S) and input samples. (C) Immunodetection of ubiquitin-conjugated proteins in two replicates by α-Ub. Precipitate (left), supernatant (middle), and input (right) samples were detected separately for better comparison. (D) quantitation of the immunoblot results. Not4 protein was quantified relative to the full-length Not4. (E) Ubiquitinated proteins were quantified relative to the signal of full-length Not4 samples. (F) Schematic representation of the protocol applied to worm lysates. (G) Immunoblot results with immunodetection of ntl-4 (α-FLAG), the α- and β-subunit of NAC (α-NAC) and the ribosomal protein Rpl10 (α-Rpl10) in the precipitate (P), the supernatant (S) and the input sample. DMSO serves as a mock control in contrast to b-isox-treated lysates (b-isox). (+) indicates additional treatment with RNaseA. (H) Accordingly, samples were silver-stained to depict the total protein content within each sample. The arrow marks the border between the stacking and separation gel.
Not4ΔRING samples showed a similar behavior regarding levels of ubiquitinated proteins as empty vector samples. Not4ΔC and Not4-L35A displayed a huge variability between the two independent experiments. With regard to b-isox precipitated samples (Figure 37 C left side), both mutants behaved similar to empty vector in the first replicate, whereas the second replicate showed more similarity to cells complemented with wild-type Not4. Both mutants displayed comparable ubiquitin levels of the input samples in both replicates. Whether or not this was merely an artifact or indicates only partial loss of function of the mutants, remained unclear and requires further investigation. Taken together, the results obtained from yeast samples showed that Not4 can be precipitated by b-isox. Furthermore, Not4 precipitation required the integrity of the protein, as the tested mutants displayed no (Not4ΔRING and Not4ΔC) or reduced precipitation (Not4-L35A) with b-isox. Between not4Δ cells complemented with empty vector (-) or wild-type Not4 (Not4), less ubiquitinated proteins were precipitated in samples lacking Not4. Although it should be noted that this reduction was already apparent in the Input samples.

Lysates obtained from FLAG-GFP::ntl-4 animals were also subjected to b-isox-mediated precipitation (Figure 37 F). Indeed, NTL-4 was strongly enriched in b-isox precipitated samples (Figure 37 G), as compared to control proteins (NAC and Rpl10), which showed comparably strong signals in Input and Supernatant samples and strongly decreased levels in precipitate samples. The precipitation of NTL-4, as well as other proteins, was unaffected by previous RNaseA-digestion, thus b-isox precipitation was highly specific, but independent on RNA integrity (Figure 37 G compare (P) samples). Taken together, both Not4 homologs revealed the ability to be precipitated by b-isox. This represents a promising starting point for future experiments aimed to identify specific interaction partners of Not4.

![Figure 38: Disorder probabilities of Not4 and variants thereof](http://prdos.hgc.jp/cgi-bin/top.cgi). A scheme of relevant domains is displayed above the graph. Probability values of the different Not4 variants were aligned accordingly. Predictions were run with default settings and a prediction false positive rate of 5%.
Discussion

6. Discussion

The results described here can be summarized as follows:

1. NOT4 deletion, though tolerated by haploid yeast cells, sporadically leads to variable phenotypes.
2. Functional investigation of Not4 by auxin-inducible Not4 protein depletion proves suitable and meaningful.
3. Not4 is implicated in transcriptional repression of stress and differentiation program-regulated transcripts during proliferation.
4. The presence of the N-terminal RING domain, as well as the C-terminal region are essential for the function of Not4.
5. Both Rubylator and biotinylated isoxazole are suitable to investigate Not4 substrates and functions.
6. NTL-4 is predominantly localized in the cytosol and is most abundant in the gonads of adult C. elegans.
7. ntl-4 knockdown affects the integrity of the vulva, but not the survival of adult worms.
8. Not4-depleted MATa cells express MF(alpha)1 and MF(alpha)2 and display a new mating-type-specific phenotype: invasive growth.

In the following sections the summarized results will be discuss in more detail.

6.1 NOT4 deletion sporadically leads to phenotypic variation

The observation that not4Δ cells displayed variation in colony size and the fact that cells from larger colonies regained the ability to grow on non-fermentative medium, led to the suggestion that suppressor mutations occur sporadically in not4Δ cells (Figure 12). This interpretation is supported by additional findings. Both, microarray data published by Cui et al., and ribosome profiling data derived from not4Δ cells, display variations between biological replicates ((Cui et al., 2008), and this work). This variation was especially apparent for pheromone-responsive transcripts. The raw data on MATa not4Δ provided by Cui et al. revealed a 7.9-fold increase in MF(alpha)2, however this data point was later omitted from their final data set due to inconsistencies between the two biological replicates. A similar observation was made regarding ribosome profiling data obtained from MATa not4Δ cells. The comparison of both biological not4Δ replicates displayed differences greater 50% for some pheromone-responsive transcripts like FIG1, FUS2, PRM1 and PRM2 (Figure 17).

Both studies together showed that deletion of NOT4 was repeatedly accompanied by discrepancies regarding the expression of pheromone-responsive genes. The varying effect on the mating response pathway in not4Δ cells is probably highly dose-dependent, and depends on how much α-pheromone is synthesized and secreted. This has a huge impact on the subsequent activation of pheromone-responsive gene expression.

The finding that complementation of not4Δ cells with plasmid-borne full-length NOT4 did not fully restore growth to wild-type level, also supports the suggestion that not4Δ cells accumulate suppressor mutations or at least different phenotypic expressions (Figure 13). Several reasons could
provide for an explanation of the failure of ectopically expressed Not4 to fully complement the chromosomal deletion of NOT4.

First, NOT4 was reintroduced into not4Δ cells on a plasmid including the NOT4 promoter sequence covering 622 nucleotides upstream of the ORF. Albeit not very likely it cannot be ruled out that additional cis-acting sequences beyond the selected promoter sequence are required for full complementation by fine-tuning NOT4 expression. This would strengthen the notion that not only the mere presence, but also exact levels of Not4 are relevant and directly affect cell growth. Second, another explanation for this observation could be (epi-)genetic adaptations taking place in not4Δ cells before reintroduction of the Not4 expression plasmid. It is conceivable that the deletion of NOT4 might require further (epi-)genetic alterations within the genome for cells to tolerate the deletion. These adaptations can occur in many ways and might not necessarily occur homogeneously in all cells. Further, reintroduction of NOT4 could lead to negative effects caused by these adaptations which become irrelevant, but might not be easily reversed.

These observations are highly relevant to the investigation of NOT4 in Saccharomyces cerevisiae. Results obtained from not4Δ cells containing suppressor mutations can be contradictory and misleading.

6.2 The functional investigation of Not4 via auxin-inducible Not4 protein depletion proved suitable and meaningful

Due to the problems associated with not4Δ strains, an alternative approach to investigate NOT4 was necessary. As RNAi-mediated gene knockdown is not applicable in Saccharomyces cerevisiae (Drinnenberg et al., 2009), this option was not considered further. Instead, the yeast-optimized auxin-inducible degron system represented an attractive alternative (Morawska & Ulrich, 2013). Accordingly, the AID*-NOT4 strain was used to investigate immediate effects associated with loss of Not4 protein and circumvent the occurrence of suppressors due to long-term loss-of-function (Baker, 2016). One disadvantage of the auxin-inducible system was that the efficiency of protein depletion varied from culture to culture, and hence needed constant monitoring. As a rule of thumb, only cell cultures displaying a reduction of at least 80% of Not4 levels were considered for analyses.

First, it was investigated whether upon Not4-depletion, AID*-NOT4 cells display a similar phenotype as not4Δ cells. Importantly, without the addition of auxin, growth of AID*-NOT4 cells is indistinguishable from wild-type cells at the temperatures tested (20°C, 30°C, 37°C). In contrast, growth of AID*-NOT4 cells was impaired at 30°C and 20°C if agar was supplemented with 1 mM auxin (Figure 21). However, the growth impairment of AID*-NOT4 cells was not as severe as for not4Δ cells. This can be most likely explained by the fact that between 10-20% of Not4 protein remained present upon depletion, either because Not4 protein was constantly synthesized or because changes in the subcellular localization of Not4 might render Not4 inaccessible for the degradation machinery, or both. Another explanation takes the stable association of Not4 with the Ccr4-NOT complex into account. Shuttling between nucleus, cytoplasm and localization into P bodies was shown for Not4 and many other components of the Ccr4-NOT complex (Cooper et al., 2012; Gupta et al., 2016; Muhlrad & Parker, 2005; Villanyi et al., 2014), while the SCF ubiquitin ligase complex - required for the auxin-induced Not4 protein depletion - is localized in the nucleus and cytoplasm (Blondel et al., 2000). Therefore, Not4 might escape auxin-induced degradation by localization into
P bodies in association with the Ccr4-NOT complex, and thus remains present within cells in small amounts.

The microarray analysis of Not4-depleted cells confirmed the differential expression of pheromone-responsive genes (e.g. *FUS1*) (Figure 23), consistent with previous findings for *not4Δ* cells (Cade & Errede, 1994; Irie et al., 1994; Leberer et al., 1994). In addition, increased sensitivity toward α-pheromone was observed in Not4-depleted cells similar to reports on *not4Δ* (Figure 26) (Leberer et al., 1994).

Two transcripts displayed increased expression throughout all three "-omic" studies which were considered here. The small Hsp-encoding genes *HSP26* and *HSP42* displayed significantly increased transcript levels in both microarray studies (Cui et al. 2008: *HSP26* 5.5-fold, *HSP42* 2.4-fold; Not4-depletion: *HSP26* 2.8-fold, *HSP42* 2.7-fold) (Figure 23) and significantly increased read density in the ribosome profiling experiment (*HSP26* 21.1-fold, *HSP42* 6.4-fold) (Figure 20). Therefore, these two genes constantly displayed increased expression, independent of whether the NOT4 gene was deleted or whether the Not4 protein was depleted. Moreover, this result was confirmed by analysis of steady-state transcript levels (microarray), as well as by read density during translation (ribosome profiling). Hsp26 and Hsp42 facilitate Hsp70/Hsp100-dependent refolding of misfolded, aggregated proteins following proteotoxic stress (Ungelenk et al., 2016). Hsp42 in addition displays aggregase function and is also involved in the active sequestration of misfolded proteins into aggregates upon proteotoxic stress (Ungelenk et al., 2016). Both genes are positively regulated by the transcription factors Hsf1, Msn2 and Msn4, which are activated by diverse stress conditions (Amorós & Estruch, 2001; Venters et al., 2011; Yamamoto, Mizukami, & Sakurai, 2005). Additional Hsf1- and Msn2/4-regulated genes displayed increased transcript abundance in Not4-depleted cells (e.g. *HSP78*, *SSA4*, *CTT1*, *HXT5*) (Figure 23). The persistent finding of increased expression of the small HSPs, alongside other stress-regulated transcripts and the observation of increased amounts of aggregated proteins in *not4Δ* cells (Preissler et al., 2015), raises the question whether increased expression of *HSP42* constitutes cause or consequence of proteotoxic stress and protein aggregation, or both. As Hsp42 displays aggregase activity, it is possible that its up-regulation facilitates aggregation and that this event is interpreted as proteotoxic stress by the cell, and thus stimulates the expression of other stress-inducible genes. A contrary finding to this idea was the finding that although many stress-responsive genes displayed increased transcript abundance, there was only a partial overlap with the set of genes usually activated in a Hsf1- or Msn2/4-dependent manner upon stress (Hahn, Hu, Thiele, & Iyer, 2004; Moskvin, Schüller, Maurer, Mager, & Ruis, 1998).

While some characteristics of *not4Δ* cells were recapitulated in cells depleted of Not4, others differed between the two. First, previous microarray studies with *not4Δ* cells revealed a distribution between transcripts with increased expression and decreased expression of approximately 1:1 (Azzouz et al., 2009; Cui et al., 2008) - in contrast to the microarray data obtained in this study from Not4-depleted cells, which displayed a ratio of approximately 4:1 (Figure 23), and an overall lower number of genes showing differential expression. This ratio was seconded by the ribosome profiling results obtained from *not4Δ* cells, where the number of transcripts displaying differential read density was distributed as 90% of transcripts with increased read density versus 10% of transcripts with decreased read density (Figure 24). In this context, it is important to consider that mRNAs are not necessarily subject to translation, hence ribosome profiling reflects more precisely which transcripts are being translated. The microarray results of Not4-depleted cells and the ribo-
some profiling results of \textit{not4}\textDelta \(\text{cells with increased rather than decreased transcripts/read densities in general fit the functional description of Not4 as a transcriptional repressor better.}

A further discrepancy between \textit{NOT4} deletion and Not4 depletion was uncovered by investigation of translation and translational repression via polysome profiling (Figure 27). The polysome profiles of Not4-depleted cells under amino acid-rich conditions displayed overall reduced levels of ribosomal monosomes and polysomes compared to non-depleted cells. However, the distribution of ribosomal particles did not differ much percent-wise between Not4-depleted cells and non-depleted cells. In contrast, \textit{not4}\textDelta \(\text{cells displayed a similarly high 80 S peak in polysome profiles compared to the non-depleted control cells under amino acid-rich conditions, albeit a mild reduction in polysomes.}

Translational repression upon amino acid withdrawal was observed in non-depleted, Not4-depleted and \textit{not4}\textDelta \(\text{cells, but the reduction in polysomes was much stronger in non-depleted, wild type-like cells. Two conclusions could be drawn from the polysome profile-based investigation of translation and translational repression. First, Not4 promotes translation under nutrient-rich conditions. This was indicated by reduced total levels of monosomes and polysomes in Not4-depleted cells, as well as reduced polysomes in \textit{not4}\textDelta \(\text{cells under amino acid-rich conditions. This promotes the idea that Not4-depleted and \textit{not4}\textDelta \(\text{cells already carry out translational repression to some extent if one compares the profiles to control cells. Second, translational repression does not seem to be complete in Not4-depleted and \textit{not4}\textDelta \(\text{cells. Assuming that Not4-depleted cells and \textit{not4}\textDelta \(\text{cells already experience translational repression under nutrient-rich conditions, one would not expect a severe change upon stress. The effect of amino acid withdrawal was further investigated regarding transcriptome-wide changes in dependency on Not4 via microarray analysis (Figure 29\(\text{A and B). The data obtained revealed that comparable changes occurred in Not4-depleted cells upon amino acid withdrawal as did in non-depleted cells. However, the extent to which transcripts were differentially expressed in Not4-depleted cells under this condition was reduced compared to non-depleted cells. This was particularly apparent for transcripts encoding proteins involved in ribosome biogenesis (Figure 29\(\text{E and F). And although the largest proportion of transcripts followed the trends of differential expression, many did not exceed the applied threshold of 2-fold. This was also reflected by the total number of ORFs displaying differential expression, which was reduced by 44\% for increased transcripts and 35\% for decreased transcripts in Not4-depleted cells.}

Taken together, the polysome profiling results in combination with the microarray analyses on cells depleted of Not4 with and without amino acid withdrawal, revealed that these cells already express a stress-related response even without the application of a stressor other than Not4 depletion. Furthermore, a response was detectable concerning transcriptional adaptations and translational repression, but with a reduced magnitude. Hence, the importance of Not4 unfolded more prominent during growth where proliferation is favored, while stress and differentiation responses are inhibited. In contrast, Not4 was not essential for the overall cellular stress response.

All in all, the investigation of Not4 function via inducible depletion of Not4 in contrast to a \textit{NOT4} deletion strain proved suitable. This was shown for the reproducibility of \textit{not4}\textDelta \(\text{phenotypes, regarding growth and the expression of pheromone-responsive genes. The depletion approach proved to be valid, as some phenotypic expressions turned out different in Not4-depleted cells compared to \textit{not4}\textDelta \(\text{cells. This included the amount of transcripts differentially expressed and analyzed by microarray (Azzouz et al., 2009; Cui et al., 2008). As well as translation and translational repression upon amino acid withdrawal which were analyzed by polysome profiling. The combination of both, auxin-inducible protein depletion and complete gene deletion proved to be a valid approach to produce reliable results in the investigation of Not4, and possibly other "sick" gene deletion strains.}
The advantages of each approach if applied correctly, have the potential to overcome the disadvantages of the other approach, and vice versa. Protein depletion is advantageous in that immediate effects can be observed without a putative accumulation of long-term side effects or the selection of suppressor mutations. On the downside, protein depletion never reached an efficiency of 100% and activity remaining might weaken the phenotypic expressions. Then again, the advantage of a gene deletion is its completeness and that it does not display residual activity. The disadvantage being that adaptations regarding Not4 and the previously argued selection for suppressor mutations can occur with high probability.

6.3 The presence of the N-terminal RING domain, as well as the C-terminal region is essential for the function of Not4

The phenotypic and biochemical examination of two truncated Not4 variants revealed, that the RING domain was essential for Not4 function. In growth assays Not4ΔRING expression in not4Δ cells did not restore wild type-like growth under all conditions tested (Figure 13). In contrast, the C-terminally truncated Not4ΔC showed that binding to the Ccr4-NOT complex - although essential for Not4 function at 30°C and 20°C - was not essential for cell growth at 37°C. Apart from that the C-terminal truncation disrupted b-isox-mediated precipitation of Not4, and affected the total ubiquitination level differently (Figure 13, Figure 37). Taken together, both truncation mutants showed that the RING domain, as well as the C terminus are both essential to the function of Not4, with the exception of the latter regarding restoration of growth at 37°C.

The finding that the Not4-L35A variant - bearing a point mutation in the RING domain - did not display any growth defect in contrast to Not4ΔRING, despite the fact that both mutant variants should be similarly defective in their ability to interact with Ubc4/5, was to be expected. Published results that made use of this point-mutation repeatedly reported a similar behavior. While in vitro studies and yeast two-hybrid assays showed the interaction between Not4 and Ubc4/5 by this point mutation to stop, Not4-L35A did not show any defects in in vivo experiments (Mulder, Inagaki, et al., 2007). For example, EGD2 was identified as a Not4-specific E3 ligase substrate by comparing the ubiquitination status of Eg2 in wild-type and not4Δ cells, but this scenario was not reproduced by the Not4-L35A mutant in vivo (Panasenko et al., 2006). Another study focused on the ubiquitination activity of the Ccr4-NOT complex demonstrated an autoubiquitination activity of Not4 in vitro, which was not detectable for the L35A mutant (Mulder, Inagaki, et al., 2007). On the other hand, growth assays conducted in the study showed a similar L35-independent restoration of growth of not4Δ at 37°C, like shown in the current study (Figure 13 B). The only in vivo result clearly showing a dependency on L35 was a plate growth assay in presence of 200 mM hydroxyurea (HU) where cells with the L35A substitution showed increased sensitivity compared to wild-type Not4 cells (Mulder, Inagaki, et al., 2007). The discrepancy between in vitro and in vivo results obtained from the L35A substitution mutant points to the interpretation that the defective interaction with Ubc4/5 observed in vitro can be overcome in vivo by additional, intact contacts between the RING domain and Ubc4/5. This notion is supported by the structural analysis of the interaction between Not4 and Ubc4, which showed additional contact sites within Not4 (inter alia: I37, E38, E69, I56, C60, N63, L70, P75) (Bhaskar et al., 2015). This notion could also explain the observation that Not4-L35A affected the ubiquitination level of b-isox precipitated proteins differently (Figure 37).

A similar observation was made for Not4ΔC, and it seems plausible that binding of Not4 to the Ccr4-NOT complex is not essential for its function, but still constitutes a prerequisite for target-ori-
Discussion

ented, spatiotemporal control of Not4 and the encounter with its substrates. Another insightful finding was the increased amount of Not4ΔC protein compared to wild-type Not4 (Figure 37). As both proteins, wild-type and C-terminally truncated Not4, were detected via an HA-epitope tag on their C terminus, it is unlikely that the difference in the detected signal strength was due to the truncation and thus missing antigen properties. Assuming that the increase in Not4ΔC levels is a result of an increase in protein stability, one could suggest that binding of Not4 to the Ccr4-NOT complex is required to restrict Not4 activity and substrate interactions by limiting the amounts of Not4. These observations encourage further examination of the subcellular localization of Not4 in dependency on the Ccr4-NOT complex. The Not4ΔC mutant could represent a suitable tool to monitor the shuttling behavior of Not4 independent on the association with the Ccr4-NOT complex.

6.4 The Rubylator and biotinylated isoxazole can be applied to investigate Not4 substrates and functions

The search for new substrates of Not4 is challenging, given that many protein ubiquitination events target substrates for proteasome-dependent degradation, and thus Not4-dependent modification by ubiquitin are highly volatile. The establishment of an in vivo Not4-dependent Rubylator could be a solution for that problem. The results presented here show that there is potential for improvement regarding specificity and cleanliness, and in the end, the protocol was limited only by the amounts investigated (Figure 15). Therefore, regarding future experiments on the Rubylator, one possibility is the experiment to be repeated and upscaled to yield sufficient protein amounts in the final eluates to allow for subsequent mass spectrometry analyses.

The investigation whether biotinylated isoxazole can be exploited for Not4 and NTL-4 yielded a positive result (Figure 37). Both, Not4 and NTL-4, could be enriched through b-isox-mediated precipitation where the N- and C-terminal truncation variants of Not4 were not precipitated. Making use of the ability to be precipitated by b-isox opens up new possibilities. As the underlying property of proteins allowing b-isox-mediated precipitation is shared by many constituents of mRNPs, future experiments might reveal the dependency of precipitated proteins on Not4/NTL-4 and give insights into new mRNP-associated substrates. Another application could be the investigation of further functional point mutations, e.g. phosphomutant or phosphomimetic Not4 mutants, to narrow down the prerequisites for precipitation of Not4 by b-isox, and possibly the transition of this information into a biological context.

6.5 NTL-4 mainly localizes to the gonads in adult worms and ntl-4 knockdown affects the integrity of the vulva

ntl-4 knockdown worms displayed an increased occurrence of the Pvl phenotype, a developmental defect (Figure 32). Pvl was mainly observable between day 5 and 10 of adulthood and was probably not aging-associated as in Avid (Leiser et al., 2016). In contradiction to a ntl-4-dependent developmental defect was the result obtained from animals only subjected to RNAi post-developmentally, that still displayed Pvl as the main reason for censorship, even though the total number of animals that required to be censored was reduced to control levels. As survival rates did not differ significantly from each other, it is unlikely that ntl-4 knockdown in adult animals increased aging and therefore induced Avid instead of Pvl in animals. These preliminary results suggest that ntl-4 function is required for the integrity of the vulva and the occurrence of Pvl in ntl-4 knockdown worms reflect defects in the gonad of the adult. This suggestion was further supported by microscopy stud-
ies on NTL-4’s cellular localization, revealing the strongest fluorescence within gonads (Figure 34, Figure 35). Somatic cells in developed C. elegans animals are strictly determined in their number, and since they are not replaced by dividing and differentiating stem cells - the way it is usually done in higher eukaryotes - they rely heavily on cellular homeostasis for survival. Accordingly, adult, somatic cells are post-mitotic and less active regarding transcription, translation and especially their spatiotemporal control compared to germline cells.

The distal tip of the gonad is mitotically active and continuously produces germ nuclei which move toward the proximal end of the gonad into the direction of the uterus. Meiosis, cellularization into oocytes and growth occur during this movement through the gonad and meiosis is completed upon fertilization within the spermatheca prior to entrance of zygotes into the uterus. The sudden decrease of NTL-4 protein observed coincided with a process termed oocyte-to-embryo transition (OET). A systematic approach to investigate the quality and quantity of transcripts and proteins could show that during the OET, thousands of maternal transcripts and proteins are rapidly degraded (Stoeckius et al., 2014). The so-called maternal-to-zygotic transition (MZT) precedes and succeeds the OET and characterizes the transition from maternal to zygotic control of development. Thus, it is plausible to assume that NTL-4 plays a role during posttranscriptional regulation of gene expression and regulation of mRNA degradation during reproduction. Posttranscriptional regulation of gene expression can be achieved, among other things, by reversible sequestration of mRNAs into mRNPs. mRNA decay was also shown to occur within specific mRNPs, the P-bodies. The finding of NTL-4 protein as granular-like structures lends further support to the previous speculation, as does the fact that NTL-4 can be specifically precipitated by treatment with b-isox, and thus displayed the chemical properties shared by many mRNP-associated proteins.

NTL-4 not only shares an extensive amino acid sequence homology and especially high conservation of the RING and RRM with yeast Not4, they both are also involved in reproductive and developmental processes and most likely control gene expression in a spatiotemporal manner. Despite the obvious similarities there are also evident differences between the two homologs. Due to the different C-terminal protein sequences it is unpredictable whether NTL-4 is stably associated with the Ccr4-NOT complex in C. elegans as Not4 is in yeast. Whereas Not4 is present within the nucleus and the cytoplasm, NTL-4 was only unambiguously detected in the cytoplasm (Figure 35). These differences could well be due to specifics of the two model organisms. In principle, both organisms share the involvement of Not4/NTL-4 in the same biological processes (e.g. regulation of gene expression, reproduction), but might differ in their preferences regarding their realization (e.g. transcriptionally versus posttranscriptionally). Therefore, differences in subcellular localization, protein sequence and protein-protein interactions could reflect this.

All in all, these findings create manifold starting points for further investigations of the organism-specific biological function(s) and molecular mechanism(s) of ntl-4 in C. elegans. First, tissue-specific and subcellular localization studies of NTL-4 during all developmental stages, including all larval stages, should be performed by microscopy of the FLAG-GFP::ntl-4 worms generated. Second, the finding that NTL-4 can be precipitated by treatment with b-isox could be exploited further. Especially if it should also prove suitable for in vitro experiments, where recombinantly expressed protein variants lacking different domains (e.g. RRM and/or PrDL) could be subjected to precipitation in order to distinguish which domains are required for this property (Figure 36). Based on that, recreation of mutant variants of interest in vivo could provide for further valuable tools. Third, the finding that knockdown of ntl-4 leads to a significant increase of otherwise heat-shock-inducible cytosolic transcripts (e.g. hsp-70 and hsp-17) can be a useful target to explore the mechanism by
which NTL-4 acts (Figure 33). Fourth, the life span experiments demonstrated that artifacts (e.g. censorship) could be reduced by induction of RNAi after the animals were fully developed (Figure 32). This is in as much important since other experiments might require larger amounts of worms where censorship is not an option (e.g. liquid culture).

Although mostly descriptive, these results can be further exploited as useful tools to know when and where to look for the investigation of the function of NTL-4 in *C. elegans*.

### 6.6 Not4-depleted MATa cells express MF(alpha)1 and MF(alpha)2 and displaying a new mating-type-specific phenotype: invasive growth

Two most surprising results in this work were the findings that Not4-depleted cells expressed the α-pheromone encoding genes MF(alpha)1 and MF(alpha)2, plus finding of a new, mating type-specific phenotype of agar invasion (Figure 23, Figure 26). The expression of MF(alpha)1 and MF(alpha)2 was surprising due to the fact that Not4-depleted cells subjected to the microarray analysis were MATa cells and therefore should not express α-specific genes. In this context, it is helpful to examine more closely the genetic background of the yeast strains used in this study. The not4Δ and AID*-NOT4 strains are both derived from BY4741, which is a derivative of the laboratory strain S288C (Brachmann et al., 1998; Mortimer & Johnston, 1986; Winston, Dollard, & Ricupero-Hovasse, 1995). The genome of yeast strain S288C is the first eukaryotic genome completely sequenced, and serves as the basis for sequence information obtainable from the Saccharomyces Genome Database (Goffeau et al., 1996). The genome’s relevant features for this work include loss-of-function alleles of the HO and KSS1 gene and the mutant flo8-1 allele (Elion, Brill, & Fink, 1991; H. Liu, Styles, & Fink, 1996; Meiron, Nahon, & Raveh, 1995). The non-functional ho allele leads to a heterothallic yeast strain, incapable of mating type interconversion due to non-functional HO endonuclease. Therefore, HO-mediated mating-type switch can be ruled out as the reason for the MF(alpha)1 and MF(alpha)2 expression detected. Furthermore, because of non-functional Kss1 and Flo8 - both being involved in the regulation of the invasive, filamentous growth pathways - pseudohyphae formation and invasive growth are highly unlikely for this strain (Granek & Magwene, 2010).

α- and a-specific genes are regulated differently, and their expression is dependent on the MAT locus (Figure 42). MATa cells express the transcription factor α1, which is required for the expression of a-specific genes with the collaboration of a transcription complex comprising Mcm1 and Ste12. On the other hand, α-specific genes are repressed by a complex formed by Hst1, Rfm1 and Sum1 in MATa cells. The participation of Sum1 in this process was demonstrated in an interspecies study and furthermore revealed a 50-fold increased expression for MF(alpha)1 in MATa sum1Δ cells (Zill & Rine, 2008). In contrast, MATa cells express the transcription factors α1 and α2, which mediate α-specific gene expression and a-specific gene repression, respectively.

In other words, α-specific genes are repressed by the Sum1-containing complex and this repression must be overcome by the presence of the α1-Mcm1-Ste12 complex in order to express α-specific genes. While a-specific genes can be induced by α1 and Mcm1-Ste12 in MATa cells, they must be repressed by α2-Mcm1 in MATa cells. In the current study, none of the transcripts encoding the major transcriptional regulators (α1, α2, α1, STE12, MCM1, SUM1, HST1, RFM1) of α- and a-specific genes were found to be differentially expressed upon Not4 depletion. Hence, the expression of α-specific genes in Not4-depleted cells was most likely not due to increased transcript levels of these transcription factors. Misregulation must therefore occur either on the level of transla-
tion or the proteins themselves. One promising candidate is SUM1, as its deletion was shown to lead to the expression of a-pheromone. LOH1, a protein involved in the assembly of the outer spore wall during sporulation in diploids was 2.3-fold increased in Not4-depleted cells, this gene being normally repressed during vegetative growth by Sum1 and Hst1 (McCord et al., 2003; Xie et al., 1999), further supporting the idea that Not4 depletion might reduce Sum1 activity.

A more likely candidate is the transcription factor Ste12. Several lines of evidence support the suggestion that the phenotypes observed in Not4-depleted cells and NOT4-deleted cells arose from increased or rather non-restricted Ste12 activity. First, cells overexpressing Ste12 display similar phenotypes, including decreased vegetative growth (Yoshikawa et al., 2011), abnormal cell cycle progression (Stevenson, Kennedy, & Harlow, 2001) and increased invasive and pseudohyphal growth (Foster et al., 2013; H. Liu, Styles, & Fink, 1993; Shively et al., 2013). Second, Ste12 is required for the expression of a- and a-specific genes in a MAT-dependent manner in haploids (Fields & Herskowitz, 1985). Third, Ste12 protein stability is negatively regulated by the Mediator kinase, Cdk8 (Nelson et al., 2003) under pheromone-uninduced conditions. Ste12 protein stability is further reduced upon pheromone stimulation in a Cdk8-dependent manner (Esch et al., 2006). Failure to restrict Ste12 activity upon pheromone exposure induces invasive growth (Esch et al., 2006; Nelson et al., 2003). The finding of Not4-dependent cyclin C destruction upon oxidative stress (Cooper et al., 2012) could provide for a link between Not4 and Ste12, as cyclin C is the cyclin partner of Cdk8. Furthermore, a Not4 dependency for the Ccr4-NOT complex-mediated regulation of the transcription factor Skn7 through Cdk8 was demonstrated and emphasizes the regulatory relationship between Not4 and transcription factors through the Mediator kinase module (Lenssen et al., 2007).

This leads to the suggestion that Not4 might be involved in the restriction of Ste12 activity under pheromone-uninduced and -induced conditions (Figure 39). Hence, Not4 depletion increases Ste12 activity which in turn leads to expression of a-pheromone and thus, autocrine stimulation of the pheromone response. The pheromone response further activates Ste12, resulting in the transcriptional activation of pheromone-responsive genes (e.g. FIG1, PRM2, PRM3). Finally, failure to restrict Ste12 activity upon pheromone stimulation induces invasive growth in cells depleted of Not4. This suggestion is furthermore supported by the finding, that invasive growth was not indicated in the microarray data obtained from cells after six hours of Not4 depletion, but was visible only in growth experiments after two or three days.

The autocrine stimulation of the pheromone-response could further provide for explanations of some of the phenotypes observed in Not4-depleted cells and not4Δ cells. Decreased vegetative growth could be a consequence of from Far1-mediated cell cycle arrest. The abnormal cell shape reported for not4Δ could also be a consequence of pheromone-induced structural rearrangements of the cell wall, which lack the directionality usually provided by the presence of a mating partner (Leberer et al., 1994). Of course, the possibility that Ste12 is a direct target of Not4 remains viable. Especially since Yap1 was shown to be a substrate of Not4 (Gulshan et al., 2012). The links described between Not4 and Cdk8-CycC could also explain the microarray result of increased transcript levels for stress-responsive, Msn2-regulated genes like the small HSPs (HSP26 and HSP42). Because Msn2 was also shown to be negatively regulated by Cdk8 (Chi et al., 2001).
Taken together, the finding of increased levels of transcripts encoding α-pheromone, pheromone-responsive genes and the invasive growth phenotype in Not4-depleted cells and not4∆ cells, strongly hint to a role of Not4 in restriction of Ste12 activity, either directly or through the regulation of the Mediator kinase module. The observation that the invasive growth phenotype was only inducible by Not4-depletion in MATa, but not MATα cells was telling. In this context, it is noteworthy to mention too that the two pheromones produced by the different mating types differ in their synthesis and post-translational modifications. While the α-pheromone is produced and secreted through the endoplasmic reticulum, Golgi apparatus and secretory vesicles (Julius, Schekman, & Thorner, 1984), a-pheromone is specifically exported by the ABC transporter Ste6 through a "nonclassical" mechanism (Kuchler, Sterne, & Thorner, 1989). Furthermore, α-pheromone precursors are glycosylated to yield mature α-pheromone, in contrast to mature a-pheromone, which requires additional modifications through prenylation and methylation. MATa and MATα cells display differences regarding the turnover of the mating type-specific G-protein-coupled receptors, Ste2 and Ste3. While Ste2 turnover is carried out by ligand-dependent endocytosis (Hicke & Riezman, 1996), Ste3 is subjected to constitutive, ligand-independent endocytosis in addition to ligand-dependent endocytosis (L. Chen & Davis, 2000). And these facts further complicate the production and secretion of α-pheromone in contrast to a-pheromone.

Early literature on NOT4 already documented a mating type-specific phenotype regarding restoration of mating efficiency in mutants of the mating response pathway, however these findings remained uncommented by the authors (Irie et al., 1994; Leberer et al., 1994). Northern analyses showed that NOT4 transcript levels were independent from the underlying mating type (Cade & Errede, 1994; Leberer et al., 1994). MATa not4Δ and MATα not4Δ cells equally displayed increased basal expression of a lacZ reporter under the control of the FUS1 promoter (FUS1::lacZ) (Cade & Errede, 1994). In contrast, experiments on mating efficiency revealed mating type-specific pheno-
Discussion

types for not4Δ mutants. While MATa not4Δ cells were capable of restoring mating in combination with different dominant-negative mutants of the Gβ-protein encoding STE4, this was not the case for MATa not4Δ cells (Leberer et al., 1994). However, MATα not4Δ cells restored mating in combination with the temperature-sensitive ste4-3 allele at the restrictive temperature, but not with the null-mutant ste4Δ allele; this was probably due to the establishment of mating competence during growth at the permissive temperature (Irie et al., 1994).

Taken together, the published results and the results presented here revealed that the Not4-dependent phenotypes are indeed mating type-specific regarding restoration of mating and invasive growth. The mating-type dependency could arise from the differences in pheromone synthesis, modification and secretion mentioned above, which appear more specialized and thus challenging in MATα cells. This suggestion is supported by the following results: first, basal expression of FUS1::lacZ was equally increased in MATα not4Δ and MATa not4Δ cells (Cade & Errede, 1994). Second, MATα not4Δ cells showed restored mating in combination with the temperature-sensitive ste4-3 allele (Irie et al., 1994). In sum, cells of both mating types lacking Not4 fundamentally display derepression (or activation) of transcripts associated with the mating response pathway. However, the phenotypic outcome is much stronger in the MATα background compared to MATa.

The effect of MATα not4Δ cells on mating efficiency was found to be independent on the presence of the GPCR-encoding STE2 (Irie et al., 1994), likewise increased basal expression of FUS1::lacZ was independent on STE2 in MATα not4Δ (Leberer et al., 1994), and the increased basal FUS3 expression was not fully dependent on the presence of STE12 (Cade & Errede, 1994). Accordingly, Not4-dependent derepression (or activation) of pheromone-responsive transcripts can not exclusively be attributed to Not4-dependent restriction of Ste12 activity and autocrine stimulation of the pheromone response pathway.

A more general effect of Not4 on transcriptional regulation could derive from another of its substrates, the histone demethylase Jhd2 (Figure 5). Jhd2 counteracts H3K4 trimethylation mediated by Set1, and undergoes Not4-dependent ubiquitination and subsequent degradation by the proteasome (Mersman et al., 2009). Additionally, both Jhd2 and the Cdk8-CycC complex were shown to be required to inhibit pseudohyphal growth under nutrient-rich conditions (Law & Ciccaglione, 2015). A detailed study on AQY1, a developmentally controlled water channel, revealed that Jhd2 and CycC contribute to the repression of AQY1 under non-fermentable growth conditions (Law & Finger, 2017). AQY1 was also among the transcripts with increased abundance in Not4-depleted cells and displayed an 2.7-fold increase upon Not4-depletion. Since both, Jhd2 and CycC, are substrates of Not4 this provides another interesting link to the Not4-dependent phenotypes described here. Importantly, neither JHD2 nor SSN8 (encoding cycline C) transcripts were differentially expressed in cells depleted for Not4.

Taken together, Not4-depletion similarly displayed increased transcript levels for pheromone-responsive genes like originally described for not4Δ cells (Cade & Errede, 1994; Irie et al., 1994; Leberer et al., 1994). The finding of significantly increased transcript levels for both genes encoding α-pheromone could be one reason why pheromone-responsive genes were expressed in cells lacking Not4. Because Ste12 mediates both, the expression of a- and α-specific genes and pheromone-responsive genes, it is the most interesting, putative target for Not4-dependent regulation that could explain the observations made here and by others. Especially, since prolonged activity or overexpression of Ste12 was also shown to result in invasive growth despite nutrient-rich conditions, similar to what was observed after prolonged depletion of Not4 (Esch et al., 2006;
Foster et al., 2013; Shively et al., 2013). Simultaneously, Not4-dependent restriction of Ste12 activity and/or inhibition of invasive growth could also occur through the Mediator kinase Cdk8 and its cyclin, and/or Jhd2. Direct proof for that model is still required. The results presented here, together with published results urge for the investigation of a direct interaction between Not4 and Ste12, as well as the functional relevance of the interaction of Not4 and CycC regarding Ste12 activity and further transcription factors, for example Msn2.
7. Outlook

The manifold results from the extensive characterization of NOT4 and its pleiotropic phenotypes yielded an overall picture where Not4 plays a prominent role in almost all aspects in the life of yeast (Figure 40). The importance of NOT4 is further highlighted by the fact that it is conserved throughout all eukaryotes.

Many phenotypes observed for not4Δ cells can be attributed to the finding that MATa cells depleted of Not4 expressed the α-pheromone-encoding genes MF(alpha)1 and MF(alpha)2, which probably results in autocrine stimulation of the mating response pathway. The activation of the mating response is characterized by Far1-mediated cell cycle arrest, Ste12-dependent transcription of pheromone-responsive genes and polarized growth. Not4-depleted and not4Δ cells both display phenotypes in line with an activated mating response. Cell cycle arrest might be incomplete and hence indirectly displayed as slowed growth, activation of pheromone-responsive genes like FUS1 has been demonstrated multiple times, and polarized growth was at least indicated by an abnormal cellular morphology of not4Δ cells (Leberer et al., 1994), as well as by the invasive growth phenotype shown in this work.

Figure 40: Graphic summary of phenotypes associated with NOT4. Colored bubbles indicate phenotypes associated with altered Not4 function.

Similar phenotypes were observed in cells overexpressing the transcription factor Ste12. These observations demand for a clarification of the relation between Not4 and Ste12. Moreover, additional transcription factors should be investigated as putative Not4-substrates, in particular the...
stress-responsive Msn2. The dominance of phenotypes associated with an activated mating response in MATα cells lacking Not4 function, is not manifested in the MATα cells, because neither mating efficiency is increased (Leberer et al., 1994), albeit similarly increased basal expression of the FUS1::lacZ reporter (Cade & Errede, 1994), nor do Not4-depleted cells display agar invasion. This fact can be potentially exploited to investigate other transcription factors which might be Not4 substrates, but whose effects might be overseen in the whirlwind of events of cells experiencing strong autocrine stimulation of the mating response pathway. Based on the microarray analysis of Not4-depleted cells the following transcription factors should be considered first: Sip4 and Cat8, Gis1, Hsf1 and Msn2/4, because together with Ste12 they were identified as transcriptional regulators of 40% of all genes which displayed increased abundance. As pointed out above these transcription factors should be investigated in cells that exclude the possibility of autocrine stimulation.

Special emphasis should also be placed on the proposed potential complex-dependent spatial control of Not4. As already noted above in the discussion of the C-terminally truncated Not4 mutant. In this context, it should be mentioned that a mutant NOT4 allele expressing a C-terminally truncated protein (Not4∆419) restored mating efficiency in a strain with a conditionally functional STE11 allele, similar to not4Δ cells (Cade & Errede, 1994).

The results presented in this work offer excellent starting points for the functional characterization of Not4 and its ortholog NTL-4 in the model organisms Saccharomyces cerevisiae and Caenorhabditis elegans in the future. The major investment in concentrating and expanding the knowledge about Not4 - including a careful consideration of the peculiarities of yeast – will elaborate how future studies approach Not4, and hopefully allow for new exciting insights once the obvious processes triggered by loss of Not4 function have been bypassed (i.e. autocrine stimulation of the mating response pathway by failure to restrict Ste12 activity).
8. Materials and methods

8.1 Materials

8.1.1 Chemicals and enzymes

All chemicals were analytical grade and purchased from Sigma-Aldrich, Roth, or Merck if not listed separately in the following list.

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<th>Name</th>
<th>Origin</th>
<th>Name</th>
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### 8.1.2 General buffers and solutions

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<th>Buffer Type</th>
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<tr>
<td>Alkaline hypochlorite solution (20%)</td>
<td>250 mM NaOH; 20% (v/v) bleach</td>
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<tr>
<td>bis-tris gel buffer (3.5X)</td>
<td>1.25 M bis-tris-HCl pH 6.5-6.8</td>
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<tr>
<td>bis-tris separation gel (7.5%)</td>
<td>2.44 ml $\text{d}_2\text{H}_2\text{O}$; 1.5 ml bis-tris gel buffer (3.5X); 1.31 ml acrylamide (37.5:1; 30% (v/v)); 37 µl APS; 10 µl TEMED</td>
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<tr>
<td>bis-tris stacking gel</td>
<td>2.44 ml $\text{d}_2\text{H}_2\text{O}$; 1.5 ml bis-tris gel buffer (3.5X); 1.31 ml acrylamide (37.5:1; 30% (v/v)); 37 µl APS; 10 µl TEMED</td>
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<td>Coomassie R$_{250}$ destaining solution</td>
<td>35% (v/v) ethanol; 10% (v/v) acetic acid; 0.1% (v/v) glycerol</td>
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<td>Coomassie R$_{250}$ staining solution</td>
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<td>DNA loading dye (6X)</td>
<td>30% (v/v) glycerol; 60 mM EDTA pH 8.0; 0.12 % (v/v) bromphenol blue; 0.12 % (v/v) xylene cyanol</td>
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<td>ECL solution A</td>
<td>100 mM Tris-HCl pH 8.6; 0.025% (w/v) luminol</td>
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<tr>
<td>ECL solution B</td>
<td>0.11% (w/v) p-hydroxycoumaric acid in DMSO</td>
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<td>ECL solution C</td>
<td>30% (v/v) $\text{H}_2\text{O}_2$</td>
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<tr>
<td>ECL working solution</td>
<td>1 ml solution A; 100 µl solution B; 1 µl solution C</td>
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<td>M9 buffer</td>
<td>85.6 mM NaCl; 42 mM Na$_2$HPO$_4$; 36.7 mM NaH$_2$PO$_4$; 1 mM MgSO$_4$</td>
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<td>MOPS running buffer (5X)</td>
<td>250 mM MOPS; 250 mM Tris base; 5 mM EDTA; 0.5 % SDS</td>
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<td>Pfu reaction buffer (10X)</td>
<td>200 mM Tris-HCl pH 8.8; 100 mM (NH$_4$)$_2$SO$_4$; 100 mM KCl; 20 mM MgSO$_4$; 1% (v/v) Triton X-100; 1 mg/ml BSA</td>
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<tr>
<td>Phusion reaction buffer (10X)</td>
<td>100 mM Tris-HCl pH 8.8; 500 mM KCl; 20 mM MgCl$_2$; 1% (v/v) Triton X-100</td>
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<td>Ponceau S</td>
<td>0.2% (w/v) Ponceau S; 5% (v/v) acetic acid</td>
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<tr>
<td>Protein sample buffer (5X)</td>
<td>255 mM Tris-HCl pH 6.8; 5% SDS; 715 mM β-ME; 0.35 % (w/v) bromphenol blue</td>
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<td>SDS protein lysis buffer (2X)</td>
<td>125 mM Tris-HCl pH 6.8; 2 mM EGTA pH 8.0; 4% (v/v) SDS; 20% (v/v) sucrose; 2x Tm complete</td>
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<td>Silver stain solution I</td>
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<td>Silver stain solution II</td>
<td>0.2% (w/v) AgNO$_3$; 0.0185% (v/v) formaldehyde</td>
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<td>Silver stain solution III</td>
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<td>Silver stain solution IV</td>
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<td>TAE buffer (50X)</td>
<td>2 M Tris base; 5.71% (v/v) acetic acid; 50 mM EDTA pH 8.0</td>
<td></td>
</tr>
<tr>
<td>TBS-T (1X)</td>
<td>20 mM Tris-HCl pH 8.0; 137 mM NaCl; 0.1% (v/v) Tween-20</td>
<td></td>
</tr>
<tr>
<td>TE (10X)</td>
<td>100 mM Tris-HCl pH 8.0; 10 mM EDTA pH 8.0</td>
<td></td>
</tr>
<tr>
<td>TFB1</td>
<td>30 mM KCH$_3$COO; 50 mM MnCl$_2$; 100 mM KCl; 10 mM CaCl$_2$; 15% (v/v) glycerol</td>
<td></td>
</tr>
<tr>
<td>TFB2</td>
<td>10 mM MOPS pH 7.0; 75 mM CaCl$_2$; 10 mM KCl; 15% (v/v) glycerol</td>
<td></td>
</tr>
<tr>
<td>Western blot transfer buffer (1X)</td>
<td>25 mM Tris base; 190 mM glycine; 0.35 mM SDS; 20% (w/v) methanol</td>
<td></td>
</tr>
<tr>
<td>Yeast gDNA buffer</td>
<td>10 mM Tris-HCl pH 8.0; 100 mM NaCl; 0.1 mM EDTA pH 8.0; 0.1% (v/v) SDS; 2% (v/v) Triton X-100</td>
<td></td>
</tr>
<tr>
<td>Yeast transformation solution</td>
<td>400 mM CH$_3$COOLi; 40% (w/v) PEG-3350; 132 mM β-ME</td>
<td></td>
</tr>
</tbody>
</table>

### 8.1.3 Co-immunoprecipitation buffers

<table>
<thead>
<tr>
<th>Buffer Type</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lysis buffer</td>
<td>50 mM Tris-HCl pH 8.0; 150 mM NaCl; 10 µg/ml Aprotinin; 5 µg/ml Leupeptin; 80 µg/ml Pepstatin A; 0.1% (v/v) nonidet P-40; 0.1 mM DTT</td>
</tr>
<tr>
<td>Low salt washing buffer</td>
<td>50 mM Tris-HCl pH 8.0; 150 mM NaCl; 10 µg/ml Aprotinin; 5 µg/ml Leupeptin; 80 µg/ml Pepstatin A; 0.1% (v/v) nonidet P-40</td>
</tr>
<tr>
<td>High salt washing buffer</td>
<td>50 mM Tris-HCl pH 8.0; 300 mM NaCl; 10 µg/ml Aprotinin; 5 µg/ml Leupeptin; 80 µg/ml Pepstatin A; 0.1% (v/v) nonidet P-40</td>
</tr>
</tbody>
</table>

Note: 20 µM NEM was added to IPs with subsequent immunodetection of ubiquitin

### 8.1.4 TAP buffers

<table>
<thead>
<tr>
<th>Buffer Type</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lysis buffer</td>
<td>100 mM NaCl; 50 mM Tris-HCl pH 8.0; 1.5 mM MgCl$_2$; 0.1% (v/v) nonidet P-40; 5% (v/v) glycerol; 100 µg/ml CHX; 1 mM PMSF; 10 µg/ml Aprotinin; 5 µg/ml Leupeptin; 80 µg/ml Pepstatin A</td>
</tr>
<tr>
<td>Washing buffer I</td>
<td>100 mM NaCl; 50 mM Tris-HCl pH 8.0; 1.5 mM MgCl$_2$; 0.1% (v/v) nonidet P-40; 5% (v/v) glycerol; 100 µg/ml CHX; 1 mM PMSF</td>
</tr>
<tr>
<td>Washing buffer II</td>
<td>100 mM NaCl; 50 mM Tris-HCl pH 8.0; 1.5 mM MgCl$_2$; 0.1% (v/v) nonidet P-40; 5% (v/v) glycerol; 100 µg/ml CHX; 1 mM PMSF</td>
</tr>
<tr>
<td>Washing buffer III</td>
<td>100 mM NaCl; 50 mM Tris-HCl pH 8.0; 1.5 mM MgCl$_2$; 0.1% (v/v) nonidet P-40; 5% (v/v) glycerol; 100 µg/ml CHX; 4 mM CaCl$_2$</td>
</tr>
</tbody>
</table>
Materials and methods

8.1.5 b-isox buffers

<table>
<thead>
<tr>
<th>Buffer</th>
<th>Composition</th>
</tr>
</thead>
<tbody>
<tr>
<td>EE buffer</td>
<td>50 mM HEPES pH 7.5; 150 mM NaCl; 0.1% (v/v) nonidet P-40; 1 mM EDTA pH 8.0; 2.5 mM EGTA pH 8.0; 10% glycerol; 1 µM DTT; 10 µg/ml Aprotinin; 5 µg/ml Leupeptin; 80 µg/ml Pepstatin A</td>
</tr>
</tbody>
</table>

Note: with subsequent immunoprecipitation EE buffer without DTT

8.1.6 Polysome profiling buffer

<table>
<thead>
<tr>
<th>Buffer</th>
<th>Composition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lysis buffer</td>
<td>20 mM HEPES pH 7.4; 100 mM KCH$_3$COO; 2 mM Mg(Ch$_3$COO)$_2$; 0.5 mM DTT; 1 mM PMSF; 1x Tm complete; 100 µg/ml CHX</td>
</tr>
<tr>
<td>15%/45% sucrose gradient buffer</td>
<td>20 mM Tris-HCl pH 7.5; 140 mM KCl; 5 mM MgCl$_2$; 100 µg/ml CHX; 0.5 mM DTT; 20 U/µl Superase-In; 7%/47% (w/v) sucrose</td>
</tr>
</tbody>
</table>

8.1.7 Ribosome profiling buffers

<table>
<thead>
<tr>
<th>Buffer</th>
<th>Composition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lysis buffer</td>
<td>20 mM Tris-HCl pH 7.5; 140 mM KCl; 1.5 mM MgCl$_2$; 100 µg/ml CHX; 1% Triton X-100</td>
</tr>
<tr>
<td>7%/47% sucrose gradient buffer</td>
<td>20 mM Tris-HCl pH 7.5; 140 mM KCl; 5 mM MgCl$_2$; 100 µg/ml CHX; 0.5 mM DTT; 20 U/µl Superase-In; 7%/47% (w/v) sucrose</td>
</tr>
</tbody>
</table>

8.1.8 Drop out mix

<table>
<thead>
<tr>
<th>Amino Acid</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>L-alanine</td>
<td>2.0 g</td>
</tr>
<tr>
<td>L-arginine</td>
<td>2.0 g</td>
</tr>
<tr>
<td>L-asparagine</td>
<td>2.0 g</td>
</tr>
<tr>
<td>L-aspartic acid</td>
<td>2.0 g</td>
</tr>
<tr>
<td>L-cysteine</td>
<td>2.0 g</td>
</tr>
<tr>
<td>L-glutamic acid</td>
<td>2.0 g</td>
</tr>
<tr>
<td>L-glutamine</td>
<td>2.0 g</td>
</tr>
<tr>
<td>L-glutamine</td>
<td>2.0 g</td>
</tr>
<tr>
<td>glycine</td>
<td>2.0 g</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Amino Acid</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>L-methionine</td>
<td>2.0 g</td>
</tr>
<tr>
<td>L-histidine</td>
<td>2.0 g</td>
</tr>
<tr>
<td>L-isoleucine</td>
<td>2.0 g</td>
</tr>
<tr>
<td>L-leucine</td>
<td>10.0 g</td>
</tr>
<tr>
<td>L-lysine</td>
<td>2.0 g</td>
</tr>
<tr>
<td>L-phenylalanine</td>
<td>2.0 g</td>
</tr>
<tr>
<td>L-proline</td>
<td>2.0 g</td>
</tr>
<tr>
<td>L-serine</td>
<td>2.0 g</td>
</tr>
</tbody>
</table>

Note: drop out mix was prepared without uracil for URA3 selection, and it was used for the preparation of yeast liquid and solid media (see below)

8.1.9 Trace metal solution

<table>
<thead>
<tr>
<th>Trace metal solution</th>
<th>Composition</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>5 mM EDTA; 2 mM FeSO$_4$; 1 mM MnCl$_2$; 1 mM ZnSO$_4$; 0.1 mM CuSO$_4$</td>
</tr>
</tbody>
</table>

8.1.10 Media

<table>
<thead>
<tr>
<th>Medium</th>
<th>Composition</th>
</tr>
</thead>
<tbody>
<tr>
<td>LB</td>
<td>0.5% (w/v) sodium chloride; 1% (w/v) BactoT tryptone; 0.5% (w/v) BactoT yeast extract</td>
</tr>
<tr>
<td>LB agar</td>
<td>0.5% (w/v) sodium chloride; 1% (w/v) BactoT tryptone; 0.5% (w/v) BactoT yeast extract; 1.5% (w/v) BactoT agar</td>
</tr>
<tr>
<td>YPD</td>
<td>1% (w/v) BactoT yeast extract; 2% (w/v) BactoT peptone; 2% (v/v) dextrose</td>
</tr>
<tr>
<td>YPD agar</td>
<td>1% (w/v) BactoT yeast extract; 2% (w/v) BactoT peptone; 2% (v/v) dextrose; 2% (w/v) BactoT agar</td>
</tr>
<tr>
<td>SC</td>
<td>0.67% (w/v) yeast nitrogen base; 0.2% (w/v) drop out mix; 2% (v/v) dextrose</td>
</tr>
<tr>
<td>SC agar</td>
<td>0.67% (w/v) yeast nitrogen base; 0.2% (w/v) drop out mix; 2% (v/v) dextrose; 2% (w/v) BactoT agar</td>
</tr>
<tr>
<td>SC-:Ura-</td>
<td>0.67% (w/v) yeast nitrogen base; 0.2% (w/v) drop out mix w/o uracil; 2% (v/v) dextrose</td>
</tr>
<tr>
<td>SC-:Ura- agar</td>
<td>0.67% (w/v) yeast nitrogen base; 0.2% (w/v) drop out mix w/o uracil; 2% (v/v) dextrose; 2% (w/v) BactoT agar</td>
</tr>
<tr>
<td>CM</td>
<td>0.67% (w/v) yeast nitrogen base; 2% (v/v) dextrose</td>
</tr>
<tr>
<td>S-medium</td>
<td>100 mM NaCl; 5.7 mM K$_2$HPO$_4$; 44 mM KH$_2$PO$_4$; 13 µM cholesterol; 10 mM tripotassium citrate; 1% (w/v) trace metal solution; 3 mM CaCl$_2$; 3 mM MgSO$_4$</td>
</tr>
<tr>
<td>NG agar</td>
<td>2% (w/v) BactoT agar; 0.25% (w/v) BactoT peptone; 0.3% (w/v) NaCl; 1 mM CaCl$_2$; 13 µM cholesterol; 25 mM KPO$_4$ pH 6.0; 1 mM MgSO$_4$</td>
</tr>
<tr>
<td>RNAi agar</td>
<td>2% (w/v) BactoT agar; 0.25% (w/v) BactoT peptone; 0.3% (w/v) NaCl; 1 mM CaCl$_2$; 13 µM cholesterol; 25 mM KPO$_4$ pH 6.0; 1 mM MgSO$_4$; 100 mg/ml ampicillin; 1 mM IPTG</td>
</tr>
<tr>
<td>HG agar</td>
<td>2% (w/v) BactoT agar; 2% (w/v) BactoT peptone; 0.3% (w/v) NaCl; 1 mM CaCl$_2$; 13 µM cholesterol; 25 mM KPO$_4$ pH 6.0; 1 mM MgSO$_4$</td>
</tr>
</tbody>
</table>
8.1.11 Antibiotics

Ampicillin (AppiChem, USA) 100 µg/ml (stock: 100 mg/ml in dH₂O)
clonNAT (Nourseothricin) (Jena Bioscience) 100 µg/ml (stock: 100 mg/ml in dH₂O)
Kanamycin 50 µg/ml (stock: 50 mg/ml in dH₂O)
Tetracycline (SERVA Electrophoresis GmbH) 10 µg/ml (stock: 10 mg/ml in dH₂O)

8.1.12 Antibodies

<table>
<thead>
<tr>
<th>Antibody/serum</th>
<th>Dilution for WB</th>
<th>Origin</th>
</tr>
</thead>
<tbody>
<tr>
<td>α-Ub antibody</td>
<td>1:1.000</td>
<td>abcam</td>
</tr>
<tr>
<td>α-Rpl17A serum</td>
<td>1:10.000</td>
<td>AG Deuerling</td>
</tr>
<tr>
<td>α-NAC serum</td>
<td>1:10.000</td>
<td>AG Deuerling</td>
</tr>
<tr>
<td>α-RPL10L</td>
<td>1:1.000</td>
<td>Biomol</td>
</tr>
<tr>
<td>α-HA.11 antibody</td>
<td>1:10.000</td>
<td>Covance</td>
</tr>
<tr>
<td>α-mouse IgG (HRP-coupled) antibody</td>
<td>1:10.000</td>
<td>Dianova GmbH, Hamburg</td>
</tr>
<tr>
<td>α-rabbit IgG (HRP-coupled) antibody</td>
<td>1:10.000</td>
<td>Dianova GmbH, Hamburg</td>
</tr>
<tr>
<td>α-CBD antibody</td>
<td>1:10.000</td>
<td>Merck</td>
</tr>
<tr>
<td>α-PGK1 antibody</td>
<td>1:10.000</td>
<td>Novex Life Technologies, USA</td>
</tr>
<tr>
<td>α-GFP antibody</td>
<td>1:5.000</td>
<td>Roche</td>
</tr>
<tr>
<td>α-FLAG® M2 antibody</td>
<td>1:5.000</td>
<td>Sigma-Aldrich Chemie, Steinheim</td>
</tr>
<tr>
<td>α-FLAG® antibody (rabbit)</td>
<td>1:5.000</td>
<td>Sigma-Aldrich Chemie, Steinheim</td>
</tr>
<tr>
<td>α-G6PDH antibody</td>
<td>1:10.000</td>
<td>Sigma-Aldrich Chemie, Steinheim</td>
</tr>
</tbody>
</table>

8.1.13 Kits

MEGAquick-spin™ Total Fragment DNA Purification Kit  INtRON Biotechnology, Korea
DNA-spin™ Plasmid DNA Purification Kit  INtRON Biotechnology, Korea
RNeasy® Mini Kit  Qiagen, Hilden
Quantitect® Reverse Transcript Kit  Qiagen, Hilden
QiAprep® Spin Miniprep Kit  Qiagen, Hilden
QiAgquick® Gel Extraction Kit  Qiagen, Hilden
RNeasy Mini Kit  Qiagen, Hilden
CircLigase™ ssDNA Ligase  epicentre
Dynabeads™ MyOne™ Streptavidin C1  Invitrogen
KAPA HiFi PCR  Kapa Biosystems
Q5® Site-Directed Mutagenesis Kit  New England BioLabs

8.1.14 Oligonucleotides

Cartridge or HPLC-purified synthetic oligonucleotides were obtained from biomers.net unless otherwise indicated. Oligonucleotides were dissolved in nuclease-free H₂O (100 pmol/µl).

8.1.14.1 Primers for cloning

<table>
<thead>
<tr>
<th>P</th>
<th>Name</th>
<th>5' -&gt; 3' sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>01</td>
<td>[SacI]-Not4 prom fwd</td>
<td>TACGGAGCTCAAGCTTCGGTGAGGAAACTG</td>
</tr>
<tr>
<td>02</td>
<td>[NotI]-Not4 prom rev</td>
<td>TACGCGGCGCCTATGACTGGATTATATACG</td>
</tr>
<tr>
<td>03</td>
<td>[NotI]-Not4ΔRING ter fwd</td>
<td>TACGGCGGCCGATGTATGATGACGAGAACGTCAG</td>
</tr>
<tr>
<td>04</td>
<td>3'Not4down(XhoI)</td>
<td>CGACTATGAGGCACGATAGCGCTAAGCTG</td>
</tr>
<tr>
<td>05</td>
<td>[SpeI]-Not4ΔC fwd</td>
<td>TACGACTAGTTCGCCGACTCAAATATTTTTC</td>
</tr>
<tr>
<td>06</td>
<td>Not4ΔC-[SpeI] ter rev</td>
<td>TACGACTAGTTTAAAGCTGAGCGATCTCCCTA</td>
</tr>
<tr>
<td>07</td>
<td>[SpeI]-Not4ΔC-HA rev</td>
<td>TACGACTAGTTTAAAGCTGAGCGATCTCCCTA</td>
</tr>
<tr>
<td>08</td>
<td>[XhoI]-GPDp fwd</td>
<td>TACGACTAGTTTAAAGCTGAGCGATCTCCCTA</td>
</tr>
<tr>
<td>09</td>
<td>GPDp-[AgeI]-[KpnI] rev</td>
<td>TACGACTAGTTTAAAGCTGAGCGATCTCCCTA</td>
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<tr>
<td>10</td>
<td>[AgeI]-ATG-3XFLAG-Rub1</td>
<td>TACGACTAGTTTAAAGCTGAGCGATCTCCCTA</td>
</tr>
<tr>
<td>11</td>
<td>Rub1-[KpnI] rev</td>
<td>TACGACTAGTTTAAAGCTGAGCGATCTCCCTA</td>
</tr>
<tr>
<td>12</td>
<td>Rub1-FusA rev</td>
<td>TACGACTAGTTTAAAGCTGAGCGATCTCCCTA</td>
</tr>
<tr>
<td>13</td>
<td>Rub1-FusB fwd</td>
<td>TACGACTAGTTTAAAGCTGAGCGATCTCCCTA</td>
</tr>
<tr>
<td>14</td>
<td>[SpeI]-Not4ΔRING ter fwd</td>
<td>TACGACTAGTTTAAAGCTGAGCGATCTCCCTA</td>
</tr>
<tr>
<td>15</td>
<td>[NotI]-Ubcl2 NoInt fwd</td>
<td>TACGACTAGTTTAAAGCTGAGCGATCTCCCTA</td>
</tr>
<tr>
<td>16</td>
<td>[SpeI]-Ubcl2 rev L6</td>
<td>TACGACTAGTTTAAAGCTGAGCGATCTCCCTA</td>
</tr>
</tbody>
</table>
17 Rub1 K3R LP_new
CCCTTCTTTTCTCTGCTGAGATGATACGAGATCTGATTCTCTCAGCTCAACAGAGATCTCTCTCCCAGTCAGTGTC
18 Rub1 K1R UP
GAACTTTTGGAGGAAAAAGAAGGGATTCCACCATCTCAACAAAGACTTATATTCCAGGGAA
19 Ntl4-sgRNA-F
TCGCAACTTTCATCGCTGCGTTTTAGAGCTAGAAATAGCAAGT
20 sgRNA-R
CAAGACATCTCGCAATAGG
21 Ntl4-HRT-F (3xFLAG)
CACTTGTTTTCCACGACTATCGCCAAAATGTCTACCTGCAGAGATTATAAAGATCATGATG
22 Ntl4-HRT-R-new
GAAATCAAACTCACTTCCTTATCGCAACTTTCATCGCTACATGTACTCATGCTTCCGCCAGATCCGCCTTTGTATAGTTCGTCCATGC
23 SacI Ntl4 For
GAACGAGCTCATGTCTACCTGCAGCGATGA
24 SpeI Ntl4 Rev
CTATACTAGTTTATCGGTTCATCACAGAAG

8.1.14.2 Depletion primers for ribosome profiling
Oligonucleotides were biotinylated at their 5' end and were a kind gift from the Judith Frydman lab (Stanford University).

<table>
<thead>
<tr>
<th>Name</th>
<th>5' -&gt; 3' sequence</th>
<th>Name</th>
<th>5' -&gt; 3' sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>ScDep1</td>
<td>GGTGCACAATCGACCGATC</td>
<td>ScDep7</td>
<td>CAAGGAGGTTTTTCAATATCAAG</td>
</tr>
<tr>
<td>ScDep2</td>
<td>GTTCTTTACTTATTCGTAAGGCGC</td>
<td>ScDep8</td>
<td>GTCGGGAATATATTAGCAATCG</td>
</tr>
<tr>
<td>ScDep3</td>
<td>TATGATGAGTACGACTATGACGCTC</td>
<td>ScDep9</td>
<td>TGATTAATAGGAGGCGGTGGA</td>
</tr>
<tr>
<td>ScDep4</td>
<td>GGGCGATCTGGCCTTTTTGAGGCCCTG</td>
<td>ScDep10</td>
<td>CCAGGATCGTTTTTGTGGCCAG</td>
</tr>
<tr>
<td>ScDep5</td>
<td>CAGCTTCTAGCTATCGCAAGCCTCC</td>
<td>ScDep11</td>
<td>ATACTGCCAGCTGGAATGAG</td>
</tr>
<tr>
<td>ScDep6</td>
<td>GATGATTCATAATACTTTTCG</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

8.1.14.3 Primers for real-time PCR

<table>
<thead>
<tr>
<th>RT</th>
<th>Gene</th>
<th>Organism</th>
<th>forward (5' -&gt; 3')</th>
<th>reverse (5' -&gt; 3')</th>
<th>amplicon</th>
</tr>
</thead>
<tbody>
<tr>
<td>01</td>
<td>gpd-1</td>
<td>C. elegans</td>
<td>ATGAAGGGAATCCTCGCCTAC</td>
<td>TTCGGGTTTGAGCGAAATGC</td>
<td>110 nt</td>
</tr>
<tr>
<td>02</td>
<td>ntl-4</td>
<td>C. elegans</td>
<td>CTGCAAAGCTGACCATCTG</td>
<td>CCGTCGGACGAAATGCA</td>
<td>87 nt</td>
</tr>
</tbody>
</table>

8.1.15 Plasmids

<table>
<thead>
<tr>
<th>Name</th>
<th>Inserted gene</th>
<th>Promoter</th>
<th>Marker (yeast/ E. coli)</th>
<th>Donor vector</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>pRS316</td>
<td></td>
<td></td>
<td>URA3/ AmpR</td>
<td>(Sikorski &amp; Hieter, 1989)</td>
<td></td>
</tr>
<tr>
<td>pSUMO</td>
<td>6xHis-SUMO</td>
<td>T7</td>
<td>-/KanR</td>
<td>pET24</td>
<td>(Andréasson, Fiaux, Rampelt, Mayer, &amp; Bukau, 2008)</td>
</tr>
<tr>
<td>pRS316-Not4</td>
<td>NOT4</td>
<td>NOT4</td>
<td>URA3/ AmpR</td>
<td>pRS316</td>
<td>AG Deuerling</td>
</tr>
<tr>
<td>pRS316-Not4-HA</td>
<td>NOT4-HA</td>
<td>NOT4</td>
<td>URA3/ AmpR</td>
<td>pRS316</td>
<td>AG Deuerling</td>
</tr>
<tr>
<td>pRS316-Not4-L35A</td>
<td>NOT4 L35A</td>
<td>NOT4</td>
<td>URA3/ AmpR</td>
<td>pRS316</td>
<td>AG Deuerling</td>
</tr>
<tr>
<td>pRS316-Not4ΔRING</td>
<td>NOT4ΔRING</td>
<td>NOT4</td>
<td>URA3/ AmpR</td>
<td>pRS316</td>
<td>This study</td>
</tr>
<tr>
<td>pRS316-Not4ΔC</td>
<td>NOT4ΔC</td>
<td>NOT4</td>
<td>URA3/ AmpR</td>
<td>pRS316</td>
<td>This study</td>
</tr>
<tr>
<td>pRS316-Not4ΔRING-HA</td>
<td>NOT4ΔRING-HA</td>
<td>NOT4</td>
<td>URA3/ AmpR</td>
<td>pRS316-Not4ΔRING</td>
<td>This study</td>
</tr>
<tr>
<td>pRS316-Not4ΔC-HA</td>
<td>NOT4ΔC-HA</td>
<td>NOT4</td>
<td>URA3/ AmpR</td>
<td>pRS316-Not4ΔC</td>
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<td>pRS316-Not4-L35A-HA</td>
<td>NOT4 L35A-HA</td>
<td>NOT4</td>
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<td>pRS316-Not4-L35A</td>
<td>This study</td>
</tr>
<tr>
<td>pRS316-GPdp-GFP-Flag-HIS3-CYC1t</td>
<td>GFP-Flag-HIS3</td>
<td>GPD</td>
<td>URA3/ AmpR</td>
<td>pRS316</td>
<td>AG Deuerling</td>
</tr>
<tr>
<td>pRS316-NΔ</td>
<td>NOT4ΔRING/3XFLAG-RUB1</td>
<td>NOT4i</td>
<td>GPD</td>
<td>URA3/ AmpR</td>
<td>This study</td>
</tr>
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</table>
### Materials and methods

#### 8.1.16 Escherichia coli strains

<table>
<thead>
<tr>
<th>Strain</th>
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<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>DH5α</td>
<td>F&lt;sup&gt;−&lt;/sup&gt;, endA1 hsdR17(rKm&lt;sup&gt;K&lt;/sup&gt;) supE44 thi-1 λ recA1 gyrA96 relA1 deoR, Δ(lacZYA-argF)U169, φ80dgalZAM15 lacIqTetR&lt;sup&gt;R&lt;/sup&gt;</td>
<td>Invitrogen</td>
</tr>
<tr>
<td>OP50</td>
<td></td>
<td>CDC Minnesota</td>
</tr>
<tr>
<td>HT115 (DE3)</td>
<td>F&lt;sup&gt;−&lt;/sup&gt;, mcrA, mcrB, IN髓E1, mc14::Tn10(DE3 lysogen: lavUV5</td>
<td>Open Biosystems</td>
</tr>
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#### 8.1.17 Saccharomyces cerevisiae strains

<table>
<thead>
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</thead>
<tbody>
<tr>
<td>BY4741</td>
<td>MATα; his3Δ1; leu2Δ0; met15Δ0; ura3Δ0</td>
<td>EUROSCARF</td>
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<td>not4Δ</td>
<td>MATα; his3Δ1; leu2Δ0; met15Δ0; ura3Δ0; ∆NOT4::CloNat</td>
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</tr>
<tr>
<td>AFB2</td>
<td>MATα; HIS3::pRS303-ADH-AFB2; leu2Δ0; met15Δ0; ura3Δ0</td>
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<tr>
<td>AID*-NOT4</td>
<td>MATα; leu2Δ0; met15Δ0; ura3Δ0; HIS3::pRS303-ADH-AFB2; 6FLAG-AIDstar-NOT4</td>
<td>AG Deuerling</td>
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<tr>
<td>AID*-NOT4</td>
<td>MATα; leu2Δ0; met15Δ0; ura3Δ0; HIS3::pRS303-ADH-AFB2; 6FLAG-AIDstar-NOT4</td>
<td>AG Deuerling</td>
</tr>
<tr>
<td>AID*-NOT4</td>
<td>MATα; leu2Δ0; met15Δ0; ura3Δ0; HIS3::pRS303-ADH-AFB2; 6FLAG-AIDstar-NOT4</td>
<td>AG Deuerling</td>
</tr>
<tr>
<td>AID*-NOT4</td>
<td>MATα; leu2Δ0; met15Δ0; ura3Δ0; HIS3::pRS303-ADH-AFB2; 6FLAG-AIDstar-NOT4</td>
<td>AG Deuerling</td>
</tr>
<tr>
<td>AID*-NOT4</td>
<td>MATα; leu2Δ0; met15Δ0; ura3Δ0; HIS3::pRS303-ADH-AFB2; 6FLAG-AIDstar-NOT4</td>
<td>AG Deuerling</td>
</tr>
<tr>
<td>AID*-NOT4</td>
<td>MATα; leu2Δ0; met15Δ0; ura3Δ0; HIS3::pRS303-ADH-AFB2; 6FLAG-AIDstar-NOT4</td>
<td>AG Deuerling</td>
</tr>
<tr>
<td>RFA1-AID*</td>
<td>MATα; his3Δ200; leu2-3; lys2-801; trp1-1 (am); URA3::TIR1-9MyC::RFA1-71-114aid-FLAG::hphB</td>
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#### 8.1.18 Caenorhabditis elegans strains

<table>
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<th>Strain</th>
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<td>CDC Minnesota</td>
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<td>FLAG-GFP-tagged ntl-4</td>
<td>This study</td>
</tr>
<tr>
<td>FLAG-GFP::ntl-4 #2</td>
<td>FLAG-GFP-tagged ntl-4</td>
<td>This study</td>
</tr>
</tbody>
</table>

#### 8.2 Methods

##### 8.2.1 Cloning

Standard protocols were used for cloning (Sambrook & Russell, 2001). The plasmids generated in this study were verified by Sanger sequencing provided by GATC Biotech (Germany) according to the service provider's instructions.
8.2.2 Preparation of chemically competent DH5α cells

500 ml LB medium were inoculated with 2.5 ml of an o.n. culture and grown to an OD₆₀₀ 0.4-0.6 at 37°C. Cells were cooled on ice for 10 min prior to centrifugation (15 min 5 krpm 4°C). The pelleted cells were resuspended in 100 ml chilled TFB1 and subjected to centrifugation (15 min 5 krpm 4°C). Cells were resuspended in 20 ml chilled TFB2, aliquoted (100-250 µl) prior to freezing in liquid nitrogen and stored at -80°C.

8.2.3 Polymerase chain reaction (PCR)

For standard PCR reactions 2 µM of each primer, 200 µM dNTP mix (10 mM each), 100-500 ng template DNA, 2% (v/v) DMSO, 1X reaction buffer and 0.5 µl DNA polymerase (Phusion or Pfu) in a total volume of 50 µl were mixed and subjected to PCR-based amplification cycles.

<table>
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<tr>
<th>Step</th>
<th>Temperature (Pfu/Phusion)</th>
<th>Time</th>
<th>Repeats</th>
</tr>
</thead>
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<tr>
<td>Initial denaturation</td>
<td>95°C/98°C</td>
<td>2 min</td>
<td></td>
</tr>
<tr>
<td>Denaturation</td>
<td>95°C/98°C</td>
<td>30 sec</td>
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<tr>
<td>Annealing</td>
<td>55°C</td>
<td>30 sec</td>
<td>30X</td>
</tr>
<tr>
<td>Elongation</td>
<td>72°C</td>
<td>0.5-1 min/kb</td>
<td></td>
</tr>
<tr>
<td>Final elongation</td>
<td>72°C</td>
<td>10 min</td>
<td></td>
</tr>
<tr>
<td>Storage</td>
<td>18°C</td>
<td>∞</td>
<td></td>
</tr>
</tbody>
</table>

8.2.4 DNA purification from agarose gel

DNA fragments were separated by size on 1% or 2% (w/v) agarose gels in 1X TAE by electrophoresis (90V). DNA samples were mixed with 6X loading dye and loaded onto the gel besides 1 kb or 100 bp DNA ladder. DNA fragments were visualized by UV illumination and fragments desired were cut from the gel for further purification by MEGAquick-spin™ Total Fragment DNA Purification Kit according to the manufacturer's instructions. Elution was carried out with ddH₂O.

8.2.5 DNA digestion with restriction endonucleases and dephosphorylation of vectors

For standard DNA digestion by type-II restriction enzymes, 1-5 µg DNA were mixed with the respective RE(s) and 1X reaction buffer according to the manufacturer's recommendations in a total volume of 20 µl. DNA fragments were purified by preparative agarose gel electrophoresis (as described above). Vectors were eluted in 40 µl ddH₂O and subjected to dephosphorylation by FastAP (5U FastAP, 1X AP buffer, total volume: 50 µl) for 30 min at 37°C, followed by heat inactivation (5 min 75°C).

8.2.6 Ligation and transformation of chemically competent DH5α

Ligation reactions were prepared from 100-200 ng vector DNA and equimolar amounts of insert DNA. DNA fragments were mixed with T4 DNA ligase and 10X T4 DNA ligase buffer in a total volume of 20 µl. Incubation was 1-2 hours at RT before heat inactivation (10 min 65°C). 10 µl ligation reaction were used for transformation of 50-100 µl chemically competent cells. After mixing, cells were incubated on ice for 10 min, transferred to 42°C for 90 sec and cooled on ice again for 10 min. 1 ml LB medium was added and phenotypic expression was performed for 1 hour at 37°C. Afterwards, cells were plated on selective media and incubated over night at 37°C.

8.2.7 Transformation of yeast cells

For the transformation of yeast cells, 30 µg heat-denatured Herring sperm DNA was mixed with 1 µg DNA and 100 µl freshly prepared yeast transformation solution; followed by resuspension of one large colony and incubating for 30 min at 37°C. Cells were pelleted (2 min 5 krpm RT), resuspended in 100 µl ddH₂O, plated on selective media and incubated for 48 hours at 30°C.

8.2.8 Cloning of truncated HA-tagged Not4 variants

pRS316-Not4 served as template for PCR-mediated amplification of various fragments (primer P01-P07, Phusion DNA polymerase standard protocol, annealing temperature: 55°C, elongation time: 1 kb/min). pRS316-Not4ΔRING was generated by PCR amplification of the Not4 promoter by primer pair P01/P02, and amplification of NOT4ΔRING and its terminator sequence by primer pair P03/P04. Both fragments were
Materials and methods

cloned into pRS316 via the restriction endonucleases SacI, NotI and XhoI. pRS316-Not4ΔC was generated by PCR amplification of the Not4 promoter and NOT4ΔC by primer pair P01/P06 and amplification of its terminator sequence by primer pair P05/P04. Both fragments were cloned into pRS316 via the restriction endonucleases SacI, NotI and XhoI. pRS316-Not4∆C was generated by PCR amplification of the Not4 promoter and NOT4∆C by primer pair P01/P06 and amplification of its terminator sequence by primer pair P05/P04. Both fragments were cloned into pRS316 via the restriction endonucleases SacI, SpeI and XhoI. The same procedure was applied for pRS316-Not4ΔC-HA, with the exception of the replacement of primer P06 with P07. All further plasmids with HA-tag sequences were cloned by AgeI/XhoI-mediated isolation from pRS316-Not4-HA and integration into pRS316-Not4ΔRING and pRS316-Not4-L35A.

8.2.9 Cloning of Rubylation plasmids

All PCRs were conducted by Phusion DNA polymerase standard protocol. For fusion PCR by overlap extension, an initial template annealing step (55°C 3 min) was added to the protocol before the cyclic amplification steps. pRS316-NΔ and pRS316-UNΔ were cloned according to Table (i) in the order described. Site-directed mutagenesis was carried out to create a RUB1 mutant with four substitutions of codons encoding lysine to arginine (K4R). Cloning was carried out according to Table (ii).

<table>
<thead>
<tr>
<th>Sequence</th>
<th>Primer</th>
<th>RE</th>
<th>Template</th>
</tr>
</thead>
<tbody>
<tr>
<td>GPDp</td>
<td>P08/P09</td>
<td>Xhol/Agef</td>
<td>pRS316-GPDp-GFP-Flag-HIS3-CYC1t</td>
</tr>
<tr>
<td>3XFLAG-RUB1(intron)-RUB1t</td>
<td>P10/P11</td>
<td></td>
<td>gDNA</td>
</tr>
<tr>
<td>RUB1 FusionA</td>
<td>P10/P12</td>
<td></td>
<td>3XFLAG-RUB1(intron)-RUB1t</td>
</tr>
<tr>
<td>RUB1 FusionB</td>
<td>P13/P11</td>
<td></td>
<td>3XFLAG-RUB1(intron)-RUB1t</td>
</tr>
<tr>
<td>NOT4p</td>
<td>P01/P02</td>
<td>SacI/Notl</td>
<td>pRS316-Not4</td>
</tr>
<tr>
<td>NOT4ΔRING-NOT4t (NΔ)</td>
<td>P03/P04</td>
<td>Notl/Xhol</td>
<td>pRS316-Not4</td>
</tr>
<tr>
<td>UBC12</td>
<td>P15/P16</td>
<td>Notl/Spel</td>
<td>gDNA</td>
</tr>
<tr>
<td>NOT4ΔRING-NOT4t (UNΔ)</td>
<td>P14/P04</td>
<td>Spel/Xhol</td>
<td>pRS316-Not4</td>
</tr>
</tbody>
</table>

Table (i): Cloning strategy for pRS316-NΔ and pRS316-UNΔ.

<table>
<thead>
<tr>
<th>Sequence</th>
<th>Primer</th>
<th>RE</th>
<th>Template</th>
</tr>
</thead>
<tbody>
<tr>
<td>GPDp</td>
<td>P08/P09</td>
<td>Xhol/Agef</td>
<td>pRS316-GPDp-GFP-Flag-HIS3-CYC1t</td>
</tr>
<tr>
<td>RUB1K4R FusionA</td>
<td>P08/P18</td>
<td></td>
<td>pRS316-NΔ</td>
</tr>
<tr>
<td>RUB1K4R FusionB</td>
<td>P19/P11</td>
<td></td>
<td>pRS316-NΔ</td>
</tr>
<tr>
<td>RUB1K4R</td>
<td>P08/P11</td>
<td>Agel/Kpn1</td>
<td>RUB1K4R FusionA + RUB1K4R FusionB</td>
</tr>
<tr>
<td>NOT4p</td>
<td>P01/P02</td>
<td>SacI/Notl</td>
<td>pRS316-Not4</td>
</tr>
<tr>
<td>NOT4ΔRING-NOT4t (NΔ)</td>
<td>P03/P04</td>
<td>Notl/Xhol</td>
<td>pRS316-Not4</td>
</tr>
<tr>
<td>UBC12</td>
<td>P15/P16</td>
<td>Notl/Spel</td>
<td>gDNA</td>
</tr>
<tr>
<td>NOT4ΔRING-NOT4t (UNΔ)</td>
<td>P14/P04</td>
<td>Spel/Xhol</td>
<td>pRS316-Not4</td>
</tr>
</tbody>
</table>

Table (ii): Cloning strategy for pRS316-NΔ-Rub1K4R and pRS316-UNΔ-Rub1K4R.

8.2.10 Generation of transgenic worms

The generation of the plasmid pDD162_sgRNA-ntl-4 was carried out by Q5 site-directed mutagenesis kit (primer pair P19/P20, template: pPD61_125-Avi-3XFLAG-GFP-TEV, annealing temperature: 55°C, elongation time: 4 min). The respective repair template for homologous recombination was generated by PCR (primer pair: P21/P22, template: pDD162_sgRNA, Phusion DNA polymerase standard protocol, annealing temperature: 57°C, elongation time: 1 min 10 sec). Plasmids and the PCR amplicon were purified with QIAprep® and QIAquick® kits for optimal purity. Young, adult N2 worms were microinjected with a mix of 50 ng pDD162_sgRNA-ntl-4, 50 ng repair template and 2.5 ng Pmyo2::mCherry::unc54. The first selection of transgenic F1 worms was carried out under the microscope based on the fluorescent emission of mCherry and GFP. Two worms were selected from the F2 generation based on GFP fluorescence.

8.2.11 Generation of the ntl-4 RNAi plasmid

The CDS of ntl-4 was PCR-amplified (primer pair: P21/P22, template: pL4440-C49H3.5, annealing temperature: 68°C, elongation time: 90 sec) and inserted into the ev RNAi plasmid via SacI and SpeI.

8.2.12 Isolation of plasmid DNA from bacteria

Isolation of plasmid DNA from bacteria was carried out with the DNA-spin™ Plasmid DNA Purification Kit according to the manufacturer’s instructions. With the exception of not cooling samples and final elution in H2O.
8.2.13 Isolation of genomic DNA from yeast

For the isolation of gDNA, 1.5 ml of stationary-phase yeast cells were pelleted (30 sec 13.2 krpm RT) and washed in 0.5 ml H₂O. Washed cell pellets were resuspended in 200 µl Yeast gDNA buffer and transferred to a tube containing 200 µl acid-washed glass beads. After addition of 200 µl phenol:chloroform:isoamylalcohol (25:24:1), cells were lysed by FastPrep (4X 20 sec 5 m/s). 200 µl 1XTE buffer was added, followed by centrifugation (8 min 13.2 krpm RT). 150 µl of the aqueous phase were transferred to a new tube and washed in an equal volume of chloroform. After centrifugation (8 min 13.2 krpm RT), the top phase containing genomic DNA was taken and 1 µl was used for PCR amplifications.

8.2.14 Isolation of total RNA from yeast

Isolation of total RNA was carried out on cell pellets obtained from 50 ml exponentially growing cells. Cell pellets were resuspended in 50 µl nuclease-free H₂O and 175 µl RLT buffer (provided by the RNeasy Mini Kit). Resuspended cells were lysed by sonification (8X, Output control level 4, 50% DutyCycle). Cell debris was removed by centrifugation (10 min 13 krpm 4°C). 200 µl lysate were transferred to a new tube and mixed with 600 µl Trizol (incubation 10 min RT) and 120 µl chloroform (incubation 5 min RT). After centrifugation (10 min 13 krpm RT), the aqueous phase was washed with an equal volume of chloroform and subjected to centrifugation again. The aqueous phase was transferred to a fresh tube and mixed with an equal volume of 100% ethanol. The mixture was immediately applied to the RNeasy Mini Kit column and washed according to the provider's manual. Elution of total RNA occurred in 35 µl nuclease-free H₂O.

8.2.15 Isolation of total RNA from worms

For the isolation of total RNA from worms, 20 µl RLT buffer (with 1% (v/v) β-ME) was prepared for each extraction and 50-100 worms collected in it. 330 µl RLT buffer (with 1% (v/v) β-ME) was added to each tube and briefly spun to transfer worms from the lid to the bottom of the tube. Lysis was carried out by sonification (2X10, Output control level 2, 50% DutyCycle). Cell debris was removed by centrifugation (1 min 13 krpm). Lysate was mixed with an equal volume of 70% ethanol. The mixture was immediately applied to the RNeasy Mini Kit column and washed according to the provider's manual. Elution of total RNA occurred in 25 µl nuclease-free H₂O.

8.2.16 cDNA synthesis and real-time PCR

1 µg purified RNA was used for cDNA synthesis by QuantiTect® Reverse Transcript Kit according to the manufacturer's manual in a total volume of 10 µl. The newly synthesized cDNA was then diluted with nuclease-free H₂O according to the total volume required for real-time PCRs. Real-time PCR was conducted in order to determine specific gene expression levels in a semi-quantitative manner. For each condition, three technical triplicates were measured with the CFX Connect™ Real-Time PCR Detection System (Bio-Rad). Each sample consisted of 1 µl freshly prepared cDNA, 3.5 µl nuclease-free H₂O, 5 µl SybrGreen and 0.25 µl of a forward and reverse primer. Samples were prepared on ice. The protocol for real-time PCR is described in the table below.

<table>
<thead>
<tr>
<th>Step</th>
<th>Temperature</th>
<th>Time</th>
<th>Repeats</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial denaturation</td>
<td>95°C</td>
<td>2 min</td>
<td></td>
</tr>
<tr>
<td>Denaturation</td>
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<td>3 sec</td>
<td>35X</td>
</tr>
<tr>
<td>Annealing</td>
<td>60°C</td>
<td>30 sec</td>
<td></td>
</tr>
<tr>
<td>Melt curve</td>
<td>65°C -&gt; 95°C</td>
<td>5 sec</td>
<td>0.5°C/step</td>
</tr>
</tbody>
</table>

For worm samples, gpd-1 served as a house-keeping gene for normalization. This gene encodes glycer-aldehyde-3-phosphate dehydrogenases. Mean Ct values were used for calculation of the fold change with the ΔΔCt method, which was performed according to the following formula:

\[
\text{Fold change} = 2^{\frac{\Delta \Delta \text{Ct}}{\Delta \Delta \text{Ct}}} = 2^{\frac{\text{Ct} (\text{control target gene}) - \text{Ct} (\text{treated target gene})}{\text{Ct} (\text{control reference gene}) - \text{Ct} (\text{treated reference gene})}}
\]
8.2.17 Microarray analysis

Affymetrix RNA microarray (Yeast 2.0) was provided by Haiping Hao and the team at Deep Sequencing and Microarray Core (via Science Exchange). Data evaluation and analysis were performed in R (version 3.4.2), including the libraries simpleaffy, affyPLM and limma provided by Bioconductor. Raw data was gcRMA-normalized.

The GO term finder function of the Saccharomyces Genome Database (https://www.yeastgenome.org), and the search for transcription factors of YeastRACT (http://www.yeastract.com/index.php) were conducted with default settings on differentially expressed genes.

8.3 Handling of model organisms

8.3.1 Cryogenic storage of *E. coli*, *S. cerevisiae*, and *C. elegans*

*E. coli* strains were stored in 10% (v/v) DMSO and *S. cerevisiae* in 20% (v/v) glycerol at -80°C after tubes were flash frozen in liquid nitrogen. For cryo-conservation of worm strains, freshly starved L1-L2 animals were washed from plates with S-buffer and transferred to a fresh tube. The same volume of S-buffer containing 30% (v/v) glycerol was added and gently mixed. 1 ml aliquots were distributed into 1.8-ml vials and slowly cooled to -80°C within a styrofoam box.

8.3.2 Growth conditions for yeast cultures

Liquid cultures were inoculated to OD₆₀₀ 0.1 from an o.n. culture in the respective medium (YPD, SC⁺, SC⁻-Ura) and grown under permanent rotation (130 rpm) at 30°C (if not indicated otherwise). For auxin-mediated protein depletion, 1 mM IAA was added after an initial growth phase of two hours. Cells were depleted for the time period indicated. For amino acid withdrawal, cells were grown in SC⁺ medium for the time indicated, then pelleted (3 min 2.500 rpm RT), resuspended in CM⁺ medium and incubated for 10 min at 30°C and 130 rpm.

8.3.3 Harvest of yeast cultures

Cells were harvested either by centrifugation or vacuum suction through a nitrocellulose filter (Whatman™ 0.45 μm). For centrifugation-mediated harvest, cells were transferred to a 50-ml Falcon tube and spun (2 min 4.4 krpm RT), washed in 1 ml ice-cold H₂O, transferred to a fresh 1.5-ml tube and pelleted again (1 min 13 krpm 4°C), before freezing in liquid nitrogen. Harvest by filter was carried out by vacuum suction through the filter and immediate scraping of the cells from the filter into liquid nitrogen. Harvested yeast cells were stored at -80°C.

8.3.4 Lysis of yeast cells

Lysis of yeast cells was either carried out by FastPrep or cryomill (Retsch MM400 mixer mill). For FastPrep-mediated cell lysis, frozen cell pellets were resuspended in 0.5-1 ml lysis buffer and transferred to 200 µl acid-washed glass beads (3X 20 sec, 5 m/s, kept on ice for 1 min in between). For cryomill-mediated lysis, 0.5-1 ml lysis buffer was flash frozen in liquid nitrogen in form of droplets and mixed with filter-harvested cell material in 25-ml cups (cooled in liquid nitrogen, 30 sec 25 Hz). Crushed cells were then thawed on ice, prior to removal of cell debris. Lysates were cleared for both lysis methods by centrifugation (5 min 13.4 krpm 4°C).

8.3.5 Growth analysis of yeast cells

Overnight-grown yeast cultures were adjusted to OD₆₀₀ 0.4 (±0.02) with H₂O and six five-fold serial dilutions were transferred onto agar plates containing the respective medium with a respective stamp. Plates were incubated as indicated in the results.

8.3.6 Maintenance of worms

Worms were grown on normal growth plates (NG) provided with 100 µl OP50 *E. coli* as a food source. Plates were kept at 15°C and worms were transferred onto fresh plates every 7-14 days.
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8.3.7 Synchronization of worms

Worms were grown on HG plates with 1 ml OP50 as a food source for 2-3 days at 20°C. Animals were washed from the HG plate with 5 ml M9 buffer and collected in a 15-ml Falcon tube. After sedimentation (1 min 1.800 x g RT), worms were treated with 15 ml Alkaline hypochlorite solution for approximately 5 min. Released eggs were centrifuged (1 min 3.000 x g RT) and washed five times in M9 buffer. Pelleted eggs were resuspended in 5 ml sterile H2O, and 5 ml 60% (w/v) sucrose was added. After centrifugation of the mixed solution (5 min 500 x g RT), eggs were transferred from the top to a fresh 50-ml Falcon and washed with M9 buffer. Pelleted (3 min 3.000 x g RT) eggs were resuspended in 15 ml M9 buffer and transferred to a flask for overnight incubation (35 rpm 20°C).

8.3.8 Cultivation of worms in liquid medium and harvest

Eggs obtained from the synchronization procedure (see chapter 8.3.7) were resuspended in 10 ml M9 buffer and transferred to an Erlenmeyer flask with four baffles containing 170 ml S-Medium and 10 ml OP50. Incubation was carried out at 23°C and 75 rpm. Worms were fed with OP50 every 1-2 days on demand. For liquid cultures OP50 were grown in larger scale (1 liter o.n. culture/pellet). Cultures were harvested after eight days of growth. Flasks were put on ice in a 45° angle for 30 min to let worms settle to the ground. After most of the medium has been aspirated off, worms were transferred to a 50-ml Falcon tube on ice and filled up to 50 ml with chilled 0.1 M sodium chloride. Worms were washed in 0.1 M sodium chloride until the supernatant was clear. Worms were then resuspended in 25 ml 0.1 M sodium chloride and 25 ml 60% (w/v) sucrose and immediately centrifuged (5 min 1.500 x g 4°C). Adult worms were collected and transferred to a fresh 50-ml Falcon tube, which was filled up with chilled 0.1 M sodium chloride to 50 ml. After one washing step with 0.1 M sodium chloride (5 min 2.300 x g 4°C), the supernatant was removed, worms were frozen in liquid nitrogen and stored at -80°C.

8.3.9 Lysis of worms

Lysis of worms was either carried out by sonification or cryomill (Retsch MM400 mixer mill). For sonification-mediated lysis see "Isolation of total RNA from worms". For cryomill-mediated lysis, harvested material from liquid cultures was placed in 50 ml cups (cooled in liquid nitrogen, 60 sec 30 Hz). Powder from crushed animals was then stored at -80°C.

8.3.10 RNAi experiments

For RNAi experiments, HT115 cells were transformed with ev RNAi as a control or ntl-4 RNAi as described for DH5α cells (see chapter 8.2.6). Selection was carried out on LB containing ampicillin and tetracycline. RNAi agar plates were prepared and 100 µl IPTG-induced HT115 bacteria were spotted onto plates the day after. For IPTG-induction, LB-amp was inoculated with HT115 bacteria to OD600 0.1 and cells were grown to OD600 0.8 (30°C 130 rpm), prior to addition of 1 mM IPTG for 2 hours. Synchronized L1 animals were placed onto RNAi agar plates on the next day.

8.3.11 Survival rate

The survival of worms in dependence on Ntl-4 was determined by RNAi experiments conducted at 20°C. Synchronized L1 animals were placed onto RNAi plates (ev or ntl-4 RNAi) and incubated for 24 hours. A total of 120 animals were distributed over four plates for each treatment (ev or ntl-4 RNAi). Worms were inspected daily and transferred onto fresh RNAi plates. Plates were changed every other day if reproduction was finalized. Worms were censored if they died of unnatural causes (e.g. dehydration) or were untraceable. RNAi plates were freshly prepared every week. Empirical data was processed into a Kaplan-Meier graph as an estimator of the survival function \( S(t) \) taking censoring into account, with the following function:

\[
S(t) = \prod_{j=1}^{k} \left( 1 - \frac{d_j}{n_j} \right)
\]

where \( d_j \) represents the number of individuals that died at time \( t_j \) and \( n_j \) represents the number of subjects at time \( t_j \).
8.4 Protein techniques

8.4.1 Protein sample preparation from yeast cells
Protein samples were either directly prepared from lysates by mixing lysate with 5X protein sample buffer, followed by heat denaturation at 95°C for 5 min or from cell pellets by NaOH lysis. For the latter, cell pellets were resuspended in 100 µl ddH2O, mixed with 100 µl 200 mM NaOH and incubated for 5 min at RT. Samples were pelleted (1 min 13.4 krpm RT) and pellets resuspended in 25-50 µl 2X protein sample buffer, prior to heat denaturation for 5 min at 95°C. Protein samples were stored at -20°C.

8.4.2 Protein sample preparation from worms
Powder from crushed animals was mixed with 1X SDS protein lysis buffer and thawed on ice. Cell debris was removed by centrifugation (15 min 13.4 krpm 4°C). Lysates were mixed with 5X protein sample buffer, prior to heat denaturation for 5 min at 95°C. Protein samples were stored at -20°C.

8.4.3 Adjustment of protein samples by Bradford assay
Bradford assays were performed to adjust protein samples to the same absorption at 595 nm. 1 ml 1X Bradford reagent was mixed with 1 µl sample at RT, prior to measurement of the absorbance at 595 nm. The resulting absorption value was then divided by 0.06 to receive an approximate value for the protein concentration (µg/µl). Samples were adjusted accordingly.

8.4.4 Adjustment of protein concentration by BCA assay
BCA assays were performed to determine the protein concentration in samples more accurately. 1 µl sample was mixed with 49 µl lysis buffer and 950 µl freshly prepared BCA solution (BCA mixed with 0.08% (w/v) copper(II) sulfate). Sample incubation (15 min 60°C 500 rpm) was followed by fast cooling on ice and measurement of the absorbance at 562 nm. A standard curve was determined as well by measurement of BSA samples of defined concentration.

8.4.5 Co-immunoprecipitation (Co-IP)
For Co-IP experiments, the protein concentration of lysates was adjusted according to BCA assay-determined protein concentrations and subjected to incubation with magnetic beads for 1 hour at 4°C while gently rotating. Magnetic beads were either already coupled to α-FLAG (Anti-FLAG® M2 Magnetic Beads) or Protein G Magnetic Beads, adjusted lysate and antibodies (1:100/1:200) were directly mixed. If preclearing with Protein A Magnetic Beads was included, lysates were previously incubated for 1 hour at 4°C while gently rotating. For each Co-IP 30 µl magnetic beads were prepared by washing four times in low salt washing buffer and final resuspension in the same buffer. After Co-IP incubation, beads were washed two times with high salt washing buffer and three times in low salt washing buffer. Native elution from beads was carried out by addition of 200 µg/ml 3XFLAG® Peptide in low salt washing buffer for 30 min at 18°C and 750 rpm. Denaturing elution was carried out by addition of 2X protein sample buffer and cooking samples (10 min 99°C 900 rpm).

8.4.6 Tandem Affinity Purification (TAP)
TAP experiments were adapted from Puig et al., 2001 and further modified. Cells were grown to an OD600 of 1, filter harvested and lysed by cryomill. The obtained powder was thawed in Lysis buffer, cell debris removed by centrifugation (30 min 30,000 x g 4°C) and samples adjusted by Bradford assay. Cleared, adjusted lysates were applied to 300 µl IgG Sepharose™ 6 FastFlow per sample (equilibrated in Washing buffer I) and incubated (2-3 h 4°C while rotating). Beads were washed three times in Washing buffer I, followed by equilibration in Washing buffer II. Beads were transferred to a 5-ml column and washed three times in Washing buffer II. Beads were incubated o.n. at 4°C with 50 µg TEV protease in 1.5 ml Washing buffer II. TEV-cleaved protein complexes were eluted into 300 µl Calmodulin Affinity Resins (equilibrated in Washing buffer III). IgG beads were washed with 0.5 ml Washing buffer II. 6 ml Washing buffer III and additionally 1 mM calcium chloride were added to the Calmodulin beads and incubated (2 h 4°C while rotating). Beads were washed with Washing buffer III before being transferred into fresh tubes and subjected to denaturing elution (2X 100 µl 2X protein sample buffer 5 min 99°C 900 rpm).
8.4.7 b-isox precipitation

For b-isox-mediated precipitation cryomill-crushed powder was thawed in EE buffer and extracted for 20 min at 4°C while rotating. Cell debris was removed by centrifugation (15 min 13.4 krpm 4°C). Samples were adjusted by Bradford assay and divided according to the number of different treatments. DMSO was included as a control, as it served as the diluting agent for b-isox. Samples were incubated with DMSO or b-isox for 90 min at 4°C while rotating. Pelleting and washing steps were performed by centrifugation (10 min 10,000 x g 4°C). Pellets were washed twice in EE buffer before resuspension in 2X protein sample buffer and heat denaturation (5 min 95°C).

8.4.8 Polysome profiling

Polysome profiles were performed as described by Koplin et al., 2010 with the following adaptations. Yeast cells were grown up to OD<sub>600</sub> 1 and lysed by cryomill. Powders were thawed in lysis buffer and adjusted based on the absorption at 260 nm. 500 µl adjusted sample (50 A<sub>260</sub> units) was loaded onto 11 ml of a linear 15-45% sucrose gradient and centrifuged (2.5 hours 39 krpm 4°C). Fractionation was performed from top to bottom with a piston gradient fractionator (Biocomp). A<sub>254</sub> signals were recorded and 500 µl fractions collected. For protein analysis collected fractions were directly mixed with 5X protein sample buffer and heat denatured (5 min 95°C).

8.4.9 Ribosome profiling

Ribosome profiling was performed as described by Ingolia, Ghaemmaghami, Newman, & Weissman, 2009, including protocol sections Extract, Ribosome Footprinting, RNA-Size Selection, cDNA Synthesis and Sequencing with the following adaptations. Cells were harvested without the addition of cycloheximide and lysed via cryomill once for 1 min at 20 Hz. Buffers were composed as described above. 20 A<sub>260</sub> units were subjected to digestion with 300 U RNase I. Ultracentrifugation was carried out for 2.5 hours at 39 krpm at 4°C. Monosome fractions were pooled and ribosome footprints were isolated by the hot acid phenol method. A TBE-Urea gel was run before dephosphorylation for size selection of ribosomal footprints. The dephosphorylation reaction was not heat-inactivated but directly subjected to RNA precipitation. A micro-RNA-linker was ligated to RNA fragments prior to cDNA synthesis. Circularization reactions were performed for 2 hours and were followed by rRNA depletion. rRNA depletion was carried out on Dynabeads MyOne after cDNA samples were mixed with a depletion master mix of biotinylated oligonucleotides (ScDep1-11). Incubation was performed for 15 min at 37°C and was followed by precipitation of the rRNA-depleted flow through. Sequencing and data analysis was carried out by Justin Chartron (Judith Frydman lab, Stanford University, Pechmann, Chartron, & Frydman, 2014).

8.4.10 bis-tris-PAGE

7.5% or 10% bis-tris gels were prepared with 1 mm thickness, comprising a stacking and separation gel. Protein samples were heat-denatured (5 min 95°C), loaded and run at constant 90V (stacking gel) and 150V (separation gel) in 1X MOPS running buffer. PageRuler™ Prestained Protein Ladder was loaded on each gel for determination of the molecular weight. After separation of protein samples, gels were subjected to Coomassie staining, silver staining or Western blot analysis.

8.4.11 Coomassie staining

Gels were covered with Coomassie R<sub>250</sub> staining solution for at least two hours under continuous shaking. And placed in Coomassie R<sub>250</sub> destaining solution until protein bands were visible. Gels were rinsed in <sub>dd</sub>H<sub>2</sub>O be-fore documentation.

8.4.12 Silver staining

Gels were fixed in Silver staining solution I for at least one hour while gently shaking. Gels were rinsed in 50% (v/v) methanol for 12 min and twice in <sub>dd</sub>H<sub>2</sub>O for 5 min. Gels were immersed in Silver staining solution III for 60 sec, followed by two rinses with <sub>dd</sub>H<sub>2</sub>O for 1 min. Gels were then immersed in Silver staining solution II for 25 min at 4°C while gently shaking. Gels were rinsed three times for 20 sec in <sub>dd</sub>H<sub>2</sub>O, followed by application of Silver stain solution IV until protein bands appeared. The development of the gels was stopped by im-
mersing gels in Silver staining solution I for 5 min. Gels were rinsed in \( \text{ddH}_2\text{O} \) three times for 1 min before documentation.

8.4.13 Western blot and immunostaining

Gels were transferred onto a nitrocellulose membrane (GE Healthcare) in a wet blot chamber with 1X Western blot transfer buffer and 100V were applied for 1 hour in order to transfer proteins onto the membrane. Proteins were fixed by incubation in Ponceau S for 5 min. Membranes were blocked in 5% (w/v) milk powder in 1X TBS-T for 1 hour. Membranes were incubated with primary antibodies diluted in 1X TBS-T o.n. (sera and polyclonal antibodies were diluted in 1X TBS-T including 3% (w/v) milk powder). Afterwards membranes were washed three times with 1X TBS-T for 10 min at RT. Secondary antibodies were applied for 2 hours at RT diluted in 1X TBS-T. After membranes were washed as before, antibody binding was detected using a Fusion SL (peqlab) chemiluminescence imaging system with freshly prepared ECL solution.

8.5 Microscopy

For microscopy worms were anesthetized with a drop of 25 mM levamisole (in M9 buffer) and placed onto a thin pad of 3% agarose on a microscope slide. Fluorescence and transmitted light images were taken with a confocal laser-scanning microscope (Leica TCS SP8). Two different microscope objectives were used in this study: 5x dry (numeric aperture: 0.15) or 63x water (numeric aperture: 1.2).
9. References


References


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10. Acknowledgments

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11. Appendix

11.1 Appendix introduction

11.1.1 The different cell types, mating and cellular differentiation of *S. cerevisiae*

*Saccharomyces cerevisiae* can proliferate as three different cell types, either as two distinct haploid cell types of different mating type (*MATα* or *MATα*), or as diploids heterozygous for both mating types (*MATα/MATα*) (*Figure 41 A*). Reciprocal transitions between the haploid and diploid states can occur. Two haploid cells of opposing mating type can fuse into a single diploid cell by mating. Diploid cells can bring forth haploid cells via an intermediate, the ascospore, which contains four haploid spores that germinate into haploid cells. Vegetative growth of cells in haplo- or diplophase is carried out by a process called budding, in which a bud protrudes and grows from the mother cell, and yields a smaller daughter cell after cell separation.

![Diagram](image)

*Figure 41*: Mating of *Saccharomyces cerevisiae*. (A) Transition between haplophase and diplophase. Diploid cells can undergo a meiotic division (MD) and form an ascospore comprising four haploid spores, from which haploid cells germinate. Haploid cells of opposing mating types can fuse and mate to generate the diploid cell type. Both, diploids and haploids, can proliferate through vegetative growth (VG). (B) Chromosome III of *Saccharomyces cerevisiae* contains the genetic information required for determination of the mating type.

Haploid yeast cells are able to change mating types and strains capable of mating-type interconversion are called homothallic, in contrast to heterothallic yeast strains incapable of switching mating types (*Figure 41 B*). The latter is the case for the widely used laboratory strain S288C, in which loss-of-function mutations in the required HO endonuclease prevent mating-type interconversion. The yeast genome carries three genetic loci on chromosome III relevant for mating-type intercon-
version and phenotypic expression of the mating type. The \textit{MAT} locus dictates the mating type of a cell and exists as one of two forms, either \textit{MATa} or \textit{MATa}. Additionally, chromosome III carries two so-called \textit{hidden MAT} loci (\textit{HML}) at each end (\textit{HML} for left, \textit{HMR} for right). These \textit{hidden MAT} loci carry the genetic information necessary for both mating types (\textit{HML} for mating-type \textit{a}, \textit{HMR} for mating-type \textit{a}) but are subjected to silencing. They serve as templates for the mating-type interconversion which is mediated by the \textit{HO} endonuclease. The genotype conferred by the \textit{MAT} locus effects phenotypic differences between both mating types, and results in differential gene expression of a subset of \textit{a}- and \textit{a}-specific genes. \textit{MATa} comprises two genes, \textit{MATa1} and \textit{MATa2}, encoding two transcriptional regulators. \(a1\) is required for the activation of \textit{a}-specific gene expression, whereas \(a2\) acts as a repressor of \textit{a}-specific genes. Both transcription factors act in conjunction with the sequence-specific DNA-binding transcription factor Mcm1, and regarding gene activation, the transcription factor Ste12. \textit{MATa} also comprises two genes, \textit{MATa1} and \textit{MATa2}, encoding the transcription factor \(a1\) and a protein of unknown function, \(a2\). Except that \(a2\) is dispensable for mating in haploids and sporulation in diploids (Dranginis, 1989) (Figure 42).

\textit{MATa}:

\textit{MATa}:

\textit{MATa} encodes the transcription factor \(a1\) responsible for the expression of \textit{a}-specific genes. \textit{MATa} encodes the transcriptional activator \(a1\) which is involved in the regulation of \textit{a}-specific genes. The transcriptional repressor \(a2\) is responsible for the repression of \textit{a}-specific genes.

\(a1\) positively regulates gene expression of \textit{a}-specific genes in conjunction with Mcm1 and Ste12. In \textit{MATa} cells, \textit{a}-specific genes are repressed in a \textit{MAT}-independent manner by a repressor complex.
consisting of the NAD⁺-dependent histone deacetylase Hst1, the transcriptional repressor Sum1, and Rfm1, which tethers Hst1 to Sum1 (Zill & Rine, 2008). Additionally, this complex is also required for silencing of HMR and HML mediated by the origin recognition complex (ORC).

The most important genes regulated in a MAT-dependent manner are involved in the intercellular communication, which answers the question of how yeast cells of opposing mating type recognize each other (Figure 43).

Figure 43: Overview of mating of two cells of opposing mating types. First, the presence of an appropriate mating partner is sensed via specific receptors, which are exposed on the surface of the respective cell, and secreted mating pheromones. Second, structural rearrangements lead to the formation of so-called shmoos, for two cells to make contact. Third, the two haploids fuse to form a diploid cell.

Both mating types express and secrete small α- and a-specific polypeptides, which act as pheromones, and bind to mating type-specific G-protein coupled receptors (GPCR) presented on the surface of a cell. Accordingly, MATa cells express and secrete the pheromone mating factor α (encoded by the genes MF(alpha)1 and MF(alpha)2), and express STE3, the mating factor a-specific receptor, whereas MATα cells express and secrete the pheromone mating factor α (encoded by the genes MFa1 and MFa2), and express STE2, the mating factor α-specific receptor. Another important question regarding mating is how do they get together, if yeast cells are incapable to actively move through their environment? This is facilitated by so-called shmoo formation, where cells make contact across a short distance through enormous reorganization of the cell wall and protrusions toward on another. The site of shmoo formation is spatially controlled by the local binding of the respective pheromone on the surface. Furthermore, mating-type specific genes are destined to decrease the sensitivity of cells toward pheromones. For example the aspartyl protease Bar1 is secreted by MATa cells. Bar1 cleaves α-pheromone and thereby increases the threshold to be overcome to activate a mating response, furthermore it modulates the pheromone gradients formed by cells to improve the directionality of pheromone sent by a putative mating partner (Barkai, Rose, & Wingreen, 1998). The binding of pheromone to the appropriate receptor causes the activation of
the mating response, a well-studied mitogen-activated protein kinase (MAPK) pathway (reviewed by Chen & Thorner, 2007).

### 11.1.2 The mating response pathway

![Diagram of the MAPK-dependent mating response pathway](image)

**Figure 44:** Overview of the MAPK-dependent mating response pathway exemplified by MATα cells.

*Figure 44* depicts the mating response in MATα cells, the same holds true for MATα cells with the exception that a-pheromone would bind to the GPCR Ste3. Binding of pheromone to the appropriate GPCR enables the receptor to act as a guanine nucleotide exchange factor (GEF) for its associated heterotrimeric G protein, comprising Gαβγ. The exchange of GDP to GTP in the inhibitory Gα-subunit triggers the release of the plasma membrane-anchored Gβγ heterodimer and subsequently allows for new protein-protein interactions to be established. Gβγ contacts Far1, a cyclin-dependent kinase inhibitor (CKI), the scaffolding protein Ste5, and the p21 activated protein kinase (PAK) Ste20. Far1 makes further contacts with the GEF Cdc24, which is associated to the inner plasma membrane, and localizes near the PAK Ste20. The protein-protein interactions made by Gβγ with Far1, Ste5, and Ste20 enable the encounter of the plasma-membrane anchored GTPase Cdc42 with its GEF Cdc24, which in turn leads to the activation Ste20. Activation of Ste20 is followed by signal transmission through the sequential activation of the MAPKKK Ste11, the MAPKK Ste7, and finally the MAPK Fus3. The scaffolding protein Ste5 provides an interaction platform to bring Gβγ, Ste11, Ste7, and Fus3 into close proximity, and thus ensures high efficacy of the signaling cascade.
Activation of the mating response pathway has three major outcomes: first, cell cycle arrest at START. Second, transcriptional activation of mating-responsive genes and third, morphological changes concerning the cytoskeleton and cell wall. Activated Fus3 facilitates all three by phosphorylation of the key factors Far1, Dig1/2, Ste12, Tec1, and Bni1. Fus3-phosphorylated Far1 leads to cell cycle arrest at START in the G1 phase. In the absence of pheromone, Far1 is subjected to Cdk28-Cln2-mediated phosphorylation, which leads to its degradation. However, Fus3-mediated phosphorylation stabilizes Far1 and unleashes its function as a CKI by binding and inactivation of Cdk28-Cln2 complexes (Peter, Gartner, Horecka, Ammerer, & Herskowitz, 1993).

Transcriptional activation of genes required for mating is facilitated by the transcription factor Ste12, which acts as homodimer and is repressed under unstimulated conditions by the binding of the repressors Dig1 and Dig2. Fus3 phosphorylates Ste12, Dig1/2, and another transcription factor required for the activation of a mating-independent differentiation program, Tec1. The activation of Ste12 is mostly facilitated by the release from its repressors, Dig1/2 (Tedford, Kim, Sa, Stevens, & Tyers, 1997). The specificity for pheromone-responsive genes is mediated by the phosphorylation of Tec1, which is subsequently being degraded. Some pheromone-responsive genes carry a so-called pheromone response element (PRE) in their promoter, which facilitates strong Ste12-binding. The performance of Ste12 is fine-tuned by immediate destruction of Ste12 after transcriptional activation. Ste12 is phosphorylated by the cyclin-dependent kinase Cdk8, and degraded in an ubiquitin-dependent manner, which is indicated by decreasing Ste12 protein levels upon pheromone stimulation (Esch et al., 2006; Nelson et al., 2003). This ensures that transcription of mating-responsive genes is not only activated in a stimulus-dependent manner, but appropriately inactivated as well.

The third relevant process involves the Fus3-dependent phosphorylation of the formin Bni1. Phosphorylated Bni1 interacts with GTP-bound Cdc42 and is recruited to the tip of the mating projection as a component of the polarisome, where it promotes actin filament assembly. Polarized growth and cell fusion is dependent on Bni1, and both processes finally facilitate the formation of a diploid cell from two haploid cells. The combined action of both MAT loci in diploid cells is required for the repression of α- and a-specific genes, as well as other haploid-specific genes. The repression of α- and haploid-specific genes is carried out by the joint activity of a1 and a2. It involves among other genes, central effectors of the mating response pathway, like the genes encoding the heterotrimeric G-protein (GPA1, STE4, STE18), the scaffolding protein (STE5), MAP kinase (FUS3), and the CKI (FAR1). Whereas the repression of a-specific genes is carried out in the same manner as in haploid MATα cells. Diploid cells can perform a meiotic division resulting in an ascospore. This process called sporulation is induced if cells encounter an environment that lacks the combination of sufficient nitrogen and carbon sources (Figure 41 A).

11.1.3 The filamentous growth response pathway

To go back to the initial figure (Figure 1), a cell’s decision to either proliferate or differentiate strongly depends on the composition of nutrients in the surrounding environment, and additionally on the presence of mating pheromone in haploid cells. Furthermore, the availability of a nitrogen and/or carbon source in sufficient quantity and of preferred quality are the major determinants for decision making. The major purpose of cellular differentiation serves survival and is established in diploids by sporulation, in case other milder forms of adaptations are futile. Under less severe nutritional conditions, cells can undergo filamentous instead of vegetative growth. Filamentous growth is characterized by a unipolar pattern of growth. Thereby, cells can overcome a distance despite
their non-motile existence to reach a site where nutrients are available, at least for their progeny. Induction of filamentous growth is characterized by changes in cell adhesion, polarity, and shape, and constitutes the prerequisite for pathogenic yeast species in order to be able to invade their respective hosts. Filamentous growth is also designated as pseudohyphal growth in budding yeast, because cells completely separate by cytokinesis, but stay attached to each other through proteins in their cell walls. Furthermore, filamentous growth is the term usually applied to describe the process of pseudohyphae formation in diploids, whereas in haploids it is mostly designated as invasive growth (reviewed by Cullen & Sprague, 2012). Despite this distinction in terminology, the cellular differentiation is controlled by the same pathways in both cell types. The switch from budding, or yeast-form growth to filamentous, invasive growth is dependent on the capacity to sense the availability of nutrients intra- and extracellularly. The concerted action of several signaling pathways finally carries out the activation of genes involved in cell polarity and morphological reconstruction.

![Diagram of filamentous growth response pathway](image)

*Figure 45: Regulation of the filamentous growth response pathway through cAMP-PKA signaling and MAPK-dependent signaling. cAMP-PKA activity impinges on gene activation through the transcription factors Flo8 and Sfl1. The MAPK-dependent gene activation is carried out through inactivation of Dig1/Dig2-dependent repression of Ste12, and activation of the transcription factors Ste12 and Tec1, which in concert activate gene expression. Black arrows indicate known molecular interactions, while gray arrows represent links of indicated interaction for which the exact mechanism is unknown. STRE means stress response element, FRE means filamentous response element.*

*non-functional genes in S288C
Filamentous growth is mainly regulated via the Ras-cAMP-PKA and a specific MAPK pathway, further signaling pathways capable to cause filamentous growth include the SNF and TOR pathways (Figure 45). One common gene expression target of all four pathways is the gene FLO11, encoding a flocculin protein required for cell aggregation via flocculation. The expression of the FLO11 gene is affected by different transcriptional regulators, and the combination of all events regulated by the different pathways contributes to the full realization of filamentous growth. There is extensive crosstalk between the pathways, and in principle, they all have in common that they are involved in sensing the nutritional status and evoke a proper cellular response accordingly. The MAPK pathway specified on the regulation of filamentous growth shares many components with the previously introduced mating response pathway. Relevant, shared pathway components include GTPase Cdc42, PAK Ste20, and MAPKKK Ste11 and MAPKK Ste7. Activation of the MAPK-dependent filamentous growth pathway (FG pathway) requires the transmembranous heterooligomeric complex formed by Sho1 and Msb2. Msb2 is highly O-glycosylated on its extracellular domain, which probably serves as a sensor of intracellular glucose availability, by which Msb2 activity is regulated. Deletion of this domain leads to the activation of the MAPK pathway independent on glucose availability (Cullen et al., 2004). The final MAPK, specific to the FG pathway, is Kss1. Activated Kss1 regulates the transcriptional activation of FG target genes by phosphorylation of Ste12, and derepression of Ste12 by phosphorylation of the repressor Dig1. In contrast to activated Fus3, Kss1 activity leaves the TEA transcription factor Tec1 unaffected. In the context of the FG pathway, Tec1 mediates DNA-binding to promoters of filamentation genes carrying a filamentous and invasion-responsive element (FRE) or a TEA consensus sequence (TCS). In addition, Ras-cAMP-PKA signaling can provide for input into the FG pathway via activated Ras1/2, and signal transmission occurs on the level of Cdc42. It can also directly impinge on filamentation and invasion, by direct activation of the flocculin-specific transcription factor Flo8 by Tpk2-dependent phosphorylation.

11.1.4 Ras-cAMP-PKA pathway

The presence of glucose is mainly sensed by the Ras-cAMP-PKA signaling network (Figure 46), and is quantitatively sensed both, extracellularly and intracellularly. Yeast cells express different hexose transporters (Hxts) which differ in their affinity toward glucose. Glucose transporters are expressed according to the concentration of glucose in the environment to ensure a steady inflow into the cell, correspondingly, low-affinity transporters are expressed under conditions of high extracellular glucose and high-affinity transporters under conditions of low extracellular glucose. Glucose influx stimulates the exchange from GDP to GTP in the GTPases Ras1 and Ras2, which is mediated by the GEFs Cdc25 or Sdc25 and can be counteracted by the GAPs Ira1 and Ira2. Extracellular glucose levels are additionally recognized by the transmembranous protein Gpr1, which in turn acts as a GEF for the GTPase Gpa2. Both activated GTPases, Ras1/2 and Gpa2, stimulate the adenylate cyclase encoded by CYR1, which catalyzes the reaction from ATP to the second messenger cAMP. Next, cAMP initiates the activation of protein kinase A (PKA) by interaction with the inhibitory subunit Bcy1 and release of active PKA (encoded by TPK1, TPK2, or TPK3). PKA consists of a heterotetramer build from two units of the regulatory Bcy1 subunit, and a homodimer formed from one of the three catalytic Tpk subunits. The three Tpk proteins display overlapping and specific functions.
Neutralization of cAMP to AMP is catalyzed by the phosphodiesterases 1 or 2 (Pde1/2), which as part of a negative feedback loop is stimulated by active PKA. Active PKA negatively regulates the kinase Rim15, which otherwise would activate the transcription activators Gis1 and Msn2/4, responsible for the gene expression of post-diauxic shift-related genes (PDS) or stress-responsive genes, respectively. Rim15 furthermore stimulates the transcriptional repressor Xbp1, which can facilitate cell cycle arrest by the repression of cyclin genes. Furthermore, PKA can directly affect Msn2/4 activity by negatively regulating its nuclear localization.

11.1.5 SNF pathway

Yeast cells are capable to grow on diverse carbon sources. Glucose is the carbon source that can immediately enter glycolysis. Yeast cells first metabolize glucose by fermentation before they rely on other carbon sources present, for example ethanol or glycerol. In the presence of glucose, genes required for respiration are transcriptionally repressed, in a process accordingly called glucose repression. Expression of these genes is positively regulated by the SNF pathway, which itself is activated when the glucose concentration declines. One way in which SNF signaling is regulated is by crosstalk with the Ras-cAMP-PKA pathway (Figure 46). Active PKA can inhibit the activation of the Snf1 kinase. This was demonstrated by genetic interactions between pathway

Figure 46: Overview of the relevant Ras-cAMP-PKA, TOR, and SNF signaling networks and the crosstalk between the respective signaling pathways.
members of both pathways, and more directly by the PKA-dependent phosphorylation of Snf1-activating kinase Sak1, which reduced SNF activity (Barrett, Orlova, Maziarz, & Kuchin, 2012).

Under conditions of declining glucose levels, SNF is activated by phosphorylation and can form three distinct complexes, all of which include the catalytic α-subunit Snf1 and the regulatory γ-subunit Snf4, as well as one of three β-subunits (Gal83, Sip1, or Sip2). The β-subunits tether the α- and γ-subunits together, and confer specific subcellular localization of the SNF complexes. Gal83 mediates localization to the nucleus, Sip1 to the vacuolar membrane, and Sip2-containing SNF complexes remain in the cytosol. Nuclear SNF acts on diverse transcriptional regulators by phosphorylation. It activates the transcription activators Hsf1 and Msn2 to evoke a stress response, the transcription activators Cat8 and Sip4 to positively regulate the expression of gluconeogenesis-associated genes (frequently carrying a carbon source-response element, CSRE), and prevents the nuclear localization of Mig1, which acts as a repressor on glucose-repressed genes (Shashkova, Wollman, Leake, & Hohmann, 2017).

It was already mentioned that the SNF signaling pathway can play a role in the activation of filamentous growth. Activated Snf1 negatively regulates four transcriptional repressors involved in the execution of glucose repression via recruitment of the global co-repressor Cyc8-Tup1. The transcription factors Mig1, Mig2, Nrg1, and Nrg2 carry out the transcriptional repression of glucose-repressed genes under conditions of sufficient glucose levels. Activation of the SNF pathway leads to the inhibition of these proteins by different, mostly unknown mechanisms. Snf1-dependent phosphorylation of Mig1 leads to its export from the nucleus to the cytoplasm. The exact mechanism by which Mig1/2 activate the FG pathway by Snf1-dependent localization to the cytoplasm is still unknown, but it is clear that Mig1/2 can directly interact with some FG pathway components (Kss1 and Ste7), and thus might increase MAPK signaling (Karunanithi & Cullen, 2012). Snf1 activity-dependent induction of filamentous growth is also carried out via direct interaction and negative regulation of Nrg1/2 (Kuchin, Vyas, & Carlson, 2002).

11.1.6 TOR pathway

TOR signaling spatiotemporally regulates cell growth in response to nutrients (reviewed in Loewith & Hall, 2011). In contrast to all other eukaryotes, Saccharomyces cerevisiae harbors two, instead of one, TOR genes, named TOR1 and TOR2 (Figure 47). The following paragraph will briefly explain the major insights into TOR signaling on the basis of the model organism Saccharomyces cerevisiae. TOR functions are realized through two complexes containing the Tor kinase. TORC1 and TORC2 differ in their composition, subcellular localization, and the signaling inputs and outputs, by which they are controlled and which they control. In yeast, TORC1 contains either Tor1 or Tor2 kinase, and is additionally characterized by Kog1 (called Raptor in mammals), Tco89, and Lst8. It is constitutively localized on the vacuolar membrane, and regulates cell growth positively and stress responses negatively. TORC1 inhibits several transcription factors by affecting their subcellular localization. These include transcriptional activators of the nitrogen catabolite-repressor, Gat1 and Gln3 (Beck & Hall, 1999), the stress-responsive transcriptional activators Msn2 and Msn4 (Monteiro & Netto, 2004), the transcriptional activators of the retrograde response, Rtg1 and Rtg3 (Dilova, Aronova, Chen, & Powers, 2004), and finally transcription factors regulating ribosome biosynthesis, including Crf1, Fhl1, and Spf1 (Marion et al., 2004; Martin, Soulard & Hall, 2004).
Figure 47: Composition and summarized information about the two TOR complexes in *Saccharomyces cerevisiae*.

TORC1 involvement in ribosome biogenesis is not restricted to RNA polymerase II-dependent transcription of ribosomal protein genes, but rather regulates all transcriptional events required for ribosome biogenesis, including RNA polymerase I- and III-dependent transcription of rRNAs and tRNAs. This is carried out in part by signaling through the cAMP-PKA pathway and its target kinase Yak1 (*Martin et al.*, 2004). The extensive crosstalk between TOR and PKA signaling pathways is no surprise given that they both report on the nutrient/energy availability and as a consequence promote cellular growth and simultaneously inhibit stress responses (*Soulard et al.*, 2010). Accordingly, TORC1 activity is nutrient-responsive, in contrast to TORC2. TOR was identified as the target of rapamycin, an immunosuppressant, which specifically inhibits the function of TORC1. The TORC2 comprises Tor2, Avo1, Avo2, Avo3/Tsc11, Lst1, and Bit61 or its paralog Bot2. It is localized on the plasma membrane and involved in the regulation of cellular polarization processes, regulation of the actin cytoskeleton, and endocytosis. In summary, TORC1 temporally controls cellular growth, whereas TORC2 is required for spatial control.
11.2 Appendix compact disc

A separate compact disc is attached to this doctoral thesis labeled “appendix”, it comprises the following files:

1. File “Ribosome_profiling_data.xlsx”
   This file contains three tables with ribosome profiling data of wild-type and \textit{not4Δ} cells. The first sheet comprises the comparison of read densities measured for wild-type and \textit{not4Δ} cells (NOT4 vs WT). The second and third sheet contain all reads for both replicates of wild-type (WT Rep1+2) and \textit{not4Δ} (NOT4 Rep 1+2).

2. File “Microarray_data_Not4-depleted.xlsx”
   This file contains the complete table of the 226 ORFs which displayed at least two-fold differential expression in Not4-depleted cells compared to non-depleted cells.

3. File “Microarray_data_amino_acid_non-depleted.xlsx”
   This file contains the complete table of the 939 ORFs which displayed at least two-fold differential expression in non-depleted cells upon amino acid withdrawal compared to amino acid-rich conditions.

4. File “Microarray_data_amino_acid_Not4-depleted.xlsx”
   This file contains the complete table of the 568 ORFs which displayed at least two-fold differential expression in Not4-depleted cells upon amino acid withdrawal compared to amino acid-rich conditions.

11.3 Appendix manuscript in preparation

Parts of this thesis are included in the following manuscript ready for submission.

Title:

\textbf{Rapid depletion of Not4 in yeast provokes transcriptional and translational reprogramming}

Authors:

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Contributions by Sina Blessing:

Data processing was performed for the following figures: Fig. 1, Fig. 3 B, Fig. 4, Fig. 5 B-D, and all supplemental figures. Experiments were performed for the following figures: Fig. 2 A, Fig. 3 ACE, Fig. 5 A, and Supplemental Fig. 1 G. Experimental design and project development, as well as writing of the manuscript in close cooperation with Stefan G. Kreft and Elke Deuerling.
**Abstract**

In yeast, the Not4 protein is a subunit of the multi-functional Ccr4-Not complex which is assumed to be involved in a plethora of cellular processes including gene expression, nuclear mRNA export, translation repression and mRNA decay. These assumptions are based mainly on gene deletion analysis. However, deletion of the NOT4 gene provokes a strong growth impairment and massive pleiotropic changes in the phenotype thus complicating the dissection of direct from indirect consequences upon loss of Not4 function.

In this study, we constructed and characterized a strain expressing chromosomally encoded Not4 with an auxin-inducible degron on its N terminus. This allowed for the controlled and rapid degradation of Not4 upon treatment with the phytohormone auxin and thus, the investigation of immediate effects caused by Not4 protein depletion. Cells depleted for Not4 showed growth impairment and comprehensive transcriptional changes e.g. of genes assigned to the biological process of reproduction and the mating response pathway. Moreover, Not4-depleted cells revealed an increased α-pheromone sensitivity and the activation of invasive growth. Upon amino acid starvation, less genes revealed changes in Not4-depleted cells compared to non-depleted cells. However, 31 transcripts were exclusively upregulated in Not4-depleted cells after amino acid withdrawal including genes associated with amino acid metabolism. Ribosome analysis revealed that translational repression under amino acid starvation conditions was affected by Not4-depletion albeit less compared to cells deleted for NOT4.

In sum, the global changes of gene expression and translation observed immediately upon loss Not4 function suggest that Not4 as part of the Ccr4-Not complex is required to control and regulate central hubs of cellular transcription and translation.

**Introduction**

The highly conserved eukaryotic Ccr4-NOT complex is a multi-functional hub within an mRNAs life (1). It is implicated in every step of gene expression, including regulation of basal and induced transcription, transcriptional elongation, nuclear mRNA export, translation, and mRNA decay. The core of the S. cerevisiae Ccr4-NOT complex is comprised of nine subunits, the deadenylase module (consisting of Caf1 and Ccr4), the NOT module (consisting Not2, Not3, Not4, and Not5), as well as the two Ccr4-associated factors (Caf40 and Caf130), all of which are arranged around the scaffolding protein Not1 (2-5). The functional characterization of the Ccr4-NOT complex has been tackled from several directions, including structural, biochemical, and genetic approaches. Single components of the complex were first discovered in genetic screens as mutants mimicking pheromone-induced cell cycle arrest (CDC36/NOT2, CDC39/NOT1) (6), or as suppressors of conditionally functional mating response pathway components (SIG1/MOT2/NOT4) (7,8). Since the initial discovery, many studies have contributed to expand the understanding of the versatile Ccr4-NOT complex (9–12). The complex localizes to both the nucleus and the cytoplasm. In the latter, it can occur evenly distributed or condensed into P bodies (13). Furthermore, Ccr4-NOT shuttles between different (pseudo-)compartments to perform its diverse condition-dependent functions (14).

The involvement of the complex in highly diverse and dynamic processes complicate the functional characterization approaches and is limited to the underlying applied methodology that does not always meet the challenges regarding dynamic interactions, and spatially and temporally controlled processes. Thus, the manifold functions emphasize the importance of this complex.
Recently, it was revealed how the Ccr4-Not complex participates in the interdependency of transcription and translation (14,15). This interdependency was previously presented by the example of two RNA polymerase II subunits, Rpb4/Rpb7, that, as a heterodimer tethered to newly transcribed mRNAs, shuttle between the nucleus and cytoplasm in order to stimulate efficient translational initiation (16). Later it was shown, that Not5 directly interacts with Rpb4, and that Rpb4-association to polysomes was Not5-dependent, as well as the de novo assembly of RNA polymerase II complex (14).

Appendage of a nuclear export signal (NES) or a nuclear localization sequence (NLS) to retain Not5 either in the cytoplasm or the nucleus, revealed the localization-dependent dual role of Not5 with regard to its functions during proper RNA Polymerase II assembly and Rpb4 shuttling (14,16). Moreover, Not5-dependent imprinting of transcripts with Not1 highlighted the involvement of the Ccr4-NOT complex within regulation of gene expression, especially that of ribosomal proteins (15). These results emphasize the mutual influence between transcription and translation and their dependency on the Ccr4-NOT complex, mostly by the example of Not5.

A genetic, functional, and structural connection between Not2, Not5, and Not4 has been repeatedly demonstrated (17–21). Crystal structures revealed that the NOT module binds to Not1 by concomitant binding of Not2 and Not5, which together stabilize binding of Not4 to the complex highlighting the intricate interplay between NOT subunits (4,5,22).

Not4 is a conserved protein that contains an N-terminal RING domain, a central RRM domain and an unstructured C-terminal domain. It harbors E3 ubiquitin ligase activity (23,24). In yeast three Not4 substrates that link Not4 to the regulation of transcription have been identified: the histone demethylase Jhd2 (25–27), which counteracts Set1-mediated histone H3K4 methylation, cyclin C (SRB11) (28), which is part of the mediator Cdk8 kinase module, and the transcription factor Yap1 (29). Not4-mediated ubiquitination of cyclin C and Yap1 was demonstrated to occur under oxidative stress conditions and was shown to be necessary to activate the oxidative stress response, as well as, to tune the activated transcriptional response according to the stimulus.

Besides a role in the regulation of transcription-associated processes, Not4 was shown to be necessary for proper translational repression upon nutrient withdrawal (30). Hitherto, yeast Ccr4-NOT complex components have been predominantly studied via respective gene deletion mutants (all corresponding genes except for Not1 are non-essential). This approach is inherently problematic since the deletion of a gene can favor the accumulation of secondary effects including selection of suppressor mutations that bypass the primary effects of a gene deletion. Even though haploid yeast cell can tolerate the deletion of NOT4 and produce viable progeny, not4Δ cells are very sick, grow slowly, and regularly display contradictory growth behavior, possibly caused by suppressor mutant formation. Induced rapid depletion of a protein of interest represents an alternative to study a protein’s function (33). Such an approach is particularly well suited to study immediate consequences of the loss of a protein of interest because it omits the risk that initial phenotypic consequences are being masked by suppressors. Besides classical temperature-sensitive mutants, a degradation signal (“degron”) is often fused to a protein of interest to allow inducible degradation of the tagged protein (34,35). The plant-derived auxin-inducible degron (AID) system (35) allows for rapid degradation of a protein of interest and has been recently optimized for protein depletion in yeast (36). Here we report on the generation of a yeast strain expressing Not4 with a 6xFLAG-tagged auxin-inducible degron on its N terminus. Controlled degradation of Not4 protein in this strain upon treatment with the phytohormone auxin allowed for the investigation of immediate
effects caused by Not4 depletion. The primary effects of Not4 depletion on cell growth, mating pheromone sensitivity, as well as Not4’s previously described involvement in inhibition of translational repression and its effect as a negative regulator on transcription on a transcriptome-wide basis were compared to the pleiotropic phenotypes reported for not4Δ cells.

Results

Comparison of phenotypes of not4Δ and Not4-depleted cells

To study the primary (immediate) phenotypic changes associated with loss of Not4 in yeast, we generated a strain that allowed for inducible rapid depletion of Not4. The yeast-optimized auxin-inducible degron (AID*) sequence with an additional 6xFLAG epitope encoding sequence (Morawska & Ulrich, 2013) was inserted at the 5’ end of the NOT4 gene resulting in expression of N-terminally 6xFLAG-AID*-tagged Not4 from the natural chromosomal locus (AID*-NOT4). In addition, an expression cassette for the A. thaliana F-box protein AFB2 (37) was integrated into the genome. In the presence of AFB2, rapid ubiquitination and subsequent proteasomal degradation of AID*-tagged protein can be induced by auxin (36).

Yeast cells lacking NOT4 (not4Δ) display a strong growth defect at 30°C and 20°C, and are inviable at 37°C (7, 8, and Fig. 1A). AID*-NOT4 cells depleted of Not4 by addition of the auxin-analog indole-3-acetic acid (IAA) also displayed reduced growth at 30°C and 20°C, but the growth impairment was generally less pronounced as for not4Δ cells (Fig. 1A). Surprisingly, IAA addition to AID*-NOT4 at 37°C did not impede on growth, whereas not4Δ cells were incapable to grow at this temperature. Growth impairment of AID*-NOT4 cells was however restored when IAA treated cells were shifted from 37°C back to 30°C (Fig. 1A lower panel).

In addition to the plate growth analysis, we monitored growth in liquid culture. Non-depleted and depleted AID*-NOT4 cells at 30°C and 37°C were followed for eight hours (Supplemental Fig. 1 A and B) and Not4 protein levels were determined in parallel (Fig. 1 B, Supplemental Fig. 1 C and D). Not4 was efficiently depleted at 30°C, with only approximately 18% and 11% remaining four and eight hours after IAA addition. Hence, the observed growth impairment of AID*-NOT4 cells upon IAA treatment is consistent with the strongly decreased Not4 levels. Analysis of variance (One-way RM ANOVA) yielded significant P values (<0.05) regarding reduced Not4 protein levels already after two hours of depletion and remained significantly low over the observed period of time at 30°C. The impact of Not4 depletion on the optical density was statistically verifiable after eight hours (30°C; P value: 0.027).

In contrast, at 37°C Not4 depletion was less efficient with 57% of Not4 remaining after eight hours exposure to IAA (as compared to 11% at 30°C) (Figure 1 B and C, Supplemental Figure 1 D). The only partial depletion at 37°C was consistent with mostly unperturbed growth at 37°C (in the presence of IAA) (Supplemental Figure 1 D). To investigate whether the auxin-inducible degron system itself is in general functional at 37°C we analyzed cells expressing C-terminally AID*-tagged replication factor Rfa1 (RFA1-AID* hereafter) (36). Rfa1-AID* depletion was comparable at 30°C and 37°C (with 27% Rfa1-AID* remaining at 30°C and 15% at 37°C, respectively) (Fig. 1 C, Supplemental Figure 1F). Differences in depletion efficiency were mirrored by doubling times (calculated from growth in the first eight hours after IAA addition). At 37°C, AID*-NOT4 cells, irrespective of whether they were exposed to IAA or not, had a doubling time of 2.7 hours. In contrast, at 30°C non-depleted and Not4-depleted cells had doubling times of 2.2 hours and 2.8 hours, respectively.
not4Δ cells displayed a doubling time of 5.6 hours (monitored over 7.5 hours at 30°C; Supplemental Figure 1 G).

Our data on Rfa1-AID* and AID*-Not4 show that the efficiency of degron-mediated protein depletion at 37°C is strongly dependent on the target protein. We suspect that subcellular localization might account for inefficient Not4 depletion at 37°C. As a constituent of the Ccr4-NOT complex, Not4 possibly shuttles between nucleus, cytoplasm and P-bodies (13,14). Presumably, sequestration of Not4 into P-bodies at 37°C could leave it inaccessible for the ubiquitination and degradation machinery. Regardless of the precise underlying reasons, the observed only incomplete Not4 depletion at 37°C precluded the use of NOT4-AID* cells to study immediate consequences of loss of Not4 at elevated temperature. Taken together, as not4Δ cells Not4-depleted cells displayed slow growth at 20°C and 30°C, only that the growth defect was less pronounced for the depleted. Since Not4 depletion was insufficient at 37°C, we conducted all further experiments at 30°C.

Not4 depletion provokes inappropriate α-pheromone and mating response target gene expression

\( \text{NOT4} \) was originally discovered independently in two genetic screens for mutants capable of restoring mating efficiency in conditional MAPK pathway mutants (7,8). We thus sought to study the direct consequences of Not4 depletion on a transcriptome-wide level by microarray analysis (Fig. 2A). To this end, cells were grown in synthetic complete medium and Not4 was depleted for six hours. Total RNA was isolated and subjected to an Affymetrix yeast genome 2.0 GeneChip. Transcripts were considered expressed significantly different if the expression was at least changed two-fold compared to non-depleted cells. Upon depletion of Not4, 183 genes exhibited significantly increased expression, 34 of which could be assigned to the biological process of reproduction (GO:0000003, Fig. 2 A and D). This confirmed the previously reported increased amounts of pheromone-responsive gene transcripts in not4Δ (8) (Fig. 2 B). In support of a role of Not4 in the modulation of reproduction-associated transcripts, FIG1, a known target of the mating response pathway, was up-regulated more than 100-fold (Fig. 2 B). Besides FIG1, additional transcriptional reporter genes for the activation of the MAPK-mediated mating response pathway like FUS1, FUS3, and PRM3 (38) were found significantly increased. Increased transcript levels were also observed for the pheromone-inducible Ty3 retrotransposon genes, encoded by YGR109W-A, YGR109W-B, and YIL082W-A (39); Fig. 2 B). Surprisingly, MF(alpha)1 and MF(alpha)2 transcripts, encoding the α-pheromone, were enriched almost five-fold and two-fold, respectively (Fig. 2 A). This was inasmuch unexpected as the cells subjected to the microarray analysis were MATa cells, and therefore should not produce the α-cell-specific pheromone. In addition to induction of mating responsive genes upon Not4-depletion, our microarray analysis further revealed an up-regulation of other genes, among them stress-induced genes SSA4, HSP26, HSP42, and XBP1 (Fig. 2 A). In contrast, 43 genes were significantly, i.e. at least two-fold, down-regulated in Not4-depleted cells (Fig. 2 C). Out of these, 17 could be assigned to the cellular component "mitochondrion" by GO: Slim Mapper (yeastgenome.org).

The number of differentially expressed genes (threshold: 2-fold) in Not4-depleted cells (226 genes) obtained in the current study substantially differs from what was reported for not4Δ cells under comparable growth conditions (21,32) (Fig. 2 C). In each of the two previous studies with not4Δ cells the ratio of up- and down-regulated genes was roughly 1:1 (Fig. 2 C). In our study with Not4-depleted cells, the number of differentially expressed genes is significantly lower as in the two not4Δ studies. Furthermore, the number of up-regulated genes is more than four times higher than
the number of down-regulated genes (Fig. 2 C). Moreover, not only the number of differentially expressed genes but also the genes themselves differed between studies (Fig. 2 E). Only a small overlap was observable in the study by Cui et al. and our study (Fig. 2 E). Notwithstanding that the two studies cannot be directly compared due to differences in sample preparation, analysis, and data evaluation, the observed small overlap likely also reflects true differences between not4Δ and Not4-depleted cells further highlighting the value of depletion studies. Genes identified in both studies (“overlapping” genes), e.g. up-regulated genes like the small heat shock proteins HSP26 and HSP42, and the pheromone-responsive genes ASG7 and PRM10, can be considered a core result. Noteworthily, in not4Δ cells Cui et al. also found the transcripts encoded by MF(alpha)2 and FUS2 enriched 7.9-fold and 15.6-fold, respectively, but did not include the genes in their final list since both genes were not found consistently. To sum up, the transcriptome analysis of Not4-depleted cells revealed a strong enrichment for genes associated with reproduction and stress-response. Compared to published not4Δ transcriptome data, our results further highlight differences between gene deletion and protein depletion systems in a quantitative and qualitative manner.

**Not4-depleted cells display increased α-pheromone sensitivity and activation of invasive growth**

The identified transcriptional activation of pheromone-responsive genes in Not4-depleted cells indicated an activated MAPK pathway-mediated mating response. To investigate the effect of Not4 depletion on the mating response pathway further, in particular to establish the sensitivity toward α-pheromone, we carried out halo experiments (7) (Fig. 3). As to be expected, haploid MATa control cells (AFB2) and non-depleted MATa AID*-NOT4 cells responded with discontinuation of growth in close proximity to an external α-pheromone source (Fig. 3 A). For Not4-depleted cells halo formation was enhanced and edges of the halos were frayed (Fig. 3 A). Comparison of halo sizes revealed a significant increase in α-pheromone sensitivity for Not4-depleted cells (Fig. 3 B).

Under certain conditions haploid yeast cells can exhibit MAPK pathway-dependent invasive growth (40). Several MAPK pathway components are shared between pheromone response and invasive growth (Fig. 3 D). Although different extracellular signals trigger the two responses (in haploids: mating pheromone or glucose depletion), target gene activation is in each case mediated by the transcription factor Ste12. Prolonged activation of Ste12, often a result of a lack of Ste12 inactivation after pheromone response, can trigger invasive growth (41,42). We hence tested whether Not4-depleted cells display invasive growth. Agar penetration is a hallmark of invasive growth which can be readily assayed. After rinsing an agar surface with water, cells that penetrate the agar remain in the agar whereas non-invasively growing cells are typically washed off (40). In context of a halo experiment, invasive growth is expected for pheromone-treated cells - that is when treated with the pheromone of the opposite mating type - within the halo, since cells in close proximity to the α-pheromone encounter permanent activation of the mating response pathway without the opportunity to productively fuse and mate, which eventually triggers the invasive growth phenotype. Upon pheromone treatment alone, control (AFB2) and AID*-NOT4 strain displayed comparable invasive growth (Fig. 3 C upper row). This was only slightly increased for AFB2 control cells when auxin was present in addition to the α-pheromone (Fig. 3 C). Strikingly, when pheromone treatment was combined with Not4 depletion, MATa AID*-NOT4 cells showed markedly increased invasive growth (Fig. 3 B and C). Moreover, Not4-depleted MATa cells (but not WT or AFB2 control MATa cells) even displayed invasive growth in absence of an external α-pheromone source (Fig. 3 E).
Appendix

Consistent with a role of Not4 in negative regulation of the MAPK-dependent invasive growth was also observed for MATa not4Δ cells (Fig. 3 E). In contrast, Not4-depleted MAT cells showed no signs of invasive growth (Fig. 3 E, compare AID*-NOT4 (a) and (α)).

With regard to the observed invasive growth phenotype of Not4-depleted MATa AID*-NOT4 cells it is surprising that the microarray analysis of these cells after Not4 depletion yielded four genes with increased expression that belong to the biological process "negative regulation of invasive growth in response to glucose limitation" (GO:2000218) (NRG2, NRG1, DIG2, SIP4; Fig. 1 D). The two paralogs NRG1 and NRG2 encode transcriptional repressors that mediate glucose repression and negatively regulate haploid invasive growth (43). DIG2 encodes an inhibitor of Ste12, and SIP4 encodes a transcriptional activator of gluconeogenic genes. Nrg1, Nrg2, and Sip4 have in common that they are regulated by the protein kinase Snf1, which itself is being activated by low glucose levels (43–45).

At the same time, as already mentioned, in our microarray analysis both transcripts encoding the α-pheromone were found to be significantly up-regulated upon Not4 depletion. Assuming that Not4-depleted MATa cells erroneously produce and secrete α-pheromone, this could possibly account for the two observed phenotypes, increased α-pheromone sensitivity and α-independent agar invasion (i.e. in absence of external α-pheromone).

Together, the halo assay and the invasive growth assay confirmed a role of Not4 in the mating response pathway, and revealed a new additional role in another, related MAPK-mediated pathway, the invasive growth pathway. Whereas an activation/derepression of the mating response pathway was previously reported for not4Δ cells (8,18), activation of the invasive growth pathway in not4Δ cells, as well as upon Not4 depletion (in MATa cells) constitutes to our knowledge a novel phenotype for such cells.

**Transcriptional changes upon amino acid withdrawal in presence and absence of Not4**

Repression of translation is a direct response to amino acid withdrawal and has been shown to depend on Not4 (30). We next determined by microarray analysis how depletion of Not4 would alter the transcriptional response to amino acid withdrawal. As reported previously (54), amino acid withdrawal triggered vast transcriptional changes in non-depleted cells. The expression of 468 genes was increased and 471 genes showed reduced expression (Fig. 4 A and C). Amino acid starvation of Not4-depleted cells led to a substantial reduction of differentially expressed genes as compared to non-deprived Not4-depleted cells (Fig. 4 A, B and C). Only about half as many genes (approx. 56%) as in non-depleted cells were up-regulated, most of which were also up-regulated in non-depleted nutrient deprived cells. Just 34 genes were increased exclusively in Not4-depleted cells upon amino acid withdrawal, three of which (STL1, YKR075C, YNL277W-A) were already significantly increased upon Not4-depletion without amino acid withdrawal. About one fifth of the transcripts exclusively up-regulated in Not4-depleted cells after amino acid withdrawal belong to biological processes associated with amino acid metabolism. From the 471 genes significantly decreased in starved non-depleted cells about 65% were decreased in Not4-depleted starved cells as well.

Gene ontology term finder was used to find terms of biological processes associated with differentially expressed genes (Supplemental Figure 2, 3 and 4). Consistent with a response to amino acid deprivation, the majority of transcripts showing an increased expression upon amino acid withdrawal belonged to biological processes concerning amino acid metabolism (Supplemental Figure...
2). Transcripts with reduced expression belonged to the biological process "ribosome biogenesis" (Supplemental Figure 3 and 4). Most genes differentially expressed in Not4-depleted cells upon amino acid withdrawal were also differently expressed in non-depleted amino acid deprived cells. It was however evident, that the number of genes associated with ribosome biogenesis was lower decreased in Not4-depleted cells (compare Fig. 4 E and F). Some of which were even below the applied threshold between non-depleted and Not4-depleted cells (Fig. 4 D). In contrast, genes assigned to the cellular component "ribosome" were not collectively affected by amino acid withdrawal (Fig. 4 G and H), although more genes were down-regulated in Not4-depleted cells. Overall, on a transcriptional level Not4-depleted cells responded in a qualitatively similar way to nutritional changes in their environment as non-depleted cells. However, the scope of the transcriptional response was generally lesser and for some genes different from that of the non-depleted control cells. One example was the transcript encoding Dal80, a general transcriptional repressor of nitrogen catabolite pathway genes. In non-depleted nutrient-deprived cells Dal80 transcript levels remained below the threshold, whereas in nutrient-deprived Not4-depleted cells, levels were increased 16-fold (Fig. 4 A and B).

Taken together, amino acid withdrawal caused extensive remodeling of the transcriptome within ten minutes of withdrawal, in non-depleted and Not4-depleted cells. Differences were less pronounced in cells depleted of Not4. For example, transcripts in non-depleted cells reached up to a 32-fold decrease, whereas Not4-depleted cells exceeded just over an 8-fold decrease (Fig. 4 A and B).

Translational repression upon amino acid withdrawal in Not4-depleted cells

In addition to our microarray analysis of cells subjected to amino acid withdrawal, we next carried out polysome profiling to investigate consequences of Not4-depletion on translational repression as has been done previously for not4Δ cells (30). An overlay of recorded polysome profiles for AID*-NOT4 control cells grown in presence of amino acids and under amino acid withdrawal conditions showed an increase in the 80 S peak accompanied by a decrease in polysomal peaks upon amino acid withdrawal (Fig. 5 A). An increase in the 80 S peak upon amino acid withdrawal was also observable for Not4-depleted cells (1 mM IAA; Fig. 5 A). However, the reduction in polysomes was in this case less pronounced as for non-depleted cells (Fig. 5 A). It was also noticeable that although total lysate samples had been adjusted based on their absorbance at 260 nm, Not4-depleted cells displayed a reduction in 80 S and polysomes compared to non-depleted cells under amino acid-rich conditions (Fig. 5 A). In not4Δ cells, translational repression after amino acid withdrawal was more seriously hampered compared to Not4-depleted cells, as manifested in an only mild increase in 80 S and an only minor decrease of polysome peaks (Fig. 5 A and B).

Together, these results confirm diminished translational repression upon amino acid withdrawal for Not4-depleted as well as not4Δ cells. However, the degree of repression was less pronounced in cells depleted for Not4 (Fig. 5 D).

Discussion

This study deals with a phenotypic comparison between NOT4 gene deletion and Not4 protein depletion. For the depletion of Not4 protein a yeast-optimized auxin-inducible degron system was established and tested (36).

Our results show that Not4-depletion was sufficient to provoke growth impairment on solid and in liquid medium at 30°C and 20°C compared to non-depleted cells. On the other hand, compared to
"not4Δ, Not4-depletion was not as severely affecting growth. This difference can be explained as Not4 protein remained detectable at extremely low amounts throughout the monitored time course, possibly due to continuation of protein expression or inaccessibility of the protein for the degron machinery. Besides the well described slow growth phenotype of not4Δ cells, we also tested for the published effect of NOT4 deletion on translational repression upon nutrient withdrawal (30). Indeed, polysome profiles displayed differences between non-depleted, Not4-depleted, and not4Δ cells under amino acid starvation conditions. While not4Δ cells displayed reduced translational repression compared to wild-type, cells depleted of Not4 still showed enhancement of the 80 S peak albeit almost no decrease of the polysome levels (Fig. 5A). However, the quantification of the decrease of polysomes upon amino acid withdrawal revealed that while wild-type cells reduced their polysome levels by nearly 30%, Not4-depleted and not4Δ cells displayed a reduction of 18% and 15%, respectively. This was in accordance with the previously described growth assays, that showed how Not4-depleted cells phenotypically approximated not4Δ cells. Likewise, amino acid withdrawal provoked a response in Not4-depleted cells which displayed more similarities to observations made in not4Δ, than wild-type cells.

Interestingly, we also observed a negative effect on translation for Not4-depleted cells under amino acid-rich conditions. This could originate from the derepression of stress-related genes. Regarding the microarray data derived from amino acid deprived samples, we showed global changes in transcript abundance for non-depleted and Not4-depleted cells. Though Not4-depleted cells differed mostly in the magnitude to which transcripts were differentially expressed, they first and for most followed the overall trend in regulation. These results could also be explained by the Not4-dependent derepression of developmental and stress-related genes discussed below in more detail, the rationale behind this being that Not4-depleted cells already carry out an environmental stress response (ESR) to some extent, which does not allow for extreme additional changes within the transcriptome upon the encounter of amino acid withdrawal.

Yeast cells are capable to adjust to rapid and harsh changes within the environment. Specialized signaling pathways allow for the detection of different stressors, examples being changes in pH, temperature, or nutrient availability. As well as, a specific response in order to protect cells from damage, which usually involves global changes within the transcriptome. Besides the highly specific nature of stress responses, a systematic, DNA microarray-based study conducted in 2000 by Gasch et al. could show that different environmental changes (inter alia: heat shock, amino acid starvation, stationary phase) resulted in an overlapping set of genes similarly affected in their expression, referred to collectively as "environmental stress response" (ESR). Interestingly, some of the genes induced in the ESR were also significantly increased in our microarray analysis of Not4-depleted cells. Increased transcript levels were detected for the stress-inducible Hsp70 SSA4, and the two small HSPs-encoding genes HSP26 and HSP42. Another interesting gene with significantly increased transcript level upon Not4-depletion was HXT5, as it was not only shown to be a member of the ESR set, but additionally found to be transcriptionally regulated by growth rates (46). HXT5 expression had been shown to correlate with decreasing growth rates, which fit our observation in Not4-depleted cells. In this context, it is tempting to suggest that Not4 constitutes a regulator of the transcriptional repression of the ESR, or at least should be considered to be studied in this context.

Transcripts of mating response target genes were increased the most in our microarray analysis of Not4-depleted cells. Pheromone-induced activation of the MAPK Fus3 not only results in activation of the transcription factor Ste12 and the regulation of other associated factors, it also stimulates
Appendix

cell cycle arrest via the activation of Far1. Therefore, one could speculate whether the activation of
the mating response pathway which is accompanied by cell-cycle arrest holds responsible for the
observed decrease of the growth rate, which in turn causes the induction of HXT5 expression. The
expression of the α-pheromone (encoded by MF(α)1 and MF(α)2) was highly unexpected, because
samples for the microarray analyses were obtained from MATα cells. Expression of the pheromone
and its secretion could provide for an autocrine stimulation of the mating response pathway, and be
responsible for the induction of mating response target genes, and subsequent agar invasion ob-
erved for Not4-depleted and not4Δ cells. We can not explain why MF(α)1 and MF(α)2 displayed
increased transcript levels in Not4-depleted cells to begin with. As invasive growth was only ob-
erved in Not4-depleted cells of mating-type a, but not α, it will be interesting to further investigate
Not4's role in this context. Interestingly, a conserved repressor of α-specific genes, encoded by
SUM1, was discovered recently, whose deletion also resulted in the expression of MF(α)1 in cells
of mating-type a (47).

Not4-depleted cells displayed invasive growth upon prolonged depletion, possibly as a conse-
quence of the proposed autocrine stimulation of the mating response pathway. To our knowledge,
we are the first to describe the invasive growth phenotype for not4Δ, as well as Not4-depleted
cells. These results were especially intriguing, as we utilized S288C as a parental strain, for which
invasive growth was highly unexpected since a naturally occurring point mutation in the transcrip-
tion factor FLO8 leaves these cells improbable to form pseudohyphae, and only few genes are de-
scribed, capable to increase the ability for invasive growth in this specific genetic background (48).
Additionally, it is improbable that activation of invasive growth occurred via the specific MAPK
pathway, as it was previously reported that the laboratory strain S288C is kss1− (49), and thus lacks
the final effector MAP kinase of this signaling pathway.

Furthermore, our microarray data obtained from exponentially growing cells displayed increased
transcript levels for genes associated with negative regulation of invasive growth that contradict the
emergence of invasive growth. However, it is questionable, whether observations made from the
microarray analysis (conducted on cells after six-hour depletion in liquid synthetic complete medi-
um (SC+)) can be compared to our growth analysis (documented after two days on solid rich medi-
um (YPD)). Taken together, the proposed autocrine stimulation of the mating response pathway by
the expression of α-pheromone could explain the majority of phenotypes of not4Δ and Not4-
depleted cells described in this study, including impaired growth, transcriptional induction of
pheromone-responsive genes, and invasive growth as a consequence of prolonged stimulation of
the mating response pathway or the lack of proper inactivation of the transcription factor Ste12
(42). Future work will shed light on how expression of α-pheromone is dependent on Not4, and
whether this can actually explain the phenotypes mentioned in this study.

A search for associated transcription factors conducted on differentially expressed genes
(Yeastract) revealed a huge overlap of the Top10 regulators between genes significantly affected
by either Not4-depletion, amino acid withdrawal, or both (Supplemental Figure 5 D). Transcripts for
these transcription factors themselves were not differentially expressed. Interestingly, Not4-depen-
dent negative regulation of the transcription factor Yap1 upon oxidative stress had already been
shown (29). Furthermore, regarding the same stress condition, Not4-dependent cyclin C degrada-
tion had also been shown (28). Cyclin C is a component of the Mediator kinase module and cyclin-
dependent kinase 8 (Cdk8)-Cyclin C-dependent regulation of transcription factors had already
been shown for Ste12 (41), Gcn4 and Msn2 (50). Additionally, Not4 had been shown to regulate
the histone demethylase Jhd2 in association with the proteasome (26,51). Based on all these dif-
ferent observations made by us and others, and our finding that a subset of different developmen-
tal and stress-related genes were derepressed in a Not4-dependent manner despite growth under
non-stress conditions, led us to the conclusion that depletion of Not4 directly and negatively affects
transcriptional repression, especially of stress-related or developmental genes.

Our finding that depletion was inefficient at 37°C in a Not4-dependent manner may point to a
change in subcellular localization leaving Not4 inaccessible for the degron machinery. A plausible
scenario would be the sequestration of Not4 into P bodies at 37°C in context of the Ccr4-NOT
complex. Localization into P bodies has previously been shown for the deadenylase subunit Ccr4,
in agreement with the findings, that P bodies constitute the site of mRNA decay initiated by de-
adenylation, and that these structures increase in size and abundance upon stress ((13,52) re-
viewed in (53)). Eviction of Not4 from the nucleus could allow for the derepression of genes re-
quired for an appropriate response to a more demanding environment. This idea would be further
supported by our microarray data that showed increased transcript levels for stress-inducible
genes upon Not4-depletion (e.g. SSA4 or XBP1). In this context it is interesting to note that yeast
cells undergo different phases of growth, from exponential, fermentative, over post-diauxic, respira-
tory, to quiescent, stationary phase. Growth phase progression demands for huge changes within
the cellular composition and structure, similar to what is required upon stress (54). Interestingly,
Not4 protein was no longer detectable after 24 hours in non-depleted cells grown in liquid culture
(Fig. 2 B). On the other hand, Not4 was present when cells were grown under respiratory growth
conditions in YP medium containing ethanol or glycerol as the sole carbon source (data not
shown), similar to what was already observed by others (55,56). We propose that the 24 hour time
point in our growth analysis (liquid culture) most likely reflected diauxic shift, whereas our growth
analysis on solid medium probably resembled the post-diauxic phase. Based on these observa-
tions, we propose that Not4 protein levels decline upon diauxic shift to allow for changes of the
transcriptome, and are reintroduced upon post-diauxic, respiratory growth to repress stress re-
sponses again in favor of proliferation. Though this needs to be proven by a continuous study on
the correlation between growth phases and the presence of Not4 protein, as well as the proposed
dynamics in subcellular localization.

In conclusion, our work presented here, provided a meaningful comparison between Not4-depleted
and not4Δ cells. Transcriptional regulators of differentially expressed genes in our microarray anal-
yses strongly overlapped between Not4-depleted and amino acid-deprived cells, emphasizing the
importance of Not4 within the repression of stress-induced target genes. The phenotypic behavior
described for not4Δ was confirmed in cells depleted of Not4, with the strongest difference within
the magnitude. This difference can be explained by the finding that Not4-depletion was incomplete
compared to the absolute gene deletion, as well as the emergence of adaptation in order to sup-
press the severe effects caused by the complete gene deletion in not4Δ cells. The combination of
both experimental systems was proven meaningful in order to distinguish immediate, primary ef-
fects caused by a loss of function, to secondary effects. And showed that protein depletion is not
only an option for loss of function studies conducted on essential genes, whose deletion is not tol-
erated by the respective model organism. Moreover, we observed that the auxin-inducible degron
system can fail at 37°C in a target protein-dependent manner.
Material and methods

Strains and plasmids

Table S1 lists the relevant data of the strains and plasmids used in this study. All strains generated during this study are derivatives of the *Saccharomyces cerevisiae* strain BY4741, with exception of *RFA1-AID*\(^\ast\) which is a derivative of DF5. The AFB2 strain was generated by transformation of BY4741 with the *MscI*-linearized plasmid pRS303-ADH-AFB2 and recombination into the *his3\(\Delta 1\)* locus. The plasmid pML104 (Wyrick *et al.* 2015 YEAST) was expanded to carry a NotI restriction site between the suggested integration sites (*SwaI*/BclI) by ligation of annealed oligonucleotides (oligonucleotides: pML104-NotI-up / pML104-NotI-lo). Genome editing was carried out by CRISPR/Cas9. Guide RNA sequences were determined with the help of the online available CRISPR Toolset (http://wyrickbioinfo2.smb.wsu.edu/crispr.html). Table S2 lists the respective oligonucleotides used for generation of pML104 plasmids and PCR-based amplification of repair templates. Gene-specific plasmid construction for CRISPR/Cas9-mediated genome editing was carried out as previously described (57). pBlsIISK(-)-6FLAG-AIDstar was generated by Gibson assembly to enable integration of the degron tag sequence at the N-terminus of target genes. PCR amplicons of 6FLAG (oligonucleotides 6xFLAG_Gibson-R/6FLAG-II-Gibson-R) and AIDstar (AID\(^\ast\)) from pHyg-AIDstar-6FLAG (oligonucleotides AIDstar_Gibson_R/AIDstar-II-Gibson-F) were inserted into *BamHI*/PstI-digested pBlsIISK(-). pBlsIISK(-)-6FLAG-AIDstar was used as a template for PCR amplification of repair templates, with exception of the re-pair template for CAF1, there the oligonucleotides were directly hybridized and completed by PCR. Deletion of the *MF(\(\alpha\))1* ORF was carried out by PCR amplification of the KanMX6 ORF with *MF(\(\alpha\))1*-specific flanking regions for homologous recombination (Primers: MF(\(\alpha\))1_KanMX6_fwd; MF(\(\alpha\))1_KanMX6_rev; Template: pFA6a-KanMX6_fwd; MF(\(\alpha\))1_KanMX6_rev; Template: pFA6a-KanMX6). Strains and plasmids were verified by PCR and sequencing of the concerned nucleotide sections.

Cell growth and Not4-depletion

Unless indicated otherwise, cells were grown in in standard YPD medium (1% yeast extract, 2% peptone, 2% dextrose) or synthetic complete media (SC; 0.67% yeast nitrogen base, 0.2% drop out mix, 2% dextrose) under steady rotation (110-130 rpm) at 30°C. YPD supplemented with 20% Bacto-Agar was used for growth experiments on solid medium. Not4 depletion was induced after an initial growth phase (1-2 hours) and addition of a final concentration of 1 mM Indole-3-acetic acid sodium salt (IAA; Sigma-Alderich; CAS No. 6505-45-9) diluted in the respective medium. Untreated and treated cells were grown over a period of another four, six, or eight hours. Monitoring of growth and depletion behavior was carried out by taking cell samples every hour and establishing the optical density at 600 nm (OD\(_{600}\)), as well as, protein sample preparation by pelleting 0.2 ODs, followed by NaOH-lysis, and resuspension in 2x protein sample buffer. Doubling times were calculated with the following formula:

\[
\text{Doubling time} = \frac{t_8-t_0}{\ln(OD_8) - \ln(OD_0)}
\]

Proteins were seperated on a 7.5% NuPAGE, transferred to a Nitrocellulose membrane, and immunostained. Growth analyses on solid medium were conducted by adjusting the OD\(_{600}\) of overnight cultures to 0.4, followed by six serial 1:5 dilutions, and spotting onto agar plates. Plates were incubated at the indicated temperatures and time periods. For amino acid withdrawal experiments, cells were grown in SC medium, cultures split, pelleted (3 min 2,500 rpm), and resuspended in either SC or complete minimum medium (CM; 0.67% yeast nitrogen base, 2% dextrose) for another ten-minute incubation. All experiments were conducted at least three times.

RNA preparation, microarray analysis and data evaluation

RNA was prepared from a 50 ml SC cultures after IAA treatment for six hours, and ten-minute shift to SC or CM medium. RNA extraction was performed with the RNeasy Mini Kit (QIAGEN) according to the provider’s protocol. Cell lysis was carried out by sonication (Branson sonifier) in the provided RLT buffer. Affymetrix RNA microarray (Yeast 2.0) was provided by Haiping Hao and the team at Deep Sequencing and Microarray Core (via Science Exchange). Data evaluation and analysis were performed in R (version 3.4.2), including the libraries *simpleaffy*, *affyPLM*, and *limma*, provided by Bioconductor. The GO term finder function of the *Saccharomyces* Genome Database (https://www.yeastgenome.org), and the search for transcription factors of Yeastactr (http://www.yeastract.com/index.php) were conducted with default settings on differentially expressed genes.
Pheromone-induced halo and invasion assay

Cells were grown to early exponential phase and spread onto YPD or YPD IAA plates. Plates were incubated at 30°C for an hour before 2 µl of alpha-factor mating pheromone (10 µg/µl, ZymoResearch) or solvent (ethanol) was applied to a 5-mm-diameter disc (made from Whatman 3-mm paper) placed in the center of the lawns. Plates were incubated at 30°C for two days. Cell lawns were washed off with water to reveal invasive growth.

Polysome profiling

For polysome profiling, 500 ml cultures were treated for six hours, then shifted to SC or CM medium for ten minutes. Cell harvest was carried out by vacuum suction through a nitrocellulose membrane (Whatman; 0.45 µm) and flash freezing in liquid nitrogen. Frozen cells were lysed by Cryomill (Retsch MM400) in the presence of 0.5 ml frozen Lysis buffer (20 mM HEPES pH 7.4; 100 mM potassium acetate; 2 mM magnesium acetate, 0.5 mM DTT; 1 mM PMSF; 1x Tm complete, EDTA-free Protease Inhibitor Cocktail (Roche diagnostics); 100 µg/ml cycloheximide) for 30 sec at 25 Hz. Cleared lysates were adjusted to 50 A260 absorption units in 0.5 ml, loaded onto an 11 ml linear sucrose gradient (15-45% in Lysis buffer), and centrifuged at 39,000 rpm for 2.5 hours at 4°C (Beckman Coulter; Ultracentrifuge Optima™; SW 41 Ti). Sample fractionation was performed from top to bottom with a piston gradient fractionator (BioComp), and polysome profiles were recorded from top to bottom with a piston gradient fractionator (BioComp). For determination of the area under the curve (AUC), the sum of all measured intensities between the respective local minima was divided through the number of measuring points.

Data evaluation and statistics

Data evaluation and presentation, unless indicated otherwise, were carried out in Microsoft Excel:mac 2011, or Adobe Illustrator CS3, and statistical analyses were performed with SigmaPlot 12.5.

References

40. Roberts RL, Fink GR (1994) Elements of a single MAP kinase cascade in Saccharomyces cerevisiae mediate two developmental programs in the same cell type: mating and invasive growth. Genes Dev 8: 2974–85
### Table S1-Strains

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### Table S1-Plasmids

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### Table S2

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Figures

Figure 1

(A) Not4-depleted cells vs. non-depleted cells

(B) Pheromone-responsive genes

(C) GO term finder: Biological Process

(D) Comparison of 2-fold differentially expressed genes

(E) Comparison of 2-fold differentially expressed genes (Cui et al. vs. this study)
Figure 2

A

YPD 30°C  

Wildtype (a)  

not4Δ (a)  

AFB2 (a)  

AID*-NOT4 (a)  

AID*-NOT4 (a)  

YPD 20°C  

YPD 20°C  

+IAA  

+IAA  

YPD 37°C  

YPD 37°C  

+IAA  

+IAA  

B

AID*-NOT4  

30°C -IAA  

30°C +IAA  

37°C -IAA  

37°C +IAA  

0 1 2 3 4  

0 1 2 3 4  

0 1 2 3 4  

0 1 2 3 4  

5 6 7 8 24  

5 6 7 8 24  

5 6 7 8 24  

5 6 7 8 24  

time [h]  

α-FLAG  

α-G6PDH  

α-FLAG  

α-G6PDH  

C

Protein relative to G6PDH  

AID*-NOT4  

RFA1-AID*  

D

Protein level relative to G6PDH  

Not4 (FLAG)  

Rpl17A  

E

AID*-NOT4  

- + IAA  

α-FLAG  

α-RPL17A  

α-G6PDH
Figure 3

A  
AFB2 (a)  
α-pheromone  
AFB2 + IAA

AID*-NOT4 (a)  
α-pheromone  
AID*-NOT4 + IAA

B  
Area [inches²]  
*** *** ***

α-pheromone sensitivity Invasive growth

C  
AFB2 (a)  
α-pheromone  
AFB2 + IAA

AID*-NOT4 (a)  
α-pheromone  
AID*-NOT4 + IAA

D  
GPA1 (Gα)  
Ste4 (Gβ)  
Ste10 (Gγ)

Ste2  
Ste5  
Ste14  
Fas3  
Ste11  
Ste7  
Klt1  
Ste12  
Ste12  
Tsc1

MAPKK  
MAPK  
PRE-gene activation  
FRE-gene activation

E  
YPD 30°C  
YPD 30°C + IAA

wildtype (a)  
not4Δ (a)  
AFB2 (a)  
AID*-NOT4 (a)  
AID*-NOT4 (a)

wildtype (a)  
not4Δ (a)  
AFB2 (a)  
AID*-NOT4 (a)  
AID*-NOT4 (a)

before wash

after wash
Figure 5

A

with amino acids
without amino acids
overlay

AID+NOT4 control

AID+NOT4 1 mM IAA

not4

soluble 40S 60S 80S polysomes soluble 40S 60S 80S polysomes soluble 40S 60S 80S polysomes

B

\%

% of total ribosomal particles

-IAA +aa -IAA -aa +IAA +aa +IAA -aa not4+1 +aa not4 -1 aa

C

Not4 protein level relative to GAPDH

\*

-IAA+aa -IAA-aa +IAA+aa +IAA-aa

D

% decrease of polysomes

-IAA +IAA not4

29 18 15

153
Figure 1: Growth phenotypes of Not-4 depleted cells.

(A) Growth of auxin-inducible degron tagged Not4 cells (NOT4-AID*) on solid medium under non-depletion (YPD) and depletion conditions (YPD 1 mM IAA) at indicated temperatures. Mating type of each haploid yeast strain is indicated in brackets. Plates were incubated at the respective temperatures for two days (30°C and 37°C), three days (20°C), or for one day at each temperature (37°C-30°C). (B) Representative immunoblot of non-depleted (control) and Not4-depleted (1 mM IAA) AID*-NOT4 cells at 30°C (left) and 37°C (right). Not4 detection was carried out using an α-FLAG antibody. G6PDH served as loading control and was detected via an α-G6PDH antibody. Protein levels were monitored and quantified over a period of eight hours and after 24 hours. α-FLAG detection yielded an unspecific signal on top of the actual Not4-specific signal (marked by an asterisk (*)). (C) Quantification of Not4 and Rfa1 proteins normalized to G6PDH of non-depleted (-IAA) and depleted (+IAA) protein samples at 30°C and 37°C after eight hours (t8). Not4 depletion was significant at 30°C (n = 3; SD -IAA ±0.20; SD +IAA ±0.04; One-way RM ANOVA: P value 0.02), but not at 37°C (n = 3; SD -IAA ±0.08; SD +IAA ±0.14; One-way RM ANOVA: P value 0.06).

Figure 2: Microarray analysis on Not4-depleted cells.

(A) Volcano plot representation of microarray data obtained from cells depleted of Not4 for six hours compared to non-depleted cells (n=3; SC medium at 30°C). log2(fold change) values are displayed against log10(adjusted P values). Data points of α-pheromone encoding transcripts (MFα1 and MFα2), as well as, the small heat shock protein encoding transcripts (HSP26 and HSP42) are highlighted. (B) Bar chart of log2(fold change) values of pheromone-responsive genes. Strongest increase for Factor-Induced Gene, FIG1 (about 109-fold), and Pheromone-Regulated Membrane protein, PRM2 and PRM3 (about 34-fold and 18-fold). Pheromone-responsive Ty3 retrotransposon genes are also included (YGR109-W-A, YGR109-W-B, and YIL082W-A). (C) Comparison of number of differentially expressed genes from other RNA-based studies conducted in not4∆ deletion strain under comparable conditions (Cui et al. 2008, Azzouz et al. 2009), to data obtained from this study. Bar chart displays total number of genes, as well as, number of up- and down-regulated genes. (D) Bar chart of GO Term Finder results for the category "Biological Process" from differentially expressed genes in Not4-depleted cells. Results are sorted by increasing P values. P value cut-off <0.05.

Figure 3: Halo assays for determination of α-pheromone sensitivity and invasive growth in dependency on Not4.

(A) Representative halo assay results for control cells (AFB2) and AID*-NOT4 cells. Circular Whatman papers were placed onto spread cell layers on YPD and YPD IAA plates, and soaked with 20 μM α-pheromone diluted in ethanol. (B) Bar chart of quantified areas affected by α-pheromone sensitivity and invasive growth (n = 3) according to A and C. One-way RM ANOVA resulted in significantly increased α-pheromone sensitivity of Not4-depleted (A, AID*-NOT4 bottom) compared to control cells (A, AFB2 top) (P value: 0.032). Invasive growth was significant in Not4-depleted (C, AID*-NOT4 bottom) compared to cells without Not4-depletion (C, AFB2 top, AFB2 bottom, and AID*-NOT4 top; P value: <0.001). (C) Same plates as in A, but after Whatman paper has been removed and cell material has been washed off to reveal agar invasion. (D) Schematic representation of the MAPK pathways carrying out pheromone response (left) and invasive growth (right). Pheromone response pathway activation upon α-pheromone binding (α) to G-protein coupled receptor (Ste2), leads to decoupling of the Gβγ heterodimer (Ste4 and Ste18) from the inhibitory Gα subunit (Gpa1), and subsequent activation of the MAPKKK Ste11, MAPKK (Ste7), and MAPK Fus3, organized by the scaffolding protein Ste5. Activated Fus3 then phosphorylates the repressors Dig1 and Dig2, and transcription factors Ste12 and Tec1. Fus3-mediated phosphorylation of Dig1/2 and Tec1 leads to their destabilization, thus leaving the Ste12 homodimer for target gene activation (PRE: pheromone-responsive element). Invasive growth in haploid cells is activated upon glucose starvation, which is sensed by the membrane-bound proteins Msb2 and Sho1. Perceived signal is transduced to the same MAPKKK and MAPKK,
but results in the activation of the MAPK Kss1, which in turn phosphorylates Dig1, Ste12, and Tec1. Kss1-mediated phosphorylation of Tec1 activates the transcription factor, in contrast to Fus3. Upon release from Dig1-repression, the Ste12-Tec1 heterotrimer activates target genes (FRE: filamentous responsive genes).

(E) Same plates as in Fig. 2 A, but after cell material has been washed off.

Figure 4: Microarray analyses on amino acid deprived samples of non-depleted and Not4-depleted cells.

(A and B) Volcano plot representations of microarray data obtained from amino acid deprived cells non-depleted (A) or depleted of Not4 (B) for six hours compared to non-depleted cells (n=3; SC medium at 30°C). log_{2}(fold change) values are displayed against -log_{10}(adjusted P values). Circle marking the most striking difference between the two sets. (C) Number of differentially expressed genes upon amino acid withdrawal for non-depleted control (total 939) and Not4-depleted cells (total 568), with respect to increased and decreased abundance. (D) Bar chart of genes assigned to GO term "ribosome biogenesis" that displayed significant decrease in either group without the other (control or Not4-depleted). (E and F) Volcano plot representations according to A and B, but with the restriction to genes assigned to GO term "ribosome biogenesis". (G and H) Volcano plot representations according to A and B, but with the restriction to genes assigned to GO term "ribosome". Ribosomal protein genes significantly depleted are marked.

Figure 5: Polysome profiling results on amino acid deprived samples of non-depleted, Not4-depleted and not4Δ cells.

(A) Representative polysome profile recordings of non-depleted (control), Not4-depleted (1 mM IAA), and not4Δ cells, with (black, SC medium) and without (gray, CM medium) amino acids, as well as, the respective overlay of the graphs. Different fractions are indicated below, and gradients increased in density from left to right. Overlay of the graphs showed translational repression upon amino acid withdrawal as indicated by a relative increase of the 80 S peak and decrease of polysomal peaks. (B) Bar chart of quantified ribosomal fractions (40 S, 60 S, 80 S, and polysomes) on a percentage basis relative to total ribosomal fractions with mean values ±SD (n = 3). (C) Bar chart of the quantification of Not4 protein levels (α-FLAG) according to loading control (G6PDH). A paired t-test yielded a significant difference for Not4 depletion (with amino acids (+aa) two-tailed P value: 0.02; without amino acids (-aa) two-tailed P value: 0.04). (D) Bar chart of detected decrease of polysomes according to (B).
Supplemental Figures

Supplemental Figure 1

A) AID*-NOT4 30°C

B) AID*-NOT4 37°C

C) AID*-NOT4 30°C

D) AID*-NOT4 37°C

E) AID*-NOT4 30°C, RFA1-AID* 30°C, AID*-NOT4 37°C, RFA1-AID* 37°C

F) RFA1-AID* 30°C, control, 1 mM IAA, RFA1-AID* 37°C, control, 1 mM IAA

G) α-FLAG, α-G6PDH
Supplemental Figure 3

A

GO term finder: Biological Process (control, down)
Supplemental Figure 4

A  GO term finder: Biological Process (Not4-depleted, down)
Supplemental Figure 5

A GO:0008152 metabolic process - control

B GO:0008152 metabolic process - Not-depleted

C YeastCyt Top10

D

Amino acid withdrawal

Not4-depletion

Swi5

Ace2
Bas1 Gen4
Msn2 Msn4 Sfp1
Ste12 Tec1 Yap1

Sok2
Appendix

Supplemental figure legends

Supplemental Figure 1
(A) Growth AID*-NOT4 (MATa) cells in liquid culture at 30°C under non-depletion (-IAA) and depletion (+IAA (1mM)) conditions. OD₆₀₀ was monitored after IAA addition over eight hours. (B) Same as in A, but at 37°C. (C) Quantification of immuno detection of Not4 protein levels (α-FLAG) normalized to α-G6PDH, and relative to t₀ of non-depleted (-IAA) and depleted (+IAA) protein samples from whole lysates prepared from samples according to A. Significant reduction upon IAA treatment was observable after two hours (n = 3; SD -IAA ±0.21; SD +IAA ±0.08; One-way RM ANOVA: P value <0.05). (D) Quantification of immuno detection of Not4 protein levels (α-FLAG) normalized to α-G6PDH, and relative to t₀ of non-depleted (-IAA) and depleted (+IAA) protein samples from whole lysates prepared from samples according to B (n = 3). (E) Determined doubling times within eight hours were 2.2 hours for untreated, and significantly increased to 2.8 hours for treated AID*-NOT4 cells (30°C; n = 3; SD -IAA ±0.01; SD +IAA ±0.03; One-way RM ANOVA: P value <0.001). In contrast to growth at 37°C, where doubling times within eight hours were 2.7 hours for untreated, and 2.7 hours for treated AID*-NOT4 cells (37°C; n = 3; SD -IAA ±0.07; SD +IAA ±0.06; One-way RM ANOVA: P value 0.669). Doubling times within eight hours of RFA1-AID* cells: 2.4 hours and 3.6 hours at 30°C (30°C; SD -IAA ±0.01; SD +IAA ±0.08), 10.2 hours, and 16.4 hours at 37°C for non-depleted and depleted cells, respectively (37°C; SD -IAA ±2.35, SD +IAA ±3.66). (F) Representative immunoblot of non-depleted (control) and Rfa1-depleted (1 mM IAA) RFA1-AID* cells at 30°C and 37°C. Immunoblot detection was carried out as described, with the exception that α-FLAG detection represented Rfa1 protein. Rfa1 protein depletion was significant at 30°C (n = 3; SD -IAA ±0.42; SD +IAA ±0.08; One-way RM ANOVA: P value: 0.006), but not at 37°C (n = 3; SD -IAA ±0.21; SD +IAA ±0.14; One-way RM ANOVA: P value: 0.256). Untreated RFA1-AID* cells displayed significantly reduced protein levels at 37°C (compared to 30°C) (One-way RM ANOVA: P value: 0.025). (G) Growth analysis in liquid medium of wildtype and not4Δ cells (YPD 30°C). OD₆₀₀ was measured every 90 minutes over a total period of 7.5 hours. Bar charts of determined doubling times were 2.7 hours for wildtype (n = 3; SD ±0.02) and significantly increased to 5.6 hours for not4Δ (n = 3; SD ±0.15; One-way RM ANOVA: P value: <0.001) cells.

Supplemental Figure 2
(A) Bar chart of GO Term Finder results for the category "Biological Process" from significantly enriched genes in non-depleted cells upon amino acid withdrawal. Results are sorted by increasing P values. P value cut-off <0.05. (B) As in A, but for Not4-depleted cells upon amino acid withdrawal.

Supplemental Figure 3
Bar chart of GO Term Finder results for the category "Biological Process" from significantly depleted genes in non-depleted cells upon amino acid withdrawal. Results are sorted by increasing P values. P value cut-off <0.05.

Supplemental Figure 4
Bar chart of GO Term Finder results for the category "Biological Process" from significantly depleted genes in Not4-depleted cells upon amino acid withdrawal. Results are sorted by increasing P values. P value cut-off <0.05.

Supplemental Figure 5
(A) Volcano plot representation of microarray data obtained from non-depleted cells after amino acid withdrawal according to Fig. 4 A, but with the restriction to genes assigned to GO term "metabolic process". (B) As in A, but for Not4-depleted cells according to Fig. 4 B. (C) Top10 results for Yeastract.com (Rank Genes by TF, default settings, all TFs) for differentially expressed genes in Not4-depleted cells (left), and amino acid deprived non-depleted (middle) and Not4-depleted cells (right). (D) Overlap of Top10 transcription factors according to C.
12. Danksagung

"Evil is the force that believes its knowledge is complete." (Jordan Peterson)

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