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The role of the peptidyl prolyl isomerase Rrd1 in the transcriptional stress response

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Cette these intitulée:

The role of the peptidyl prolyl isomerase Rrd1 in the transcriptional stress response

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RÉSUMÉ

La régulation de la transcription est un processus complexe qui a évolué pendant des millions d'années permettant ainsi aux cellules de s'adapter aux changements environnementaux. Notre laboratoire étudie le rôle de la rapamycine, un agent immunosuppresseur et anticancéreux, qui mime la carence nutritionelle. Afin de comprendre les mécanismes impliqués dans la réponse a la rapamycine, nous recherchons des mutants de la levure *Saccaromyces cerevisiae* qui ont un phenotype altérée envers cette drogue. Nous avons identifié le gène *RRD1,* qui encode une peptidyl prolyl isomérase et dont la mutation rend les levures très résistantes à la rapamycine et il semble que se soit associé à une réponse transcriptionelle alterée. Mon projet de recherche de doctorat est d'identifier le rôle de Rrd1 dans la réponse à la rapamycine. Tout d'abord nous avons trouvé que Rrd1 interagit avec l'ARN polymérase II (RNAPII), plus spécifiquement avec son domaine C-terminal. En réponse à la rapamycine, Rrd1 induit un changement dans la conformation du domaine C-terminal *in vivo* permettant la régulation de l'association de RNAPII avec certains gènes. Des analyses *in vitro* ont également montré que cette action est directe et probablement liée à l'activité isomérase de Rrd1 suggérant un rôle pour Rrd1 dans la régulation de la transcription. Nous avons utilisé la technologie de ChIP sur micropuce pour localiser Rrd1 sur la majorité des gènes transcrits par RNAPII et montre que Rrd1 agit en tant que facteur d'élongation de RNAPII. Pour finir, des résultats suggèrent que Rrd1 n'est pas seulement impliqué dans la réponse à la rapamycine mais aussi à differents stress environnementaux, nous permettant ainsi d'établir que Rrd1 est un facteur d'élongation de la transcription requis pour la régulation de la transcription *via* RNAPII en réponse au stress.

Mots clés: ARN polymérase II, rapamycine, peptidyl-prolyl isomérase, Immuno-precipitiation de chromatine sur micropuce, régulation transcriptionelle, élongation

ABSTRACT

Transcriptional regulation is a complex process that has evolved over millions of years of evolution. Cells have to sense environmental conditions and adapt to them by altering their transcription. Herein, we study the role of rapamycin, an immunosuppressant and anticancer molecule that mimics cellular starvation. To understand how the action of rapamycin is mediated, we analyzed gene deletion mutants in the yeast *Saccharomyces cerevisiae* that have an altered response to this drug. Deletion of *RRD1*, a gene encoding a peptidyl prolyl isomerase, causes strong resistance to rapamycin and this was associated with a role of Rrd1 in the transcriptional response towards rapamycin. The main focus of my PhD was therefore to unravel the role of Rrd1 in response to rapamycin. First, we discovered that Rrd1 interacts with RNA polymerase II (RNAPII), more specifically with its C-terminal domain and we showed that in response to rapamycin, Rrd1 alters the structure of this C-terminal domain. This phenomenon was confirmed to be directly mediated by Rrd1 *in vitro*, presumably through its peptidyl prolyl isomerase activity. Further, we demonstrated that Rrd1 is capable of altering the occupancy of RNAPII on genes *in vivo* and *in vitro*. With the use of ChIP on chip technology, we show that Rrd1 is actually a transcription elongation factor that is associated with RNAPII on actively transcribed genes. In addition, we demonstrate that Rrd1 is indeed required to regulate the expression of a large subset of genes in response to rapamycin. This data let us propose a novel mechanism by which Rrd1 regulates RNAPII during transcription elongation. Finally, we provide evidence that Rrd1 is not only required for an efficient response towards rapamycin but to a larger variety of environmental stress conditions, thus establishing Rrd1 as a transcriptional elongation factor required to fine tune the transcriptional stress response of RNAPII.

Keywords: RNA polymerase II, transcriptional regulation, peptidyl prolyl isomerase, Chromatin immunoprecipitation and chip analyis, elongation, rapamycin

TABLE OF CONTENTS

RÉSUMÉ......IV

ABSTRACT......V

TABLE OF CONTENTS......VI

LIST OF FIGURES......VIII

ABREVIATIONS......IX

DEDICATION......XII

ACKNOWLEDGMENTS......XII

1 INTRODUCTION......1

1.1 TRANSCRIPTION......1
1.1.1 Gene conservation during evolution......1
1.1.2 The transcriptional machinery......4
1.1.2.1 RNAPII structure......5
1.1.2.2 Transcription factors......6
1.1.3 Transcription initiation......8
1.2.4 Transcription elongation......9
1.1.3.1 SAGA......9
1.1.3.2 TFIIS......9
1.1.3.3 TFIIF, Elongin, Ell and Csb......10
1.1.3.4 PTEFb......10
1.1.3.5 The Paf1 complex......10
1.1.3.6 DSIF and NELF......10
1.1.3.7 Elongator and FACT......11
1.1.3.8 CTD-phosphatases......11
1.1.4 Transcription termination and polyadenylation......12
1.1.5 A central role of the RNAPII CTD during transcription......14
1.1.6 RNAPII arrest during transcription......15
1.1.6.1 Transcriptional blocks caused by DNA lesions......16
1.1.6.2 RNAPII ubiquitylation during transcriptional arrest......17
1.1.7 Transcriptional regulation of RNAPII......18
1.1.7.1 The target of rapamycin (TOR) signaling pathway......18
1.1.7.1.1 TORC1 regulates transcription of specific regulons......19
1.1.7.1.2 TORC1 regulates ribosome biogenesis......20
1.1.7.1.3 Differences between mammalian and yeast cells......21
1.1.7.2 The environmental stress response (ESR)......21
1.2 RRD1 AND ITS BIOLOGICAL ROLE......23
1.2.1 Introduction......23
1.2.2 Discovery of the RRD1 gene in yeast......24

1.2.3 Discovery of PTPA, the human homologue of Rrd1 .... *25*
1.2.4 Structure and function of PP2A phosphatase complexes....... *25*
1.2.5 Rrd1 is a peptidyl prolyl isomerase *26*
1.2.6 RRD2 .... *30*
1.2.7 Structure of Rrd1....... *31*
1.2.8 Rrd1 interacts with phosphatases in vivo....... *33*
1.2.9 Rrd1 is involved in transcriptional regulation in response to rapamycin *34*
1.2.10 Role of Rrd1 in rapamycin resistance....... *35*

2 ARTICLES.......36

2.1 Rrd1 isomerizes RNA polymerase II in response to rapamycin....... 36
2.2 The yeast peptidyl prolyl isomerase Rrd1 is an elongation factor required for transcriptional stress responses 67

ABSTRACT 69

3 DISCUSSION 112

3.1 Rrd1 acts independently of the Sit4-Tap42 complex 112
3.2 Rrd1 interacts with RNAPII 113
3.3 Rrd1 isomerizes the CTD of RNAPII 114
3.4 Rrd1 modulates transcription in response to rapamycin....... 115
3.5 Rrd1 is a transcription elongation factor 116
3.6 Rrd1 is required for a broad range of stresses....... 118
3.7 Model of RNAPII regulation 119
3.8 The catalytic activity of Rrd1 122
3.9 Alternative targets of Rrd1....... 123
3.10 Involvement of Rrd1 in regulatory pathways....... 124
3.11 S.cerevisiae as a model....... 127
3.12 Is the TORC1 complex regulated by superoxide anions? 128

4 CONCLUSION 131

4.1 Rrd1 is a transcription elongation factor required for the ESR in yeast....... 131
4.2 The reason why rrd1Δ mutants have pleiotropic phenotypes....... 132
4.3 Rrd1 is the second PPIase that isomerizes the CTD of RNAPII....... 133
4.4 The new role of Rrd1 might be conserved during evolution....... 134
4.5 Rrd1 as a potential drug target 135
4.6 Rrd1 has multiple cellular roles 135

5 PERSPECTIVES 137

6 REFERENCES 142

LIST OF FIGURES

FIGURE 1 PRE-INITIATION COMPLEX FORMATION AT THE PROMOTER 7

FIGURE 2 TRANSCRIPTION CYCLE OF RNAPII...15

FIGURE 3 REGULATION OF TRANSCRIPTION BY TOR1 SIGNALING.....................20

FIGURE 4 MODEL OF PROLINE CIS- TRANS ISOMERISATION BY PPIASES 27

FIGURE 5 PHYLOGRAM OF PEPTIDYL PROLYL ISOMERASE FAMILY 30

FIGURE 6 CRYSTAL STRUCTURE PTPA...33

FIGURE 7 SEQUENCE ALIGNMENTS OF PTPA HOMOLOGUES...............................33

FIGURE 8 MODEL OF RNAPII REGULATION..122

FIGURE 9 THE RRD1 CATALYTIC MUTANT (D200G) 123

FIGURE 10 RRD1 REGULATES RIBI GENES AND RP GENES....................................125

ABREVIATIONS

4NQO	4-nitro quinolone oxide
5.8S	5.8 Svedberg
6-4PP	6-4 photoproduct
6AU	6-azauracil
ATP	Adenosine triphosphate
Ca^{2+}	Calcium
CD	circular dichroism
ChIP	chromatin immunoprecipitation
CPD	cyclobutane pyrimidine dimers
Crf1	corepressor of Fhl1
CSA	cocayne syndrome factor A (yeast gene is Rad28)
CSB	cocayne syndrome factor B (yeast gene is Rad26)
CTD	C-terminal domain of RNAPII
CUT	cryptic unstable transcript
Def1	RNAPII degradation factor
DNA	desoxyribonucleic acid
DSB	double strand break
DSIF	Elongation factor formed by Spt4 and Spt5
EF	elongation factor
Ell	elongation factor (eleven–nineteen-lysine-rich leukemia)
ESR	environmental stress response
Ess1	Peptidyl prolyl isomerase in yeast homologue of Pin1
FACT	**fa**cilitates **c**hromatin **t**ranscription
Fcp1	CTD phosphatase in yeast
Fhl1	transcripion activator
FK506	Tacrolimus (an immunosupressant)
FKBP	peptidyl prolyl isomerase family
Gal4	Transcription factor that activates GAL genes
GGR	global genomic repair
Gln3	Transcription factor that regulates NCR genes
GST	glutathione S-transferase
GTF	general transcription factor
GTP	guanine triphosphate
HAT	Histone acetyl transferase
HDAC	Histone deacetylase
Ifh1	interactor of Fhl1
Mep2	ammonium permease induced by NCR
MMS	Methyl-Methane sulfonate
MPA	Mycophenolic acid
mRNA	messenger RNA
Msn2/Msn4	Transcription factor activating STRE
NaAs	sodium arsenite
NaCl	sodium chloride
NAD+	oxydized form of nicotinamide dinucleotide
NCR	nitrogen catabolite repression
NELF	**n**egative tanscription **el**ongation **f**actor (not found in yeast)

NER	nucleotide excision repair
NMR	nuclear magnetic resonance
Npr1	kinase that prevents nitrogen permease degradation
PAF	RNAPII associated factor
PI3K	phosphoinositide-3 kinase
PIC	pre-initiation complex
Pin1	PPIase, human homologue of Ess1
PP2A	phosphatase 2A
Ppg1	PP2A like phosphatase involved in glycogen metabolism
Pph3	PP2A like phosphatase involved in DNA repair
PPIase	peptidyl prolyl isomerase
PTPA	PP2A phosphotase activator
RNA	ribonucleic acid
RNAPII	RNA polymerase II
ROS	reactive oxygen species
Rpb1	yeast major subunit of RNAPII
Rpb2	yeast second largest subunit of RNAPII
Rrd1	resistant to rapamycin deletion 1
Rrd2	resistant to rapamycin deletion 2 paralogue of Rrd1 in yeast
rRNA	ribosomal RNA
RTG	retrograde signalling genes
Rtg1/2	Transcription factor regulating the RTG
Sit4	PP2A related phosphatase
snoRNA	small nucleolar RNA
snRNA	small nuclear RNA
Spt15	the yeast gene of TBP
Ssu72	Supressor of Ua7 gene 2 (serine 5 phosphatase in yeast)
STRE	Stress regulated genes
TAF	TBP associated factor
Tap42	PP2A associated protein involved in Tor1 signalling (yeast)
TBP	Tata box binding protein
TCA	tricarboxylic cycle (crebs cycle)
TF	transcription factor
TFIIS	transcription elongation factor (dst1 in yeast)
TOR	target of rapamycin
Tor1	target of rapamycin 1 gene in yeast
tor2	target of rapamycin 2 gene in yeat
TORC1	TOR complex contains Tor1,Kog1, Tco89 and Lst8
TORC2	TOR complex contains Tor2, Avo1, Avo2, Avo3, Bit61, and Bit2,
tRNA	transfer RNA
TSS	transcription start site
UAS	upstream activation sequence
Ub	Ubiquitin
UV	ultra violet light
XPA	NER factor recognizes and binds damaged DNA
XPF	NER factor single strand endonuclease
XPG	NER factor single strand endonuclease

DEDICATION

This thesis is dedicated to my parents and my aunts (Theresia, Christel and Magda)
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1 INTRODUCTION

The transcription of mRNA encoding for proteins from template DNA is a phenomenon that is required for the most basic forms of life, from simple to complex organisms. Transcription has to be regulated, meaning that the right mRNA has to be produced in the right amount, at the right moment and if possible without mistakes. Thus, in response to every possible condition, a cell must precisely control the production of specific mRNAs. To perform such an enormous task, various levels of transcriptional regulation have evolved, where multiple factors influence transcription at virtually every step of the process. One can imagine that a failure of this highly regulated process is not compatible with life and can lead to a multitude of diseases including different forms of cancer. Therefore, studying this fundamental process will lead to further understanding of life as well as pathologies and disease treatment.

An outline of the basic steps of transcription, its general regulation as well as more specific regulatory mechanisms in response to different cellular conditions will be described in this section. The second part of this article will then focus on the functions of the peptidyl prolyl isomerase Rrd1, which we have now shown to play a role in transcription.

1.1 Transcription

1.1.1 Gene conservation during evolution

During the long evolutionary process from yeast to man, the signaling pathways that mediate transcription and other relevant processes described here, have undergone some changes and as genes evolved, some were replaced or new genes with additional functions were created. Since the thesis focuses only on work in yeast, a table detailing the conservation of the genes (comparison of the *S.cerevisiae* and human genome) that are described in the introduction is provided below.

If the gene is not found in one or the other genome it is labeled as not determined (N.D.), which could mean that this gene is not (yet) discovered, that it is not conserved, or it was created in later stages of evolution and is not present in *S.cerevisiae*.

Class	Mammalian gene	Yeast gene	brief description
RNAPII			RNA polymerase II consists of 12 subunits
	hRpb1	Rpb1	yeast major subunit of RNAPII contains the C-terminal domain
	hRpb2	Rpb2	yeast second larbest subunit of RNAP II , is also part of the active site
	hRpb3	Rpb3	orthologue of the alpha factor of the bacterial RNAP together with Rpb11
	hRpb4	Rpb4	forms a subcomplex with Rpb7 and is involved in the stress response
	hRpb5	Rpb5	shared subunit of RNAPI, II and III is required for transcription activation
	hRpb6	Rpb6	shared subunit of RNAPI, II and III is required for assembly and stability of the complex
	hRpb7	Rpb7	form a subcomplex with Rpb4
	hRpb8	Rpb8	shared subuit of RNAPI, II and III, binds to oligonucleotides
	hRpb9	Rpb9	contains a zinc binding motif, supposed to be involved in elongation and start site selection
	hRpb10	Rpb10	shared subunit of RNAPI, II and III
	hRpb11	Rpb11	orthologue of the alpha factor of the bacterial RNAP together with Rpb3
	hRpb12	Rpb12	shared subunit of RNAPI, II and III
GTFs			
TFIID	TBP	Spt15	TATA-binding protein
	TAFII250	TAFII145/130	Involved in promoter binding, G1/S progression; histone acetyltransferase; kinase (human)
	CIF150	Tsm1	Involved in promoter binding; mutations arrest in G2/M of cell cycle (yeast)
	TAFII130/135	N.D.	Involved in interaction with activators
	TAFII100	TAFII90	Mutations can cause arrest in G2/M of cell cycle (yeast)
	TAFII70/80	TAFII60	Similar to histone H4; binds downstream promoter elements (DPEs) (Drosophila)
	TAFII31/32	TAFII17	Similar to histone H3; interacts with p53
	TAFII20	TAFII68/61	Similar to histone H2A
	TAFII15	N.D.	Similar to histone H2A; highly similar to TAFII20
	TAFII28	TAFII40	Similar to histone H3; contains atypical histone fold motif seen in Spt3-like transcription factors
	TAFII68	N.D.	Contains consensus RNA binding domain; can bind RNA and ssDNA;
	TAFII55	TAFII67	Interacts with numerous activators
	TAFII30	TAFII23/25	Mutations can cause arrest in G1/S of cell cycle (human)
	N.D.	TAFII47	No homologous subunit identified in metazoans
	N.D.	TAFII30	Shared with TFIIF (yeast), no homologous subunit identified in metazoans
	TAFII18	TAFII19	Similar to histone H4; contains atypical histone fold motif seen in Spt3-like transcription factors
	TAFII105	N.D.	B-cell specific; related to TAFII130; co-activator for NF-kappaB
TFIIA	TFIIAα	Toa1	Involved in transcriptional coactivation; involved in stabilizing TBP-DNA interactions
	TFIIAβ	N.D.	TFIIAα and TFIIAβ result from processing of a single peptide that is homologous to Toal.
	TFIIAγ	Toa2	Involved in activator interactions, TFIIA-mediated antirepression, stabilizing TBP-DNA interactions
TFIIB	TFIIB	Sua7	Involved in start site selection, promoter binding and promoter bending during initiation
TFIIF	RAP74	Ssu1	Makes extensive contacts with DNA to position the template during initiation
	RAP30	Tfg2	Binds RNA polymerase II and suppresses non-specific DNA binding
	N.D.	Anc1	Common component of yeast TFIID, TFIIF, and Swi/Snf; similar to ENL and AF-9 proteins
TFIIE	TFIIEa	Tfa1	Interacts with TFIIH; involved in recruitment, stimulation of TFIIH and promoter opening
	TFIIEb	Tfa2	Double strand DNA binding activity
TFIIH	p62	Tfb1	Required for nucleotide excision repair; target for activators
	p52	Tfb2	Required for nucleotide excision repair
	MAT1	Tfb3	Required for nucleotide excision repair; MAT1/Cdk7/cyclin H form the CAK subcomplex (human)
	p34	Tfb4	Required for nucleotide excision repair
	XPD/ERCC2	Rad3	5′–3′ DNA helicase; ATPase; required for DNA repair
	p44	Ssl1	Required for nucleotide excision repair; involved in DNA binding and stimulation of XPD activity
	XPB/ERCC3	Ssl2	3′–5′ DNA helicase; ATPase; essential for promoter opening and promoter escape
	Cdk7	Kin28	Kinase subunit of cyclin-dependent CTD kinase; Kin28 & Cell form the TFIIK subcomplex (yeast)
	CyclinH	Ccl1	Cyclin subunit of cyclin-dependent CTD kinase
SAGA	PCAF	Gcn5	histone acetyl transferase catalytic unit, acetylates histone H2B and H3
	ADA1	Ada1	adaptor protein required for structure of SAGA
	ADA2b	Ada2	component of SAGA
	ADA3	Ada3	transcriptional regulator of SAGA

	ND	Spt8	component of SAGA
	SPT20	Spt20	component of SAGA
	SPT7/	Spt7	component of SAGA, required for assembly
	SPT3	Spt3	component of SAGA required for transcriptonal activation
	TAFII70/80	TAFII60	TBP associated factor present in SAGA and TFIID
	TAFII100	TAFII90	TBP associated factor present in SAGA and TFIID
	TAFII32	TAFII17	TBP associated factor present in SAGA and TFIID
	TAFII30	TAFII23/25	TBP associated factor present in SAGA and TFIID
	TAFII20	TAFII61	TBP associated factor present in SAGA and TFIID
	TRRAP	Tra1	coactivator protein of SAGA and NuA4 histone acetylase complex
	SGF29	Sgf29	component of SAGA
	USP22	Ubp8	ubiquitin protease required for deubiquitination of histone H2B
	ATXN7L3	Sgf11	associates Ubp8 to SAGA
	ENY2	Sus1	required for mRNA export and transcription elongation component of SAGA
	ATXN7	Sgf73	component of SAGA required for PIC assembly
	ND	Chd1	chromatin remodeling factor associated with SAGA
TFIIS	TFIIS	Dst1	Transcription elongation factor, restarts RNAPII after arrests, mRNA cleavage stimulatory activity
DSIF	DSIF	Spt4, Spt5	elongation factor composed of Spt4 and Spt5
	NELF	N.D.	**n**egative tanscription **el**ongation **f**actor
FACT	SSRP1	Spt16	**fa**cilitates **c**hromatin **t**ranscription , heterodimer, remodels chromatin during transcription
	SUPTH16	Pob3	**fa**cilitates **c**hromatin **t**ranscription , heterodimer, remodels chromatin during transcription
	Paf1	Paf1	RNAP II associated factor, assists in transcription elongation
		Def1	RNAP II degradation factor
CTD			kinases and phosphatases of the RNAPII CTD
	Cdk7	Kin28	RNAPII CTD kinase phosphorylates serine 5 and serine 7 , part of TFIIH
	PTEFb	Ctk1	RNAPII CTD kinase phosphorylates serine 2
	Fcp1	Fcp1	RNAPII CTD phosphatase that dephosphorylates serine 2
	Ssu72	Ssu72	RNAPII CTD phosphatase that dephosphorylates serine 5
DNA repair			
	CSA	Rad28	cocayne syndrome factor A
	CSB	Rad26	cocayne syndrome factor B
	XPA	Rad14	NER factor recognizes and binds damaged DNA
	XPF	Rad1	NER factor single strand endonuclease
	XPG	Rad2	NER factor single strand endonuclease
PP2A			
	α4	Tap42	PP2A associated protein involved in Tor1 signalling (yeast)
	PPP4C	Pph3	PP2A like phosphatase involved in DNA repair
	PTPA	Rrd1	PP2A phosphotase activator
	PPP6C	Sit4	PP2A related phosphatase
	Pin1	Ess1	PPIase in yeast homologue of Pin1
	N.D.	Rrd2	resistant to rapamycin deletion 2 paralogue of Rrd1 in yeast
	PPP2CA	Pph21	PP2A catalytic subunit redundant with PPH22
	PPP2CA	Pph22	PP2A catalytic subunit redundant with PPH21
	N.D.	Ppg1	PP2A like phosphatase involved in glycogen metabolism
PPiases			
	Pin1	Ess1	Parvulin family member, isomerizes phospho-prolines, involved in multiples diseases
	PTPA	Rrd1	the new familiy of PPIases, so far known to regulate PP2A phosphatase complexes
	FKBP1A	Fpr1	FKBP binding protein, binds to FK506 and Rapamycin , involved in protein folding
	CypA	Cpr3	Cyclophilin family member, binds to cyclosporin, involved in protein folding
TOR			TOR signaling pathway
Torc1	mTOR	Tor1	PI3like kinase that controls growth in response to nutrient , is inhibited by rapamycin
	N.D.	Tco89	part of Torc1 complex
	MLST8	Lst8	part of Torc1 and 2 complex
	Raptor	Kog1	controler of growth protein 1 part of the Torc1 complex
Torc2	mTOR	Tor2	PI3like kinase that controls spatial growth is not inhibited by rapamycin
	hSin1	Avo1	Torc2 associated factor

	N.D.	Avo2	Torc2 associated factor
	rictor	Avo3	Torc2 associated factor
	mLST8	Lst8	part of Torc1 and 2 complex
	N.D.	Bit61	binding partner of Tor2
Npr1	N.D.	Npr1	kinase that prevents nitrogen permease degradation
Mep2	N.D.	Mep2	ammonium permease induced by NCR
TFs			Transcription factors
	N.D.	Msn2/Msn4	Stress response transcription factors , binds to STRE elements
	N.D.	Rtg1/2	retrograde signaling transcription factors
	N.D.	Gal4	Galactose regulation transcription factor
	N.D.	Gln3	Nitrogen discrimination transcription factor
	N.D.	Fhl1	binds to ribosomal genes and activates transcription
	N.D.	Ifh1	transcriptional activator for ribosomal gene expression
	N.D.	Crf1	transcriptional repressor for ribosomal gene expression

1.1.2 The transcriptional machinery

Transcription is the process by which RNA is synthesized in a DNA-dependent manner. It is similar to the replication of DNA, as it makes use of a polymerase, synthesis is unidirectional and DNA is used as a template. The process can be divided into three distinct phases: initiation, elongation and termination. Unlike DNA replication, the polymerase does not need a primer but binds to specific sequences called promoters. These promoters will then drive the transcription of the gene downstream of the promoter [1].

The RNA polymerase dissociates the DNA duplex and forms a transcription bubble in which one of the separated DNA strands becomes the template for the RNA strand, forming a short DNA-RNA hybrid. This hybrid dissociates as the polymerase advances and the DNA strands reassociate, closing the bubble. In eukaryotes three different RNA polymerases (RNAP) have been identified and were named RNAPI, RNAPII and RNAPIII. Each RNAP produces specific types of RNAs: RNAPI drives the expression of only rRNA, namely 5.8S, 28S and 18S rRNA, which is required for the synthesis of the large and small subunit of the ribosome [1]. The eukaryotic rRNA genes are arranged in tandem repeats and form a few clusters within the genome. rRNA transcription accounts for about 50% of the total RNA produced in a cell [2]. RNAPIII transcribes tRNA and 5S rRNA. tRNAs are cruciform transfer RNAs which become covalently linked to a specific amino acid. They enter the ribosome and add this amino acid to a growing chain of polypeptides in a sequence specific manner during the translation of mRNA. The 5S rRNA is another part of the ribosomal complex [1]. RNAPII transcribes mRNA as well as different small RNAs including spliceosomal small nuclear RNAs (snRNA), small nucleolar RNAs (snoRNA), microRNA precursors and cryptic unstable transcripts (CUT) [3]. mRNAs are

processed and driven to a ribosome to be translated into a protein, whereas snRNAs have multiple functions in mRNA maturation, formation of heterogenous nuclear ribonucleoproteins (hnRNP), ribozymal activity, transcriptional regulation and telomere maintenance [1].

1.1.2.1 RNAPII structure

Eukaryotic RNAPII is a holoenzyme consisting of 12 individual proteins. It is highly conserved among eukaryotes and the crystallization of the yeast RNAPII has provided deep insights into the structure and function of this enzyme [4-6].

The largest subunit in yeast is called Rpb1, is homologous to the bacterial RNA polymerase and with Rpb2, the second largest unit, forms the central core of RNAPII. Within this core is located the active center, surrounded by a mobile clamp and the inner structure termed the cleft. In addition, a pore provides access from the outside to the active center [4-6].

During transcription initiation, the double stranded DNA is separated and the template strand is inserted into the cleft of RNAPII. In the active center the ribonucleotide complementary to the open DNA strand is then linked to the growing mRNA chain [4-6]. The resulting DNA-RNA hybrid leaves the active center through the pore, where it is then separated. The two loose DNA strands reform a duplex and the mRNA strand will be processed by additional factors [4-6]. Additionally, Rpb1 contains a mobile structure called the jaw that is required for efficient binding to the DNA strands. Finally, Rpb1 also contains a unique C-terminal domain (CTD) which consists of a highly conserved heptapeptide (YSPTSPS) that is repeated 26 times in yeast and up to 52 times in mammalian cells [4, 7-9]. This CTD has multiple roles throughout the transcriptional process which will be discussed in detail in section 1.1.6.

It is noteworthy that, overall, the three different RNAPs have a similar structure within their catalytic center as was revealed by structural experiments [10, 11]. However, they acquire different transcriptional properties because they associate with different subunits attached to their core enzyme. Notably, RNAPIII and RNAPI have additional subunits that are required for their transcriptional initiation and recruitment to the specific promoters [10, 11]. These different compositions also allows for the specificity of the type of RNA transcribed by each RNAP. The transcription factors (specific for each type of RNA) recruit only the RNAP which is associated with its unique subunits that recognize these transcription factors [10, 11].

1.1.2.2 Transcription factors

RNAPII itself does not recognize promoters and is instead recruited by a series of accessory proteins called transcription factors during transcription initiation [12]. Transcription factors are proteins that modulate the transcriptional process. One can distinguish between general transcription factors (GTFs), positive transcription factors (activators) and negative transcription factors (repressors). GTFs are required for the assembly and recruitment of RNAPII at the promoter as well as for transcription initiation. Stemming from 30 years of *in vitro* transcriptional studies, a model for the stepwise recruitment of the RNAPII machinery has been established. The first step is the formation of the pre-initiation complex (PIC), in which a GTF called the TATA box binding protein (TBP) binds to the promoter (Figure 1). TBP specifically recognizes the TATA box, a sequence rich in thymine and adenine upstream of the transcriptional start site (TSS) of the gene. Around 20 % of genes contain a TATA box, but the position of this box with respect to the TSS varies. The consensus sequence of the TATA box in yeast is TATA(A/T)A(A/T)(A/G) [13]. However, TBP also binds to promoters which do not contain a TATA box. TBP is part of a multiprotein complex composed of TBP associated factors (TAFs) collectively termed TFIID. This DNA-protein complex recruits TFIIA, which stabilizes the complex and recruits TFIIB, which is required for the recognition of the TSS. TFIIF, TFIIE and TFIIH are then recruited [12, 14] (Figure 1). TFIIE reorganizes the structure of RNAPII by modifying the jaw-like structure from a closed to an open position. TFIIH has three important functions: (i) its helicase activity unwinds and separates the DNA duplex; (ii) it ensures that the correct DNA strand is transcribed, and (iii) it phosphorylates the CTD of RNAPII on serine 5 of heptapeptide repeats. This forms the complete PIC, which is ready to initiate transcription [12, 14] (Figure 1).

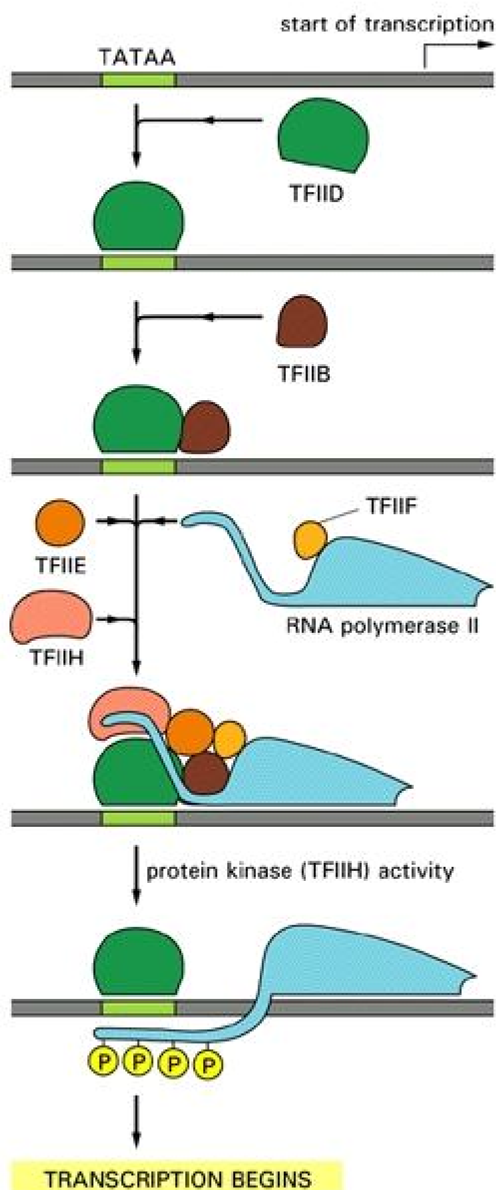


Figure 1 Pre-initiation complex formation at the promoter

(Molecular Biology of the Cell)

Activators and repressors regulate the recruitment of the PIC complex. Most activators are DNA binding proteins that recognize specific sequences within the promoter or within upstream activation sequences (UAS), which are located distantly from the promoter. These activators recruit large co-activator complexes as well as ATP dependent nucleosome remodeling complexes, both of which remodel and render the local DNA accessible. This can be done by two mechanisms, one by weakening the nucleosome-DNA interactions through acetylation of the

histone tails, and second by actively removing the histones from the DNA. As a consequence the TATA box and the transcriptional start site become more accessible to GTFs, allowing assembly of the PIC [15]. Activators can also directly bind to GTFs to promote PIC assembly, although both mechanisms of GTF recruitment are probably interrelated [12, 14].

Transcriptional repressors can inhibit the PIC formation in different ways, by competing with activators for the same sequence, by directly inhibiting GTF recruitment, or by recruiting chromatin modifying enzymes such as histone deacetylases (HDAC) to the promoter region. These HDAC complexes deacetylate histones, resulting in a more compact and less accessible form of chromatin.

In addition, the mediator, a large complex of 20 polypeptides, mediates the interaction of activators with RNAPII. It bridges distant activator sites with the PIC at the promoter and stimulates transcription. More precisely, it interacts directly with the CTD of RNAPII and can therefore mediate signaling from the activator directly to RNAPII. It is composed of several modules, which can vary depending on the cellular conditions and can provide an interface for integration with additional signaling pathways. Thus, the mediator provides an additional target for transcriptional regulation [12, 14]. The balance between activators and repressors allows for the integration of different signaling pathways towards the decision of PIC assembly. Once the PIC has been assembled successfully, transcription initation can take place [12, 14].

1.1.3 Transcription initiation

Once the PIC is assembled, TFIIH, with the help of TFIIE, recruits RNAPII onto the DNA template. This conformation is termed the open complex and once it is formed, RNAPII becomes ready to initiate transcription. Upon phosphorylation of serine 5, RNAPII clears the promoter. During transcription of the first 2-15 nucleotides, the process is often abortive and RNAPII restarts a new round of initiation. However, once the first 15 nucleotides are transcribed, RNAPII escapes the promoter and enters the processive phase of transcription elongation.

In addition, RNAPII dissociates from the GTFs TFIID, TFIIA, TFIIB and the mediator, which remain bound to the promoter. These GTFs then allow for formation of a new PIC, leading to rapid subsequent rounds of transcription [12, 14]. Elongation factors (EFs) then associate with RNAPII to regulate transcription elongation [12, 14].

1.2.4 Transcription elongation

The elongation process is complex and involves multiple factors, which are exchanged during elongation. For simplicity, the role of each elongation factor will be described separately.

1.1.3.1 SAGA

SAGA stands for Spt–Ada–Gcn5 acetyltransferase and is a multiprotein complex which has multiple roles during transcription. First, like TFIID, it can bind to TBP and be recruited to promoters [16-18]. Also, like TFIID it contains histone acetyl transferase activity and is able to recruit RNAPII and initiate transcription. Both complexes contain shared subunits necessary to associate with TBP, the TAFs [17]. TFIID and SAGA together are essential for gene expression of RNAPII but each of them is required for different gene sets. Notably, TFIID expresses house keeping genes and dominates up to 90% of all genes expressed whereas SAGA is required for stress regulated genes and is important for only 10% of the genes [16]. Interestingly, SAGA has additional roles which include regulating transcriptional elongation and linking transcription to mRNA nuclear export. It is thought that SAGA associates through the serine 5 phosphorylated CTD of RNAPII, remains associated during elongation and enhances this step through different mechanisms, notably by acetylating histones and deubiquitinating histone H2B [18]. Furthermore, SAGA is thought to assist in mRNA export through some of its subunits that recruit mRNA export factors and bring the transcription site close to nuclear pores, favoring a rapid export [18].

1.1.3.2 TFIIS

TFIIS is the first elongation factor that was found to interact with elongating RNAPII and is thought to promote elongation. TFIIS co-localizes with elongating RNAPII throughout the gene and can stimulate the intrinsic mRNA cleavage activity of RNAPII by reaching into its active center and altering its structure [19-21]. This activity is important when RNAPII stalls. RNAPII may arrest at each step of nucleotide addition. The time of arrest is variable and depends on nucleotide availability and the sequence of the DNA template. Drugs that diminish the overall nucleotide pool, such as mycophenolic acid (MPA) and 6-Aza uracil (6AU) (both of which inhibit GTP synthesis,) increase RNAPII arrests. If GTP synthesis is inhibited, the nucleotides UTP and GTP are depleted and RNAPII cannot insert the complementary nucleotides and

therefore stops transcribing the genes [22, 23]. In addition, the sequence context influences RNAPII arrest by affecting the stability of the RNA-DNA hybrid. AT-rich sequences are particularly weak and cause RNAPII arrest[22]. Upon arrest, RNAPII may backtrack 2-4 nucleotides. When RNAPII backtracks more then 7-15 nucleotides, it typically stalls irreversibly, unless TFIIS stimulates the restart of RNAPII by cleaving the mRNA [20-22]

1.1.3.3 TFIIF, Elongin, Ell and Csb

TFIIF, Elongin, Ell and CSb are additional factors that also influence the rate of transcription elongation by regulating the pausing of RNAPII during elongation [12]. These factors will be discussed in more detail in section 1.1.7.

1.1.3.4 PTEFb

PTEFb is a cyclin dependent kinase (named Ctk1 in yeast). It is a positive elongation factor that phosphorylates RNAPII during elongation on serine 2 of the CTD. PTEFb phosphorylates serine 2 after RNAPII enters the processive phase of elongation and, serine 2 remains phosphorylated until termination [7-9]. Serine 2 phosphorylation has several consequences which will be described in more detail in section 1.1.6.

1.1.3.5 The Paf1 complex

The Paf1 complex is another positive elongation complex associated with RNAPII that was initially found as being essential for the expression of some genes. It is present in yeast and higher eukaryotes including mammalians. Paf1 physically and genetically interacts with other elongation factors such as FACT and DSIF, and has multiple roles including chromatin modifications during elongation and mRNA processing [24]. It was proposed that Paf1 acts as a platform for the recruitment of other elongation factors to RNAPII [25].

1.1.3.6 DSIF and NELF

In higher eukaryotes, DSIF consists of a heterodimer of Spt4 and Spt5, and is thought to negatively regulate elongation. NELF is a multiprotein complex that interacts with DSIF and is also required for DSIF function. Together, they slow down the elongation rate *in vitro*, counteracting the positive elongation effect of PTEFb [26]. In addition, they inhibit the mRNA cleavage activity of TFIIS when RNAPII stalls. In yeast, DSIF is named and composed of Spt4 and Spt5 and they positively influence transcription. However, NELF is not found in yeast suggesting that it has evolved later during evolution [26].

1.1.3.7 Elongator and FACT

DNA is wrapped around nucleosomes and this inhibits transcription elongation, since it acts as a physical barrier for RNAPII processivity. To overcome this nucleosome barrier, the evolutionarily conserved complexes Elongator and FACT promote elongation by remodeling the chromatin as RNAPII slides along the DNA. Elongator contains a histone acetyltransferase that co-transcriptionally acetylates histones H3 and H4, which in turn diminishes the histone-DNA interaction and opens the chromatin [27].

FACT is a chromatin remodeling complex which removes the H2A-H2B dimers of the nucleosomes ahead of RNAPII in order to facilitate RNAPII movement. After passage of RNAPII, the nucleosomes are then restored by FACT [28].

1.1.3.8 CTD-phosphatases

During transcriptional initiation at the promoter, the CTD of RNAPII is highly phosphorylated on serine 5 by Kin28 which is part of TFIIH (see section 1.1.2.2), but serine 5 becomes progressively dephosphorylated in the body and end of the gene. In contrast, PTEFb phosphorylates serine 2 progressively after RNAPII leaves the promoter and until it reaches the end of the gene (see section 1.1.4.4). After the round of transcription RNAPII becomes dephosphorylated on serine 2. Several phosphatases mediate these dephosphorylations. The Ssu72 phosphatase is a component of the mRNA processing machinery that dephosphorylates serine 5 once RNAPII is processively elongating [7-9, 26]. More recently, another phosphatase, Rtr1, was shown to be required to dephosphosphorylate the serine 5-phosphorylated form and thus favor the serine 2-phosphorylated form of the CTD.

Finally, to restore the unphosphorylated form of the CTD at the end of the gene, Fcp1 phosphatase dephosphorylates serine 2 during transcription termination. This allows RNAPII to reinitiate a new round of transcription [7-9].

It was long thought that transcription elongation was a simple processive step regulated only by PIC assembly. However, during the last decade, research in this field has clearly shown that multiple factors regulate the transcription elongation process at multiple levels. Most studies have been performed *in vitro*, and only recently has chromatin immunopreciptiation (ChIP) allowed gene-specific analysis *in vivo*. The precise mechanisms underlying how these factors interact together *in vivo* remains to be elucidated [29]. However, it seems that their concerted action regulates at least two crucial events during elongation:

a) mRNA processing

The processing and maturation of nascent mRNA occurs cotranscriptionally. For example, the capping enzyme is recruited to the CTD upon serine 5 phosphorylation and mediates the cotranscriptional capping of the 5' end of the mRNA chain [30]. 3' mRNA processing also takes place during transcription and is regulated by proteins which are recruited to the CTD, such as the PAF complex [24]. In addition, the spliceosome, a ribonucleoprotein complex, is recruited during the elongation process to mediate alternative splicing of the mRNA [30]. Finally, it has even been shown that mRNA export from the nucleus and translation are regulated during elongation [31, 32]. Taken together, it seems that all known mechanisms of mRNA processing are linked to active transcription.

b) **Cotranscriptional chromatin modification**

As discussed above, the positive elongation factors FACT and Elongator facilitate the passage of RNAPII through the chromatin. However, once RNAPII has moved through the gene, the initial chromatin state must be restored; otherwise, the transcriptional machinery can be inappropriately recruited to "open chromatin" within the body of the gene. This is referred to as cryptic initiation [33]. To restore the initial chromatin state and to protect against cryptic initiation, an HDAC complex is recruited to de-acetylate the nucleosomes after passage of RNAPII. To accomplish this, the histone methyltransferase Set2 is recruited by phosphorylated serine 2 on the CTD and methylates histone H3 on lysine 36. This methylation allows the recruitment of the Rpd3 HDAC complex, which then deacetylates histones to restore a closed chromatin state [34, 35].

Chromatin modifications during transcription are also important for the recruitment of mRNA processing factors during transcriptional initiation. At every actively transcribed gene, lysine 4 of histone H3 is heavily methylated at the promoter by the histone methyltransferase Set1, which is recruited by phosphorylated serine 5 on the CTD. This mark is thought to recruit mRNA processing factors to the initiating RNAPII. [34].

1.1.4 Transcription termination and polyadenylation

The end of a gene is marked by a transcription termination site (TTS), which is specifically recognized by the ribosomal machinery [1]. However, at this site, RNAPII continues

transcribing the template strand and since there is no conserved signal for transcription termination, it can occur variably anywhere ranging from a mere few to thousands of nucleotides after the 3' end of the mature mRNA [3, 36]. As well, shortly after the TTS lies a specific polyadenylation element (AAUAAA). This and a second (GU rich) element, determine where the mRNA is cleaved off from RNAPII and where the poly(A) tail will be added [37]. For this, the cleavage specificity and polyadenylation factor (CSPF) travels along with elongating RNAPII. At the end of the gene a large polyadenylation complex is recruited, that contains the poly(A) polymerase (PAP), the poly(A) binding protein (PABP) as well as additional factors required for efficient cleavage (CstF, CFI and CFII) [37, 38]. This complex mediates the cleavage of the pre-mRNA from the still transcribing RNAPII. PAP, then, extends the poly(A) tail by adding adenines to it. PABP binds to this elongating poly(A) tail. This poly(A) tail and the associated factors have the important function of regulating mRNA stability by inhibiting 3' exonucleases that chew off the tail in the cytoplasm [37-39].

The RNAPII continues transcribing after the mRNA has been cleaved off and needs to be released from the DNA. For this, two models of termination have been proposed and current evidence suggests that termination might occur through a combination of both. First, the anti-terminator or allosteric model postulates that transcription of the polyadenylation signal induces a structural change in RNAPII and the elongation complex, causing them to dissociate and recruit termination factors. The second model is called the torpedo model and is based on the observation that the 3' mRNA is rapidly degraded after cleavage of the polyadenylation site from the mRNA. The cleavage recruits a 5'-3' exonuclease which degrades the 5' end of the uncapped mRNA associated with RNAPII, and when it rejoins the elongating RNAPII, this exonuclease dissociates the complex from the DNA and terminates transcription [3, 36].

A different termination mechanism occurs during snoRNA and snRNA transcription, since these RNAs are not polyadenylated. This mechanism involves Nrd1, a protein complex that binds to the serine 5-phosphorylated form of the CTD. Nrd1 regulates recruitment of termination factors as well as the exosome, an mRNA processing complex [3].

1.1.5 A central role of the RNAPII CTD during transcription

As discussed previously, the CTD is phosphorylated differentially along the transcription cycle. The CTD phosphorylation pattern distinguishes between the different states of transcription. A hypophosphorylated form of RNAPII is recruited to the promoter and is found during PIC assembly (figure 2). Then, during open complex formation, the CTD becomes hyperphosphorylated on serine 5 and RNAPII enters initiation. Once processive elongation is underway, serine 2 becomes phosphorylated and serine 5 becomes dephosphorylated. Finally, when termination occurs, serine 2 is then also dephosphorylated to regenerate the hypophosphorylated form and prepare for a new round of transcription (figure 2) [7-9, 14, 26].
More recently, it was shown that the CTD is also phosphorylated on serine 7. This phosphorylation was found to be enriched within the promoter region as well as towards the 3' end of the gene, following a pattern similar to that of serine 2 [40, 41]. Recent publications suggest that this phosphorylation is mediated by TFIIH and by components of the mediator complex [42, 43]. A precise role has not yet been characterized; however, enrichment towards the end of the gene suggests a role in 3' mRNA processing. Additional research will be required to understand the precise function of this phosphorylation.
The CTD has also been shown to be isomerized by peptidyl prolyl isomerases (PPIases). These enzymes catalyze the cis-trans isomerization of proline residues (see section 1.2.5 and figure 4). Since the CTD is rich in prolines, their isomerization might affect the binding of proteins to the CTD and alter the ability to phosphorylate or dephosphorylate the CTD [7-9, 44, 45]. As discussed above, numerous proteins have been shown to bind to the CTD and any of these interactions might be regulated by PPIases. Since the heptapeptide is repeated multiple times, this allows for a high number of different structural possibilities. Therefore it has been postulated that the RNAPII regulates transcription through a 'CTD-code' that is dynamic and changes as elongation goes on. This also allows for tight regulation of the transcription elongation process as well as integration of the multiple events, such as mRNA processing and chromatin remodelling, taking place during mRNA production [7-9].

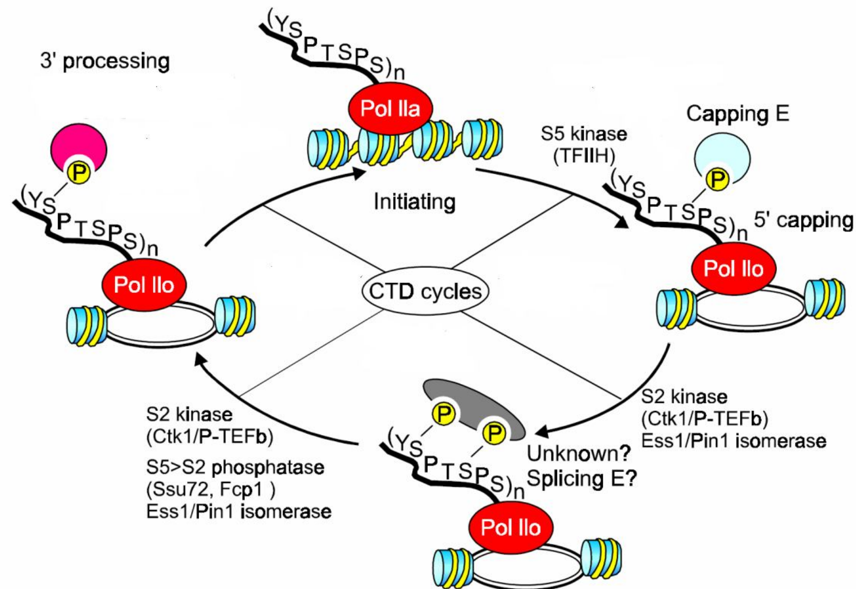


Figure 2 Transcription cycle of RNAPII

Modified from [8]. RNAPII is recruited as an unphosphorylated form during initation, becomes phosphorylated on serine 5 during the early elongation phase, then phosphorylated on serine 2 and dephosphorylated on serine 5 during the late elongation phase. The last step is the transcription termination where RNAPII is dephosphorylated on serine 2.

1.1.6 RNAPII arrest during transcription

As RNAPII transcribes the gene, various obstacles can slow down or arrest it. Several phenomena may cause RNAPII blockage: the intrinsic DNA sequence can cause slippage of RNAPII, which then backtracks and ultimately arrests [22]. Also, if the chromatin structure in front of elongating RNAPII is in a repressive state, RNAPII progression can be slowed or even blocked [28]. Finally a variety of DNA lesions can cause an irreversible block of RNAPII progression, such as cyclo butane pyrimide dimers or 6-4 photoproducts generated by UV light (see also section 1.2.1) [46].

A blocked polymerase on a crucial gene can have deleterious effects, if this gene product has vital cellular roles. Cells have therefore developed multiple mechanisms to assist RNAPII in overcoming these blocks. To counter those blockages and continue transcription, RNAPII recruits different factors, depending on the specific situation [47]. As mentioned earlier, the elongation factor TFIIS is very important for restarting backtracked and stalled RNAPII by

stimulating its intrinsic mRNA cleavage activity. Once this mRNA end is clipped off, RNAPII can restart elongation where it stopped. The importance of TFIIS is emphasized when cells are challenged with the drug 6-azauracil (6AU). This uracil analog inhibits GTP and UTP synthesis, decreases their cellular concentration and causes RNAPII to slow down during elongation [48, 49]. TFIIS mutants are highly sensitive to 6AU because TFIIS is crucial for RNAPII to restart after stalling. In fact, 6AU was used to discover a variety of elongation factors that are required for RNAPII progression, as mutants of these factors are hypersensitive to this drug [22, 23]. Besides TFIIS, there are other elongation factors such as Elongin, TFIIF and ELL that stimulate the restart of paused RNAPII as shown by *in vitro* transcriptional systems [12, 47].

1.1.6.1 Transcriptional blocks caused by DNA lesions

DNA integrity is constantly challenged by lesions caused by endogenous and exogenous factors, including reactive oxygen species (ROS) generated during normal cellular metabolism, UV light from the sun, radiation or genotoxic agents. Besides causing gene mutations, deletions and single strand or double strand breaks that are a major threat to cellular survival, certain types of lesions also block the progression of RNAPII during transcription. Throughout evolution, cells have developed mechanisms to protect themselves against these deleterious lesions. These include a variety of DNA repair pathways as well as induction of apoptosis when the damage is not reparable [46, 50] . As mentioned earlier, several lesions can cause a RNAPII blockage, for example, UV can create cyclobutane pyrimidine dimers (CPD) as well as 6-4 photoproducts (6-4PP) in DNA, which block RNAPII progression [51]. Also, bulky adducts in DNA can cause RNAPII arrest as they block the active center of RNAPII. Even byproducts of ROS: for example, malondialdehyde generated through lipid peroxidation can form guanine adducts that block elongation [46, 50]. In addition, abasic sites are known to block transcription. Abasic sites are produced during a step of the base excision repair pathway [52]. Some lesions that cause RNAPII arrest may simply be bypassed by RNAPII, although this can lead to transcriptional errors [46, 50]. To protect against lesions that cause the stalling of RNAPII during transcription, a specific DNA repair pathway is activated. This pathway is called transcription coupled repair (TCR), and is a sub-pathway of nucleotide excision repair (NER). TCR is one way to recruit the NER, which alternatively can be recruited by global genomic repair (GGR). In fact GGR and TCR are two separate mechanisms of lesion recognition which then use the NER pathway to repair the lesion [46, 50]. TCR is initiated when CSB (Rad26 in yeast) recognizes and binds to

stalled RNAPII at the lesion. CSB is loosely associated with elongating RNAPII, and upon stalling, this association becomes tighter and a second TCR factor, CSA, is recruited. Then, the NER factors that excise the lesion, XPF, XPG and XPA, are recruited. More precisely, these factors incise the lesion at the 5'end, then the intact DNA strand is replicated by a DNA polymerase while the 3' end of the DNA strand containing the lesion is cleaved off. Finally, the newly synthesized strand is ligated and the intact double stranded DNA is repaired [46, 50].

1.1.6.2 RNAPII ubiquitylation during transcriptional arrest

If RNAPII restart fails, an ultimate mechanism is activated that consists of ubiquitylation and subsequent proteasomal degradation of RNAPII. Ubiquitin (Ub) is a highly conserved polypeptide of 76 amino acids that is covalently linked to lysine residues of the target protein. The process of ubiquitylation is highly regulated via three factors, the Ub-activating enzyme that associates to Ub (E1), the Ub-conjugating enzyme E2 that recieves Ub from E1 and adds the Ub to the substrate, and finally, the Ub ligase E3, which provides substrate specificity and ligates Ub to the substrate [53]. The substrate often becomes polyubiquitylated, meaning that additional Ubs are linked to the first, eventually forming an Ub chain. This Ub chain is then recognized by the proteasome, a large complex responsible for protein degradation. RNAPII contains two lysine residues in its major subunit Rpb1 that can be ubiquitylated: K330 and K695 [54]. The ubiquitylation process originally observed in response to DNA damage seems to be a general mechanism in response to RNAPII stalling [53]. For example, mutant cells lacking the TFIIS gene accumulate high levels of ubiquitylated RNAPII when treated with 6AU [53]. One key factor required for efficient RNAPII ubiquitylation is Def1 [55]. The double deletion of *DEF1* and *TFIIS* is lethal, suggesting that both mechanisms-- RNAPII-Ubiquitylation and TFIIS-mediated mRNA cleavage-- are essential for clearing RNAPII arrest. This is further confirmed by the fact that the K330R mutation of *RPB1* is also synthetic lethal with *TFIIS*, and indeed strains containing the single deletion of *DEF1* or the K330R mutation of *RPB1* are hypersensitive to 6AU [47].

During TCR, RNAPII is also ubiquitylated by Def1. Interestingly, CSB inhibits ubiquitylation when engaging the NER pathway. RNAPII becomes ubiquitylated and degraded only if this repair fails [55]. Taken together, data suggests that RNAPII ubiquitylation and degradation is a "last chance" mechanism that is only used when other RNAPII release mechanisms fail [46, 47].

1.1.7 Transcriptional regulation of RNAPII

Cells constantly need to sense and adapt to environmental changes so that they can modify their transcriptional program accordingly. Multiple signaling pathways sense the external conditions and signal the nucleus to induce transcription of the appropriate genes for each condition. To achieve this, specific transcription factors (TFs) are required to regulate gene expression. Genes belonging to a similar pathway are generally regulated by the same TF, and these groups of genes are called regulons. For example, the galactose regulon consists of multiple genes regulated by the Gal4 TF in response to galactose. This regulon is then expressed and allows the cell to produce proteins required for galactose metabolism [56].

1.1.7.1 The target of rapamycin (TOR) signaling pathway

Cells must constantly adapt to the availability of nutrients in the environment. Nutrient levels are critical for the decision to grow and multiply or to limit consumption and metabolism. A signaling pathway conserved from yeast to human senses nutrients availability via the TOR protein kinases. When nutrients are readily available, TOR becomes active and stimulates transcription of genes involved in anabolic processes, including translation, ribosome biogenesis and gene transcription. Additionally, catabolic processes such as protein degradation and autophagy are inhibited. When nutrients are limited, TOR becomes inactive and catabolic processes, stress response genes, and G1 cell cycle arrest is activated whereas anabolic processes are repressed [57-59]. The TOR kinases are PI3K like kinases and two isoforms (Tor1 and Tor2) have been identified in yeast. Tor1 was originally discovered as being inactivated by the immunosuppressant rapamycin, a macrocyclic lactone that was isolated from the bacteria *Streptomyces hygrocopicus* on the Rapa Nui islands. Tor1 is associated with several cofactors including Kog1, Tco89, and Lst8, and together they form the rapamycin-sensitive TORC1 complex. Tor2 is found in another complex, where it is associated with additional factors Avo1, Avo2, Avo3, Bit61 and Bit2. Together they form the rapamycin-insensitive TORC2 complex [57, 59]. It is thought that the TORC1 complex regulates growth temporally (i.e. it makes the decision of whether the cell should grow and divide), whereas TORC2 regulates growth in a spatial context, meaning that it decides in which direction the cell will grow [57, 59].

In yeast, TOR signaling is controlled by amino acid levels, such as the nitrogen rich amino acid glutamine. In multicellular organisms, additional upstream signals include the insulin signaling

pathway and the platelet derived growth factor [58]. There are several downstream targets of Tor1, which will be discussed in detail below. In yeast, Tor1 signaling, like most signaling pathways, results in translocation of TFs into the nucleus to alter gene expression.

1.1.7.1.1 TORC1 regulates transcription of specific regulons

The first pathway, downstream of TORC1 signaling that was characterized includes the PP2A-like phosphatase Sit4 and its regulator Tap42. Under high nutrient conditions, TORC1 maintains Tap42 in a phosphorylated state. Upon amino acid depletion or rapamycin treatment, TORC1 becomes inactivated and Tap42 is dephosphorylated by PP2A, activating several classes of genes including stress-regulated (STRE) genes, nitrogen catabolite repressed (NCR) genes and retrograde signaling (RTG) genes [59, 60] (see figure 3). For example, Tap42 dissociates from the Sit4 phosphatase, which in turn becomes activated and dephosphorylates the cytoplasmic TF Gln3. Gln3 is retained in the cytoplasm in a phosphorylated state when TOR is active. When Gln3 becomes de-phosphorylated, it moves into the nucleus and activates NCR genes, which generate nitrogen from proline or urea [61] (see figure 3).

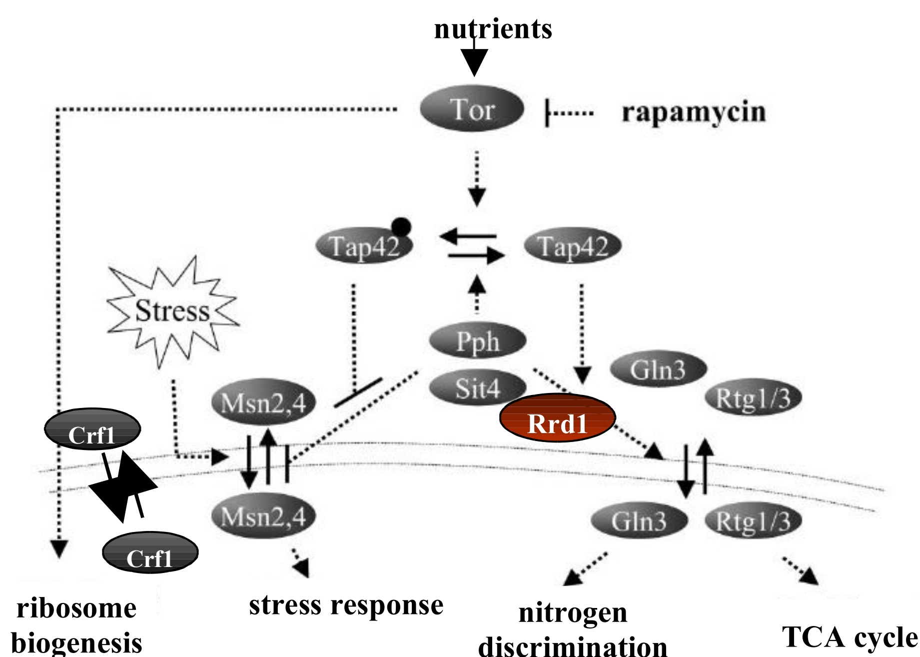
Similarly, the TFs Rtg1 and Rtg3 are retained in the cytoplasm in a phosphorylated form and upon activation of the Sit4 phosphatase; they are dephosphorylated and move into the nucleus to activate the RTG genes (see figure 3) [59, 60]. RTG genes are important for the TCA (tricarboxylic acid) cycle, which in turn is crucial for respiration and to mediate the conversion of nutrients into energy in the mitochondria. In addition, it provides molecules that are important for biosynthetic pathways. For example, α-ketoglutarate is a precursor of glutamate and glutamine, which in turn are used for nucleotide biosynthesis as well as for nitrogen containing molecules including NAD+ [62].

Finally, Tap42 is also implicated in the transcription of the STRE genes by regulating the translocation of the TFs Msn2 and Msn4. When Tap42 is active, it inhibits Sit4 and the Msn2/4 TFs are exported from the nucleus. When Tap42 is inactivated, the Sit4 phosphatase can dephosphorylate Msn2/4, causing them to stay in the nucleus [60] (see figure 3).

Msn2/4 binds to specific DNA sequences called stress response elements (STRE). STRE-containing genes are divided of several sub-classes including carbohydrate metabolism, genes required to scavenge reactive oxygen species, protein chaperones such as heat shock proteins and DNA repair proteins. Additionally in response to stress, genes are induced that regulate the transcriptional stress response with negative or positive feedback loops. For example, upon

induction of stress, transcripton of the Msn4 gene is activated, thus contributing to a faster and stronger stress response [63].

Tor1 is implicated through its regulation of Tap42 in the nuclear retention of Msn2/4, which keeps STRE gene transcription active. However, Tor1 does not regulate the translocation of Msn2/4 from the cytoplasm to the nucleus, as has been shown for other transcription factors. Therefore, additional upstream signaling pathways are required to first mediate the translocation of Msn2/4 from the cytoplasm to the nucleus in order to activate the STRE response induction [60]. Next, Tor1 may keep this STRE response active by retaining Msn2/4 in the nucleus.



Modified from Duevel et al. 2003

Figure 3 regulation of transcription by Tor1 signaling

Transcription factors are regulated by translocation from the cytoplasm to the nucleus in yeast.

1.1.7.1.2 TORC1 regulates ribosome biogenesis

Ribosome biogenesis is crucial for cellular anabolism and growth, which is in turn a prerequisite for cell cycle progression. At least 100 protein-coding genes are required as well as the combined action of the three RNA polymerases. This requires tight regulation, as the process

expends a large amount of energy [57, 59]. Upon TORC1 inactivation, *via* rapamycin treatment or in response to nutrient limitation, Tor1 moves into the nucleus and inhibits RNAPI and RNAPIII by binding to the promoter of the rDNA genes [64]. To regulate RNAPII ribosomal gene expression, Tor1 controls the nuclear translocation of Ifh1, a transcriptional activator, and Crf1, a transcriptional repressor [65-67]. When Tor1 is active, Ifh1 is associated to Fhl1, a ribosomal gene TF, and together they are bound to ribosomal gene promoters and stimulate their transcription. When Tor1 becomes inactive, Crf1 is dephosphorylated and enters the nucleus to compete with Ifh1 for binding to Fhl1 (see figure 3). Once Crf1 binds to Fhl1, it represses transcription of the ribosomal genes [67]. This mechanism seems to be specific to some strain backgrounds since it has been demonstrated that in a *crf1Δ* mutant from a W303 background, ribosomal genes are still repressed upon rapamycin treatment, suggesting that alternative mechanisms of ribosomal repression via the Tor signaling pathway must exist [68].

1.1.7.1.3 Differences between mammalian and yeast cells

In mammalian cells, anabolism is regulated through translation initiation rather than transcription. The S6K kinase and 4EBP-1 are regulated by TORC1 and are responsible for the regulation of translation initiation and mRNA production [58]. In yeast, there is no homologue of 4EBP-1 and the majority of Tor signaling is regulated through transcription [60]. Only recently has Sch9, the homologue of the S6K kinase, been identified [69]. Sch9 is required for optimal ribosomal gene expression and translation of mRNA [70].

1.1.7.2 The environmental stress response (ESR)

Yeast cells must constantly adapt to various extracellular conditions, which can cause cellular stress. Such conditions include heatshock, pH variations, changes in osmolarity, an increase in reactive oxygen species as well as toxins. To adapt to these conditions, yeast have developed a rapid response termed the ESR [63]. Within this ESR, various sensors signal changes to an intracellular signaling pathway, resulting in changes to the transcriptional program. Around 300 genes are upregulated in response to stress and 600 are repressed [71]. There is a correlation between the ESR and the severity of the stress, indicating that the ESR is tightly regulated [63, 71]. Downregulated genes include those associated with cellular anabolism and cell cycle progression as well as genes required for ribosome biogenesis. In contrast, upregulated genes include the STRE genes induced by the Msn2/4 transcription factors. Interestingly, not every condition induces exactly the same response, and not every branch of the signaling

pathway is required for every condition [63, 71]. For example, Sko1 is only activated during osmotic changes via the Hog1 signaling branch, and induces genes required for osmotic regulation. Alternatively, upon oxidative stress, the Yap1 TF is activated to regulate oxidative stress genes [63, 71]. For DNA damage response, the Mec1 kinase is activated which in turn activates Dun1 to trigger the ESR [63]. Together this suggests a network of overlapping signaling pathways that allow for a precise response to each condition [63, 71].

1.2 Rrd1 and its biological role

1.2.1 Introduction

Our laboratory is interested in the cellular responses to exogenous stresses caused by drugs used in clinical therapies. These drugs include various DNA damaging agents as well as agents causing oxidative stress or the starvation mimicking drug rapamycin. It is crucial to understand how cells respond to drug treatment in order to understand drug resistance mechanisms of cancer cells. In addition, a better understanding of cellular responses to drug treatment might provide insights into new drug targets and prevent inappropriate treatments. To study these cellular responses, yeast is an ideal model as genes can be easily deleted, allowing the analysis of their functions. Through yeast genetics, major cell activities such as transcription, cell cycle regulation, replication and DNA repair have been studied [72]. Throughout evolution, these pathways have been conserved from yeast to man, which validates the use of yeast as a model system in current molecular biology research. The yeast strain library harbouring each viable gene deletion (approx. 4800 genes of a total of 6125 genes) which is now available for the research community, allows for genome wide identification of genes important for cellular responses to stresses [73-76]. In order to study these responses, several 'model' drugs are commonly used and will be briefly described in this section:

4-nitroquinoline 1-oxide (4NQO)

This carcinogen is not used as a chemotherapeutic agent in clinics but widely in research. It is thought to cause cellular damage through two different modes of action. After entering the cell, 4NQO is activated via chemical modifications and becomes 4-hydroxyaminoquinoline, which reacts with purines and forms stable bulky adducts [77]. These bulky adducts resemble lesions caused by 254 nm UV radiation, namely cyclobutane pyrimidine dimers and 6-4 photoproducts [51, 74, 77]. Similarly to UV lesions, 4NQO-dependent bulky adducts are also recognized and repaired via the NER pathway.

The second mode of action by which 4NQO causes cellular damage is thought to be through the generation of reactive oxygen species (ROS), thereby causing intracellular oxidative stress [74,

77]. ROS cause cellular damage by altering DNA as well as through lipid peroxidation and protein oxidation [78].

Ultraviolet radiations

UV radiations have wavelengths ranging from 400 nm to 100 nm. They are divided into UVA (400-315 nm), UVB (315-280 nm) and UVC (280-100 nm); their toxicity differing depending on the wavelength. UVC radiation is mostly used at a wavelength of 254 nm for research purposes as a standard and it mainly induces cyclobutane pyrimidine dimers as well as 6-4 photoproducts, whereas UVA generates reactive oxygen species. UVB causes both types of stress, ROS becoming more prominent as the wavelength increases [51].

Gamma rays

Gamma irradiation is used in clinical oncology to treat several types of cancer. It is also used in molecular biology as it is known to induce double-strand breaks (DSB) as well as oxidative stress [79]. DSB are of high interest in research as they are very toxic to the cells and their repair is highly regulated and complex [79, 80].

Hydrogen peroxide

Hydrogen peroxide (H_2O_2) reacts with metal ions and generates ROS *in vivo,* such as superoxide anions, which are known to react with lipids, proteins and DNA [81]. It is widely used in research to induce oxidative stress in cells.

1.2.2 Discovery of the *RRD1* gene in yeast

RRD1 (rapamycin resistant deletion 1) was originally identified in a genome-wide screen for mutants hypersensitive to 4NQO but not to UVC, suggesting that this gene product might be important for the response to increased ROS but not to lesions repaired by the NER pathway. This hypothesis was further confirmed since *rrd1Δ* mutants showed sensitivity towards UVA and diamide, which both cause oxidative stress but showed no increased sensitivity to γ-radiations, MMS, UVB or UVC [74]. Taken together, these phenotypes suggest that the function of Rrd1 would be in the cellular response to oxidative stress but not to other stresses such as those caused by DNA lesions [74]. Subsequently, *rrd1Δ* mutants were shown to be highly resistant to rapamycin as well as to caffeine but sensitive to vanadate, Ca^{2+}, ketokonazole and cycloheximide. Both caffeine and vanadate are also thought to influence cellular oxidative stress,

though, through different mechanisms. It is believed that caffeine directly inhibits cellular signalling pathways including oxidative stress response pathways, but does not generate intracellular reactive oxygen species [82]. Vanadate also stimulates the stress response pathways but does so by increasing the ROS levels [83]. The difference in the resistance could therefore be explained by the hypothesis that *rrd1Δ* mutants do not adequately respond to stresses. In the case of caffeine, *rrd1Δ* mutants grow better then wild type cells since they don't activate the ESR (see section 1.1.8.2). In contrast, in response to vanadate, *rrd1Δ* mutants still do not activate the ESR but in this case, ROS will lead to cellular damage and ultimatively to cell death [82, 83]. Ketokonazole is an anti-fungal agent that interferes with ergosterol biosynthesis [84], whereas cycloheximide inhibits protein biosynthesis by interfering with ribosomal function.

1.2.3 Discovery of PTPA, the human homologue of Rrd1

The *RRD1* gene is evolutionary conserved and its human homologue is called PTPA. PTPA was originally identified as being an **A**ctivator of the *in vitro* **P**hospho-**T**yrosyl **P**hosphatase activity of PP2A phosphatases without affecting their serine/ threonine dephosphorylation activity [85, 86]. PP2A phosphatases are a class of phosphatases which have multiple cellular roles and will be described in more detail in the next section. To date, the *in vitro* phospho-tyrosyl phosphatase activity was not shown to be relevant *in vivo* [87, 88]. Rather, it was found that PTPA is crucial for the reactivation of inactive phosphatase complexes as well as for their substrate specificity and holo-enzyme assembly *in vivo* (see next section). PTPA was therefore renamed **P**P **T**wo A **P**hosphatase **A**ctivator [89, 90] .

1.2.4 Structure and function of PP2A phosphatase complexes

PP2A phosphatases complexes (PP2A) are serine/ threonine phosphatases involved in numerous cellular pathways including cell cycle, cellular morphology, DNA repair and transcription [91, 92]. PP2A is constituted of three distinct subunits, the structural A subunit which is bound to the catalytic C subunit to form a core dimer that associates with the third and regulatory B subunit [91, 92].

The structural A subunit exists in two isoforms, α and β, which share high sequence similarity. Subunit A binds tightly to the catalytic C subunit and serves as a scaffold for the recruitment of

the regulatory B subunit. Subunit C also has two isoforms (α and β) which are differentially expressed within the cell and are highly conserved throughout evolution. There are at least 4 different families of B subunits that can mutually exclusively bind to the core A-C dimer. They are named according to their molecular size: PR55, PR61, PR72 and PR93, all expressing various isoforms within their family and the different isoforms contribute to substrate specificity. The various associations between A, B and C subunits give rise to multiple different compositions of PP2A which allow for specific cellular localisation, phosphatase activity and most importantly, substrate specificity [91, 92]. Besides holo-enzyme composition, PP2A can also be regulated by post-translational modifications such as phosphorylation or methylation [91, 92]. In addition, it has been shown that although PP2A is a serine/ threonine phosphatase *in vivo,* PTPA can stimulate the low phosphotyrosyl phosphatase activity of AC dimers but not of ABC trimers in *vitro* [85, 86]. This activity requires Mg^{2+} and ATP, but it is not known wether the phosphotyrosyl stimulating activity of PTPA plays a role *in vivo* [87, 88, 91].
Subsequently, it was shown that PTPA is required to activate PP2A *in vivo* and contributes to its substrate specificity in yeast cells [89]. In addition, when PTPA is not present, the catalytic C unit is less stable and its *in vitro* phosphatase activity becomes dependent on bivalent metal ions. This suggests that PTPA might alter the structure of PP2A catalytic units [89].

1.2.5 Rrd1 is a peptidyl prolyl isomerase

Rrd1 was recently shown to posess peptidyl prolyl isomerase (PPIase) activity [93]. As mentioned above, PPIases are enzymes that catalyze the conversion of proline residues from the cis to the trans conformation. Proline isomerisation can occur spontaneously (albeit slowly) and is often a rate-limiting step during protein folding. However it can be stimulated by a PPIase [94, 95]. In addition, PPIases can switch the proline conformation when the target protein is already in a folded form. This induces a conformational change which may alter the activity or function of the target protein (see figure 4).

The molecular basis of proline isomerization

The extreme rigidity of prolines compared to other amino acids has several structural implications. Notably, prolines are normally excluded from secondary structures like alpha helices and beta strands but are found in turns. Also, most amino acid bonds are form in a trans

position, because of steric hindrance of the cis conformation. However, for an aa-Pro bond, both the cis and the trans conformations are possible. Thus both conformations are possible and each will result in a distinct structure of the same peptide. It is thought that 5 % of these bonds are in cis form when the protein is folded [96]. Since prolines are positioned mostly within turns, they are often exposed to solvent and a switch from cis to trans will result in a drastic change of the structural conformation with consequences like alteration of activity and function [96]. This has been termed a molecular switch, where cis conformation can change to trans and vice-a-versa, thereby altering the protein structure. This switch has a high energy barrier. It can occur slowly and spontaneously, or rapidly with PPIase catalysis. How exactly does PPIases mediate this switch is not yet known [96]. It has been postulated that PPIases might act as a molecular timer whereby they rapidly promote one conformation of prolines with a timely effect on the activity of this target protein. Due to conformational restriction this proline will slowly switch back to the alternate conformation causing the target protein to stop its activity [96].

To date, three classes of PPIases are known; cyclophylins, FKBP's and parvulins (see figure 5).

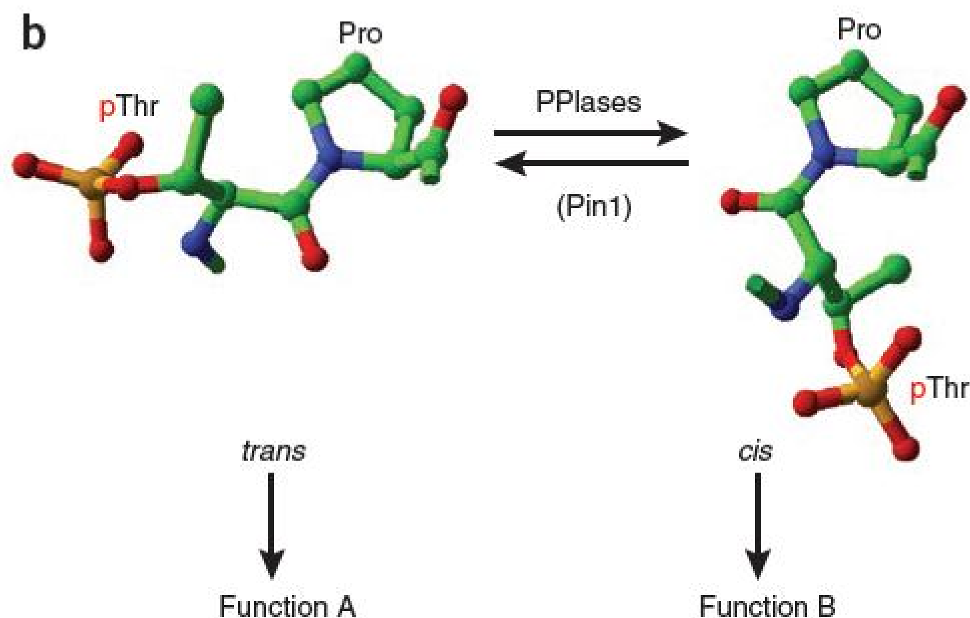


Figure 4 Model of proline cis- trans isomerisation by PPIases [96]

Cyclophilins

These are present in all living organisms. Members of this family share a conserved domain of 109 amino acids called cyclophilin like domain (CLD) [97]. Originally, cyclophilin A was identified as binding the immunosuppressant cyclosporine A from the fungi *Tolyplocadium inflatum*. In mammals this binding leads to inhibition of calcineurin, a serine /threonine phosphatase, which is required to translocate the NF-AT transcription factor in T-cells and induces transcription of interleukins including IL2 [97, 98]. Interestingly, the binding of cyclosporine A to cyclophilins is conserved throughout evolution. For example, in the yeast *S.cerevisiae,* the homologue of cyclophilin A (Cpr1) binds cyclosporine A and this is thought to inhibit growth from alpha-factor arrested cells [98]. Besides this role, cyclophilins are localized mainly in the cytoplasm and in the endoplasmatic reticulum where they are thought to assist in protein folding by isomerizing prolines. In yeast, a simultaneous knockout of all cyclophilins is still viable, suggesting that their function in protein folding is not essential [99]. Additional roles for this family have also been described; for example, Cpr1 is found in the nucleus and regulates meiosis [100].

In mammalian cells, cyclophilins are required for proper protein folding, and this is also crucial for the formation and infectivity of HIV-1 virions [101]. In addition, cyclophilins have been found to be associated with transcriptional regulators such as YY1 and steroid receptors, suggesting that besides protein folding, cyclophilins may also be involved in regulatory functions of signaling pathways [97].

FK506 Binding Proteins

These proteins were initially discovered because they bind to the immunosupressants FK506 isolated from *Streptomyces tsukubaensis* and rapamycin from *Streptomyces hygroscopicus*. Whereas binding of FKBP with rapamycin results in the inhibition of the Tor signaling pathway, the binding of FKBP to FK506 leads (similar to cyclophilin and cyclosporine binding) to the inactivation of calcineurin and inhibition of T-cell activation [102, 103]. Similar to cyclophilins, they isomerize proline residues during protein folding. FKBPs are characterized by a 108 amino acid long FK506 binding domain but vary in their other domains. Some FKBPs are additionally thought to act as chaperones, binding and sequestering misfolded proteins [104].

The fact that both the cyclophilins and FKBPs are involved in immunosuppressive activity --through binding of exogenous factors-- has lead to their common appelation of immunophilins

[104]. This also suggests that their evolutionary conservation is maintained, thus underscoring their importance in cellular function. The yeast FKBPs and cyclophilins are, however, dispensable for survival [99]. Both families are under investigation for their role in disease treatment as they can be easily inhibited by drug treatments [104]. Thus cyclophylins and FKBP's are PPIases required for proper protein folding and can isomerize a broad spectrum of peptides [93, 94].

Parvulins

Parvulins have high substrate specificity as they only isomerize prolines that are preceded by a phosphorylated serine or threonine residue [93, 94, 96, 105]. The prototype of the parvulin PPIase family is Pin1 in mammalian cells and Ess1 in *S.cerevisiae*. Numerous cellular targets of Pin1 have been identified so far, and Pin1 is thought to regulate a number of important cellular pathways, including transcription, cell cycle, DNA damage stress responses and immune responses as well as developmental roles such as germ cell maturation and neuronal differentiation [96]. In the yeast *S.cerevisiae,* Ess1 was found to isomerize the CTD of RNAPII and influence its phosphorylation status (see also section 1.1.6) [44, 45, 95, 106-108]. This mechanism is conserved during evolution and Pin1 overexpression leads to hyperphosphorylation of the serine 5 of RNAPII, its dissociation from the chromatin and accumulation into speckle like structures [95]. More recently, it was discovered that Ess1 is also involved in transcriptional termination of snoRNAs by altering the phosphorylation status of the RNAPII CTD and thereby promoting the recruitment of the NRD complex which, in turn, terminates transcription (see section 1.1.5) [44]. Interestingly an Ess1 knockout deletion is lethal and suggests that besides these roles, Ess1 might perform additional functions as well, as was found for Pin1 in mammalian cells [44].

The importance of Pin1 is further underscored by its involvement in numerous diseases, including cancer, asthma and Alzheimer's disease [96, 105].

The exact role of Pin1 in cancer is not clear yet but Pin1 is known to interact with transcription factors Jun and Fos which are both involved in cellular proliferation. It stabilizes the tumour suppressor P53 and P73 during checkpoint arrest in response to DNA damage and Pin1 knockout mice are less prone to certain types of cancer [109]. For asthma, aberrant expression or activity of Pin1 is thought to increase cytokine release, by enhancing the stability of mRNA of these

cytokines [109]. In Alzheimer's disease Pin1 plays a role by interacting with and regulating Tau and amyloid precursor protein, both of which are important in this disease occurrence [109].

RRD1

Rrd1 can isomerize prolines and has substrate specificity similar to FKBP12 and cyclophilin (Cpr7 in yeast). This PPIase activity is stimulated by ATP and Mg^{2+} [93]. Rrd1 does not isomerize substrates preceded by a phosphorylated residue, suggesting that it has different substrate specificity than Pin1 and might belong to a different family (see figure 5). Originally Rrd1 was found to isomerize a specific proline residue in PP2A (186-LQEV**P**HEGAMCDL-198), which induces a conformational change, thereby affecting its activity [93].

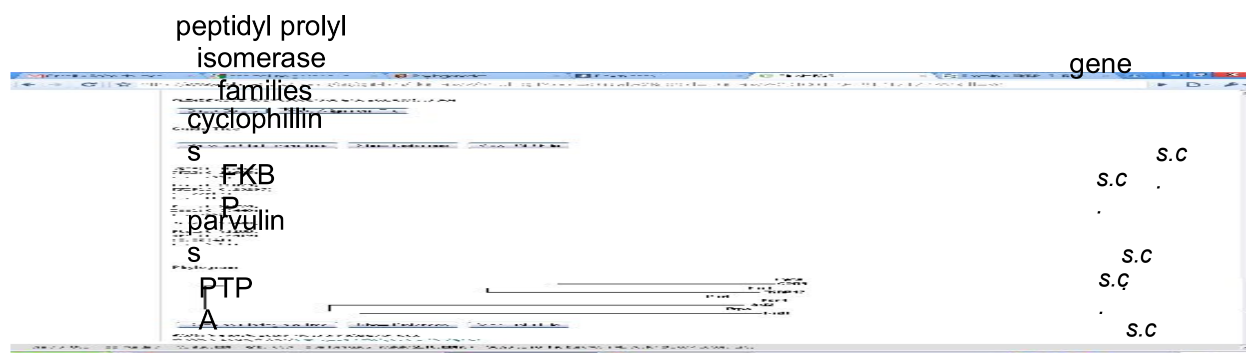


Figure 5 Phylogram of peptidyl prolyl isomerase family

The sequence alignment was generated by clustal W 2.0. s.c indicates that the gene is from *S.cerevisiae*, if not gene it is from *H.sapiens*.

1.2.6 *RRD2*

RRD2 is the yeast paralogue of *RRD1* (figure 5) and it shares 40 % of sequence identity with Rrd1 [74, 110]. *rrd2*Δ mutants are also resistant to caffeine and rapamycin (although to a lesser extend) but display no phenotype towards 4NQO, MMS and UVA [74, 110]. Rrd2 interacts with different PP2A phosphatases then Rrd1, notably Pph21 and Pph22 but might also share some overlapping function with Rrd1 [87, 93, 111]. However, when our lab performed the genome wide screen for 4NQO hypersensitive mutants that are resistant to UV radiation, *rrd2*Δ mutants were not identified and it was subsequently shown that *RRD2* is not required for the response to 4NQO [74]. Since our lab only found *RRD1* to be sensitive towards 4NQO, the

RRD2 gene was not further investigated. Interestingly, *rrd1Δ- rrd2Δ* double mutants are lethal, a phenotype which can be rescued with the overexpression of PTPA [90, 110, 112]. This suggests that they both have important cellular functions and the fact that PTPA can rescue this phenotype suggests that these functions are conserved throughout evolution. Since Rrd1 and Rrd2 both are activators of a variety of phosphatases (Sit4, Pph3, Ppg1, Pph21 and Pph22) loss of both isomerases could lead to inactivation of all these phosphatases and ultimately to cell death, a phenotype which might be suppressed if only one of these genes is deleted, as one Rrd protein might take over some of the functions of the other missing protein. This is consistent with the fact that they have some overlapping functions notably in the reactivation of inactive PP2A complexes and substrate specificity [74, 87, 93, 111].

1.2.7 Structure of Rrd1

In 2006, two research groups independently published the characterization of the crystal structure of PTPA [87, 88]. These analyses revealed important cues about the structure and function of this enzyme. Although there are some structural differences in the crystals analyzed, similar general conclusions were drawn from both publications. The PTPA structures were described differently by the two groups and only one will be elaborated here. PTPA contains 17 alpha helices and 4 beta strands. These secondary structures were further divided into three distinct subunits, the core, lid and linker (figure 6); the linker connecting the core to the lid. A large cleft is found between the lid and the core and a deep pocket is formed between the core and the linker (figure 6). This deep pocket is important for the catalytic activity of PTPA as shown by the high evolutionary conservation of the residues as well as through mutational analysis of these residues (figure 6 and 7) [87, 88]. To analyze the previously characterized ATPase activity of PTPA, Chao and coworkers co-crystallized ATP with PTPA and showed that ATP binds within the deep pocket and is maintained there by the highly conserved residues of the pocket (eg. D205G). They further showed that the ATP binding of PTPA is required for its *in vitro* phosphotyrosil phosphatase activity. Finally, they demonstrated that binding of PTPA to PP2A is required for PTPA's ATPase activity which is mediated by a surface patch of conserved residues at the lid-linker border [88]. Taken together, their results suggest that PP2A and PTPA form a composite ATPase that is required to modulate the substrate specificity of PP2A for the

dephosphorylation of phosphotyrosyl peptides [88]. The modulation of substrate specificity by Rrd1 is further confirmed by the fact that Rrd1 binding to PP2A inhibited the serine/threonine phosphatase activity of PP2A *in vitro* [88].

Leulliot and coworkers additionally demonstrated that PTPA, Rrd1 and Rrd2 have nearly identical structures [87]. They also described the deep pocket, which contains the highly conserved residues but, surprisingly, they demonstrated that this pocket is required to bind a peptide from PP2A that was previously shown to be isomerized by PTPA [87, 93]. The crystal structure further revealed that when PTPA binds this peptide, it forms a homodimer that binds two molecules of the peptide.

Finally, they concluded similarly to Chao et al., that the conserved residues in the deep pocket are required for the tyrosyl phosphatase activity of PP2A and, additionally, that this domain is also required for the resistance to rapamycin *in vivo*.

No structural homologue of PTPA has been identified to date. Interestingly, the structure of PTPA is distinct from all characterized peptidyl prolyl isomerases (see figure 5). Although it seems surprising that the same pocket binds ATP in one case and peptides in the other it clearly suggest that the highly conserved pocket is essential for PTPA catalytic activity, whether this activity is ATP hydrolysis or proline isomerisation [87, 88].

Both publications concluded that PTPA has a highly conserved and unique structure which puts it into a novel class of peptidyl prolyl isomerases [93] (figure 5).

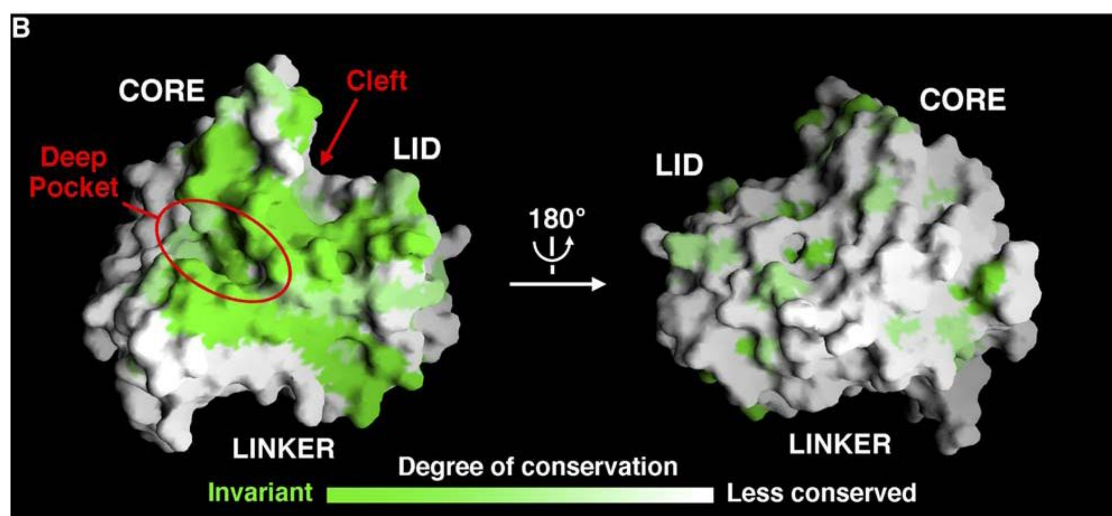


Figure 6 crystal structure PTPA, conserved residues are painted green, showing the deep pocket, the linker core and lid [88].

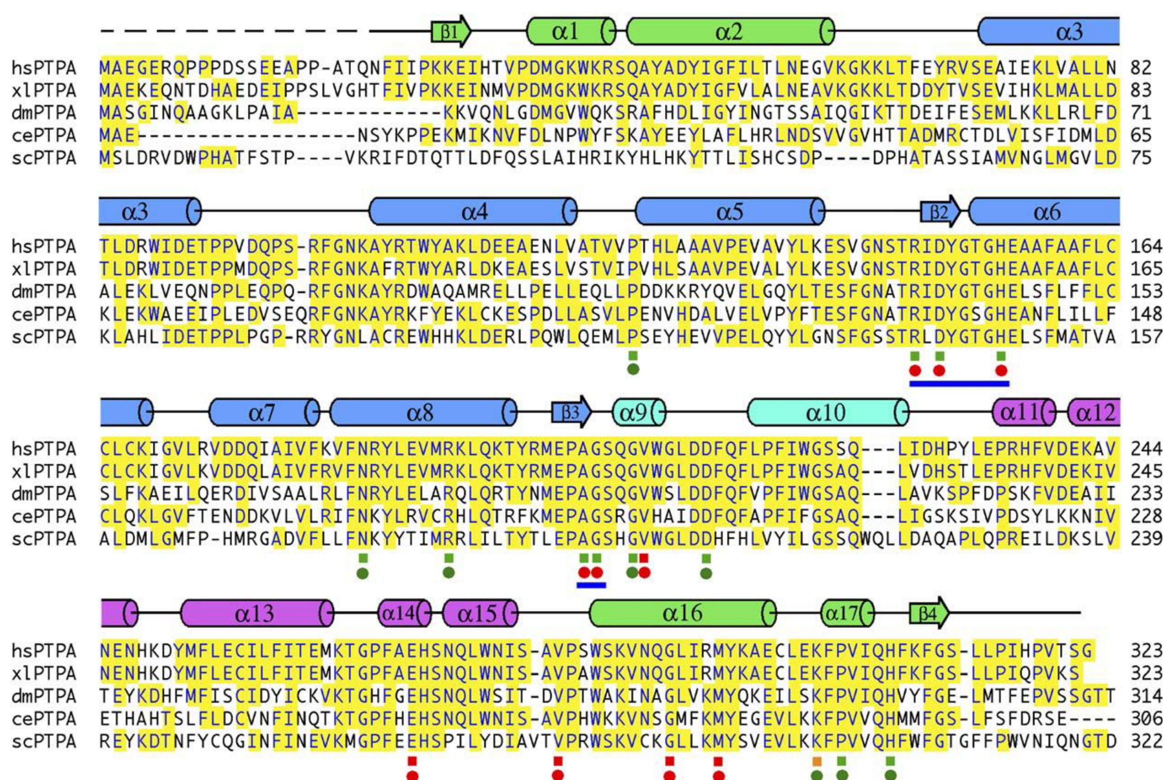


Figure 7 Sequence alignments of PTPA homologues

Mutational analysis was performed and analyzed for two distinict functions: 1. Binding of Rrd1 to PP2A as indicated by squares below the alignment if it is affected by mutation of the residue. 2. Catalytic activity of the PPIase as indicated by circles if it is affected by mutation. Colour indicates the severity , green= mild, orange= severe and red = completely abolished [88].

1.2.8 Rrd1 interacts with phosphatases *in vivo*

In yeast, it has been shown that Rrd1 interacts with different phosphatases, such as Pph3, Ppg1 and Sit4 [111-114]. The yeast PP2A catalytic C subunit is encoded by *PPH21* and *PPH22* genes whereas the catalytic A subunit is encoded by *TPD3*. Only two families of regulatory B subunits are found in yeast; PR55, that is encoded by *CDC55* and Pr61, encoded by *RTS1* [91]. There are three PP2A like phosphatases in yeast, namely Pph3 which is known to dephosphorylate H2AX [115], Ppg1, which is involved in glycogen metabolism and Sit4, which is known to be involved in the rapamycin response [113, 114, 116, 117].

Rrd1 was found to form a ternary complex with Tap42 and Sit4, and this complex dephosphorylates the TF Gln3 as part of the Tor signaling pathway [117, 118]. This would explain why *rrd1Δ* mutants are resistant to rapamycin; in the absence of Rrd1, Sit4 cannot dephosphorylate Gln3; therefore, the NDG signaling pathway is not activated. Since *gln3Δ* mutants are resistant to rapamycin, the lack of Gln3 activation might confer resistance to rapamycin to *rrd1Δ* mutant cells. However, it was found that Gln3 can be activated independently of the Sit4 phosphatase [119, 120]. In addition, we have shown that expression of a target gene of Gln3, *MEP2* was still induced in response to rapamycin in *rrd1Δ* mutants [113]. Moreover, Sit4 was able to dephosphorylate the kinase Npr1 independently of *rrd1Δ* deletion in response to rapamycin. [113]. It was previously shown that Pph3 and Rrd1 interact together [111]; however, we showed that *pph3Δ* mutant are resistant to rapamycin and that the *rrd1Δ-pph3Δ* double mutant is synergistically resistant, suggesting that they function in different signaling pathways to mediate the response to rapamycin [113]. These findings indicate that Rrd1 might have additional functions that are required for the response to rapamycin.

1.2.9 Rrd1 is involved in transcriptional regulation in response to rapamycin

We have shown that Rrd1 is required to modulate gene expression in response to rapamycin. More precisely, it was demonstrated that Rrd1 is required to not only activate gene expression of the diauxic shift genes *CPA2* and *PYC2*, but also to inhibit the expression of at least two ribosomal genes, *RPL26A* and *RPL9A*, in response to rapamycin. This suggests that more than these four genes are regulated by Rrd1. This is the case especially for ribosomal genes, since ribosome biogenesis is a coordinated and highly regulatored network which involves all three RNAPs [121]. Therefore it is likely that more then a couple of ribosomal genes are influenced by Rrd1. Further, if Rrd1 plays a role in transcriptional regulation of ribosomal genes, Rrd1 would have additional roles since it has been shown that the Tap42-Sit4-Rrd1 complex is not involved in regulation of ribosomal genes [60]. Most interestingly, and for the first time, it was shown that RNAPII was degraded in response to rapamycin over time. This could be a mechanism to drastically reduce excess RNAPII and reduce metabolic gene expression. However, when Rrd1 was deleted, RNAPII was not degraded anymore, suggesting that Rrd1 may play a direct role in RNAPII transcriptional regulation [113].

1.2.10 Role of Rrd1 in rapamycin resistance

To date Rrd1 has been shown to interact with phosphatases, an interaction that requires biochemical isomerase activity. Notably, Rrd1 associates with Sit4 and Tap42, which form a complex crucial for TORC1 signaling. Rapamycin inactivates TORC1, causing the dissociation of Tap42 from Sit4-Rrd1 and the activation of Sit4 phosphatase, which then dephosphorylates Gln3 or Npr1 kinases [113, 118]. Deletion of Rrd1 could cause aberrant activation and/or loss of Sit4 substrate specificity; therefore, causing rapamycin resistance. However, several data indicate that Gln3 can be activated independently of Sit4 and, even more interestingly, independently of Rrd1 [113, 119, 120]. This clearly suggests that Rrd1 might have additional regulatory roles in the rapamycin response. Indeed, we have shown that Rrd1 is required to regulate the expression of rapamycin responsive genes [113]. This clearly indicates that Rrd1 has a role independent of its Sit4 phosphatase activator function, since ribosomal genes are regulated independently of the Tap42- Sit4 complex [60]. Since the catalytically inactive mutant of Rrd1 is also resistant to rapamycin and this resistance does not seem to depend on the interaction of Rrd1 with Sit4, it is very possible that Rrd1 has additional targets for its isomerase activity, and those could be important for the response to rapamycin.

Consistent with this notion, the PPIase Pin1 also has multiple cellular targets and is involved in various different cellular activities, including the isomerisation of RNAPII and its transcriptional regulation [94].

The fact that Rrd1 is required to modulate gene expression in response to rapamycin, independently of its Sit4 associated function, led us to the hypothesis for my Ph.D project:

Rrd1 regulates RNAPII transcription in response to rapamycin through its peptidyl prolyl isomerase activity.

The two subsequent articles will describe, in the form of manuscripts for publication, how the project developed, the methods that were used and the results that were obtained during my Ph.D.

2 ARTICLES

2.1 Rrd1 isomerizes RNA polymerase II in response to rapamycin

Nathalie Jouvet[ψ], Jeremie Poschmann[ψ], Julie Douville, Lisa Bulet, Xiaoming Yang, and Dindial Ramotar

My contribution to this article:

I would evaluate my contribution to the experimental data to about 35 %. I produced figure 1, figure 3A, figure 7, figure 8 and supplemental figure S1. NJ produced figure 2 and figure 3B. Figure 3C was done by LB. Figure 4, 5 and 6 were produced by NJ and JD. Protein purification for assays was done by XY.

The entire manuscript has been written by NJ, DR and myself.

This article has been rejected by the reviewers for NAR submission.

However, based on their comments, we believe that we can do the required experiments and re-submit to NAR soon.

Rrd1 isomerizes RNA polymerase II in response to rapamycin

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Running head: Rrd1 isomerizes the CTD of RNA pol II

ψN.J and J.P are equal contributors

Keywords: transcriptional regulation, rapamycin, RNA polymerase II, C-terminal domain, chromatin, isomerase, circular dichroism, ChIP assay

Abstract

In *Saccharomyces cerevisiae*, the immunosuppressant rapamycin engenders a profound modification in the transcriptional profile leading to growth arrest. Mutants devoid of Rrd1, a protein possessing in vitro peptidyl prolyl cis/trans isomerase activity, display striking resistance to the drug, although how Rrd1 activity is linked to the biological responses has not been elucidated. We now provide evidence that Rrd1 is associated with the chromatin and it interacts with RNA pol II. Circular dichroism reveals that Rrd1 mediated structural changes of the C-terminal domain (CTD) of the large subunit of RNA pol II (Rpb1) in response to rapamycin, although this appears to be independent of the overall phosphorylation status of the CTD. In vitro experiments, revealed that recombinant Rrd1 directly isomerizes purified GST-CTD and that it releases RNA pol II from the chromatin. Consistent with this, we show that Rrd1 is required to alter RNA pol II occupancy on rapamycin responsive genes. We propose as a mechanism, that upon rapamycin exposure Rrd1 isomerizes Rpb1 to modulate transcription.

Introduction

Rapamycin is an immunosuppressant that was recently approved for treating kidney carcinomas [122]. It is known to inhibit the Tor1 (Target of Rapamycin) kinase signalling pathway leading to growth inhibition [123]. In *S. cerevisiae*, several factors have been identified through genome-wide screens that when deleted cause resistance to rapamycin [124]. One of these proteins is Rrd1 (Rapamycin Resistance Deletion 1) that was first reported to play a role in protecting cells against oxidative DNA damage caused by the carcinogen 4-nitroquinoline-1-oxide (4-NQO) and by UVA [74]. Mutants deficient in Rrd1 are also unable to undergo rapamycin-induced growth arrest and therefore exhibit marked resistance to the drug [110]. Rrd1 is conserved in eukaryotes and shares 35% identity with the human phosphotyrosyl phosphatase activator, hPTPA, which was initially isolated as a protein that stimulated the weak phosphotyrosyl phosphatase activity of the type 2A Ser/Thr phosphatase PP2A [125, 126]. We and others reported that Rrd1 can physically interact with the Ser/Thr phosphatase Sit4, a PP2A like phosphatase [111, 114, 127]. In *S. cerevisiae*, rapamycin binds to the peptidyl-prolyl cis/trans isomerase Fpr1 and this drug-protein complex inactivates the Tor1 kinase causing a profound modification in the transcriptional profile, and culminating in G1 growth arrest [57, 128, 129]. Inhibition of Tor1 leads to the activation of Sit4, by virtue of its dissociation from the inhibitor complex Tap42-Sit4, which in turn dephosphorylates several targets including the nutrient-responsive transcriptional activator Gln3 that translocates to the nucleus to activate *GLN1* and *MEP2* expression [57, 61, 130]. However, these Sit4-dependent processes do not require the function of Rrd1, suggesting that the latter protein might execute a function downstream in the Tor1 signaling pathway [113, 129, 131, 132].

Recent data indicate that Rrd1 exerts an effect at the transcriptional level [113]. Genes known to be upregulated (e.g., the diauxic shift genes *CPA2* and *PYC1*) and down-regulated (e.g., the ribosomal protein genes including *RPS26A, RPL30*, and *RPL9*) following rapamycin exposure showed an altered transcription pattern in *rrd1Δ* mutants [113, 129, 131, 132]. To date, the exact function executed by Rrd1 causing alteration in transcription has not been investigated. Rrd1 and its mammalian counterpart PTPA have been shown to possess an in vitro peptidyl prolyl cis/trans isomerase (PPIase) activity on model substrates [133]. PPIases are ubiquitous proteins

that catalytically facilitate the cis/trans isomerization of peptide bonds N-terminal to proline residues within polypeptide chains [134, 135]. Both Rrd1 and PTPA can independently change the structure of short peptides including the synthetic substrate (186LQEPHEGPMCDL198) representing a conserved sequence amongst PP2A phosphatases [133]. As such, it has been suggested that Rrd1/PTPA could activate PP2As via this PPIase activity [133]. So far, neither the in vivo target nor the biological function of the PPIase activity of Rrd1 has been elucidated, although this is not the case for other PPIases. For example, the PPIases Ess1 and Pin1 from S. cerevisiae and mammalian cells, respectively, possess the ability to associate with the C-terminal domain (CTD) of Rpb1 [95, 108]. In yeast, the CTD consists of 26 repeats of the YS2PTS5PS7 heptad sequence and Ess1 has been shown to stimulate the dephosphorylation of Ser-5 to efficiently terminate transcription of a subset of genes [44].

In this study, we show that Rrd1 is associated with RNA pol II and isomerizes the CTD of Rpb1 in vivo and in vitro. Our data suggest a model whereby this isomerization leads to the dissociation of RNA pol II from the chromatin resulting in transcriptional changes. This study provides insight into a possible new mechanism by which RNA pol II could rapidly respond to transcriptional changes.

MATERIALS AND METHODS

Strains, media and plasmids

The strains used in this study were the parents BY4741 (*Mat a*, *his3-1*, *leu2-0*, *met15-0*, *ura3-0*), YDL401 (*MAT*a *his3-200 leu2-1 trp1 ura3-52 gal2 gal-108*), and the isogenic mutants *rrd1*Δ and *gln3*Δ. Strains were endogenously and independently tag at the following loci *APN1*, *RAD52*, *RRD1*, *SWE1* and *YAP8* as previously described [136]. Strains bearing Rpb1-TAP was provided by Tom Begley (Albany, USA). Strains were grown in either rich (YPD) or selective (SD) media. Construction of pGFP-SIT4, pGFP-RAD52, pGFP-RRD1, GST-APN1 was previously described [114]. pGST-CTD was constructed by amplifying the murine CTD from plasmid pGCTD [137] and subcloned into pTW340 (provided by Tom Wilson, Michigan, USA). Construction of the plasmid pGAL-HIS-RRD1 and purification of HIS-Rrd1 fusion protein were done as previously reported for pHIS-BLH1 [138].

Spot test analysis

The assay was done as previously described, except for plates containing rapamycin [139].

Extraction of chromatin-associated proteins

Extraction of proteins bound to chromatin was done as previously described [139]. Exponentially growing cultures (50 ml) were spun down, resuspended in 6.25 ml of 100 mM PIPES/KOH, pH 9.4, 10 mM DTT, and incubated at 30ºC for 10 min with agitation. Cells were spun down and resuspended in 2.5 ml of YPD containing 0.6 M sorbitol, 25 mM Tris-HCl pH 7.5 and 100 µl of 25 mg/ml lyticase. Cells were incubated at 30ºC for 30 min with agitation, spheroplasts were spun down, resuspended in 2.5 ml YPD containing 0.7 M sorbitol, 25 mM Tris-HCl pH 7.5 and incubated at 30ºC for 20 min with agitation. The spheroplasts were washed 3 times with 1 ml of lysis buffer (0.4 M sorbitol, 150 mM KoAc, 2 mM $MgCl_2$, 20 mM PIPES/KOH pH 6.8, and the protease inhibitor cocktail (Roche, 1 tablet per 10 ml). The washed spheroplasts were resuspended in 300 µl of lysis buffer containing 1% Triton X-100. To a 90 µl aliquot of the lysed spheroplasts was added 45 µl of 2X protein loading buffer and used as the “whole cell extract”. Another 100 µl of the lysed spheroplasts was spun at 14,000 rpm for 5 min in a microcentrifuge at 4ºC. To the supernatant was added 45 µl of 2X protein loading buffer and labelled the “SOL”

fraction, while the pellet was resuspended in 90 µl of lysis buffer and 45 µl of 2X protein loading buffer and labelled the "CHR" fraction.

Co-Immunoprecipitation experiments

Co-immunopreciptation was done as previously described [114], except using 8WG16 covalently coupled to AminoLink matrix (Pierce) and total extracts [140] prepared from cells expressing either MYC- or GFP-tagged form of the indicated proteins or from the untagged parent or *rrd1*Δ cells. The matrix with bound proteins was washed four times with a buffer containing 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, and 0.1% NP40. The input (5%) used in the co-immunoprecipitation experiment as well as half the volume of the matrix were assessed by Western blost using either anti-MYC, -GFP (Clonetech), or -ubiquitin (Rockland). The remaining half of the matrix was analyzed separately by Western blot probed with 8WG16 antibody.

Western blot analysis of GST, GST-CTD and Rpb1-TAP

BY4741 parent or *rrd1*Δ mutant cells expressing the GST-CTD or carrying the endogenous Rpb1-TAP tag were subcultured in the appropriate media and treated with rapamycin (200 ng/ml for 30 min). Whole cell extracts or where indicated affinity purified proteins (GST, GST-CTD or Rpb1-TAP using manufacturer's protocol (Stratagene, USA)) were analyzed by Western blot with anti-GST (Sigma), H5 (anti-Ser2 phosphorylated) and H14 (anti-Ser5 phosphorylated) antibodies (Covance) or anti-PAP (Sigma). For purification of GST-CTD, 500 ml cultures grown in selective media to an OD_{600} of ~ 1.0 were used, which yielded ~1 to 2 mg of purified protein which was stored in phosphate buffer.

Interaction between Rrd1-MYC and GST-CTD

Total protein extracts derived from parent cells (100 ml) expressing GST-CTD or GST-Apn1, untreated or treated with rapamycin (200 ng/ml for 2h) were allowed to bind to 1 ml GST affinity matrix slurry as described for the purification, except samples were not eluted from the columns. A second protein extract (1 mg) derived from a strain expressing Rrd1-MYC or Yap8-MYC was applied and allowed to bind for 1 h at room temperature on a rotating platform. The columns were then washed with 20 bed volumes of PBS and an aliquot of the beads (30 µl) was loaded onto an 8% SDS-PAGE and processed for Western blot. The presence of GST-CTD on both columns was detected using polyclonal anti-GST (Sigma) and the bound Rrd1-MYC was revealed anti-MYC monoclonal antibody (SantaCruz).

Circular dichroism spectroscopy

Continuous far-UV circular dichroism spectra (197-250 nm) of the GST and the GST-CTD fusion protein (2.0 g and 4.32 g, respectively, in 100 l of 10 mM phosphate buffer pH 7.0, 50 mM NaCl) were collected using a Jasco-810 spectropolarimeter. The measurements were carried out at room temperature using a 1 mm path-length cuvette (Hellma) and a 1 nm bandwidth. Three spectra were collected for each sample and averaged. The spectral contribution of the buffer was corrected for by subtraction. Relative ellipticity was converted to mean residue molar ellipticity [Θ] according to Fasman [141].

Limited chymotrypsin digestion assay

The purified GST-CTD (~100 ng) derived from parent cells untreated or treated with rapamycin (200 ng/ml for 2h) was subjected to digestion with 5 ng chymotrypsin (Roche) in the presence of 1 mM $CaCl_2$, and incubated at 37°C for the indicated time. Digestion was stopped by the addition of SDS-PAGE loading buffer and boiling of the samples. Processing of the GST-CTD was analyzed using 8% SDS-PAGE followed by staining with silver.

In vitro isomerase assay

Purified HIS-Rrd1 was added to the purified GST-CTD in sodium phosphate buffer (10 mM $NaPO_4$ pH 7.0, 50 mM NaCl) without or with 1 mM $MgCl_2$, and 1 mM ATP in a final volume of 200 l. The proteins were incubated for 1 h at 30ºC the GST-CTD was recovered by GST-affinity purification and then subjected to CD analysis.

In Vitro Rpb1 release assay

Exponentially growing culture (200ml) of the BY4741 *rrd1*Δ Apn1-MYC strain was prepared and lysed as above for the extraction of chromatin associated proteins. Supernatant was discarded and the pellet was washed once in 1 ml of isomerization buffer (10 mM $NaPO_4$ pH 7.0, 50 mM NaCl, 1 mM $MgCl_2$, and 1 mM ATP). Supernatant was discarded again and pellet was resuspended in 600 l of isomerization buffer and equally divided in three tubes. Increasing amounts of purified HIS-Rrd1 were added and samples were rocked for 1 h at 30ºC. Samples were then spun down and supernatant was kept for subsequent western blot analysis. The remaining pellet was resuspended in benzonase buffer (50 mM Tris pH 8.0, 1 mM $MgCl_2$) and 1 l of benzonase (Novagen) was added and tubes were incubated for 30 min at 37ºC. Supernatant (SOL) and chromatin (CHR) fractions were loaded onto SDS-PAGE gels for western blot analysis with 4H8 and anti-MYC antibodies.

ChIP assay

The ChIP assay was done as previously described [142]. Primers are available upon request. *ACT1* was used as an endogenous control and relative quantity was calculated using the ΔΔCT method (Applied Biosystems). IP's were normalized to the respective input. Untreated IP samples were given an arbitrary unit 1 and increase or decrease folds were calculated. At least three independent experiments were done for each gene and Student T test was used to calculate the p-value.

RESULTS

Rrd1 is associated with the chromatin and interacts with Rpb1

We previously demonstrated that Rrd1 is required to modulate the expression of a subset of rapamycin-regulated genes independently of Sit4 [113]. To corroborate our earlier findings that Rrd1 acts separately from the Sit4-Gln3 signaling pathway, we deleted the *RRD1* gene in the *gln3Δ* background (known also to be resistant to rapamycin) and examined the resulting *gln3*Δ *rrd1*Δ double mutant for the level of resistance to the drug [143]. This genetic analysis revealed that the *gln3*Δ *rrd1*Δ double mutant was significantly more resistant to rapamycin than either of the single mutants (Fig. 1), suggesting that Rrd1 performs a distinct role to regulate response to the drug.

To investigate this potentially novel role of Rrd1, we first checked whether Rrd1 binds to chromatin in light of its involvement in gene regulation [113]. Chromatin fractions were derived from strains expressing MYC-tagged Rrd1, as well as the control proteins Swe1, Rad52 and Apn1 from the endogenous loci and subjected to Western blot analysis probed with anti-MYC antibody. As shown in Fig. 2A, a significant amount of Rrd1-MYC was found in the chromatin fraction (lane 3), suggesting that Rrd1 is associated with the chromatin and consistent with an earlier study showing that Rrd1 is also present in the nucleus [114]. In contrast, the control protein Swe1-MYC was only found in the soluble fraction (lane 2), while Rad52-MYC and Apn1-MYC, two DNA repair proteins known to bind chromatin, were present in the chromatin fraction (lane 3) [144, 145].

Since Rrd1 is bound to the chromatin and is involved in regulating gene expression, we tested if it is associated with RNA pol II by performing co-immunoprecipitation analysis. For this experiment, we used total extracts derived from cells expressing either Rrd1-MYC or Swe1-MYC and checked for the pull-down with anti-Rpb1 (8WG16). Rrd1-MYC was co-immunoprecipitated with Rpb1, but not the control protein Swe1-MYC (Fig. 2B). Since only a small amount of Rrd1-MYC was co-immunoprecipitated with anti-Rpb1, the association between Rrd1 and RNA pol II may be weak or transient. There was no alteration in the amount of Rrd1 co-immunoprecipitated by anti-Rpb1 when cells were treated with rapamycin (200 ng/ml for 30 min) (Fig. 2B).

Anti-Rpb1 also co-immunoprecipitated Rrd1 from parent cells carrying a plasmid expressing GFP-tagged Rrd1 (Fig. 2C). In addition, the Sit4 phosphatase known to physically interact with Rrd1 [114] co-immunoprecipitated with Rpb1 from parent cells expressing this protein as GFP fusion (Fig. 2C). Two additional GFP fusion proteins, GFP-Imp2 and GFP-Rad52, which do not interact with Rrd1, were not co-immunoprecipitated with anti-Rpb1 antibody, although a minute amount of GFP-Rad52 non-specifically interacted with the beads used for immunoprecipitation (Fig. 2C, and data not shown). Thus, Rpb1 associates with proteins known to bind Rrd1, suggesting that Rrd1 could exist in a complex with Rpb1. We note that the reverse co-immunoprecipitation with Rrd1-MYC did not pull down Rpb1 under the same reaction conditions, raising the possibility that the size of the RNA pol II complex might impede the pull down although we cannot exclude other alternatives such as a weak or indirect interaction via another protein.

Rrd1 associates with the CTD of Rpb1 and alters its structure in response to rapamycin

Since the C-terminal domain (CTD) of Rpb1 is a repeated sequence (YSPTSPS) rich in proline residues, and has previously been shown to bind the isomerases Ess1 and Pin1 [95, 108, 146], we reasoned that Rrd1 could function to isomerize the CTD. As such, we assessed whether the CTD is a substrate for the PPIase activity of Rrd1 *in vivo*. The CTD was expressed as a GST fusion protein from a previously described plasmid (see Materials and Methods) and has been shown to undergo post-translational modifications including Ser-5 and Ser-2 phosphorylation, isomerization and ubiquitylation [44, 137, 147, 148]. Introduction of this plasmid into the parent and *rrd1Δ* strains directed the expression of the GST-CTD fusion protein with the expected size (95-kDa) as determined by Western blot analysis probed with anti-GST antibodies (Fig. 3A, see also Supple. Fig. S1). The GST-CTD contained both phosphorylated Ser-5 and Ser-2 as detected by anti-H14 and anti-H5 antibodies, which specifically recognize Ser-5 and Ser-2 phosphorylation, respectively (Fig. 3A), consistent with previous studies that the GST-CTD can be functionally modified *in vivo* [137, 147-149]. From these analyses, we observed no differences in the (i) size, (ii) level of expression, and (iii) phosphorylation of the GST-CTD whether it was derived from the parent or the *rrd1Δ* mutant or from cells that were pretreated with rapamycin (Fig. 3A. Supple. Fig. S1).

We next prepared GST-CTD affinity beads from parent cells and determined whether these could pull down Rrd1. Total extract derived from the parent strain expressing Rrd1-MYC (Fig. 3B, lane 2) was incubated with the GST-CTD affinity beads. The beads were recovered, washed and an aliquot examined for retention of Rrd1-MYC by Western blot analysis. As shown in Fig. 3C and D, Rrd1-MYC was pulled down by the GST-CTD affinity beads. In contrast, the GST-CTD affinity beads did not pull down the transcriptional activator Yap8, also tagged with MYC (Fig. 3B, C and D). As expected, the empty beads did not pull down Rrd1-MYC from the total extract nor did the control beads carrying GST-Apn1 (Fig. 3C and D). These data support the notion that Rrd1 associates with the CTD of Rpb1, consistent with the above observation that Rpb1 co-immunoprecipitated Rrd1.

We next investigated whether Rrd1 could induce conformational changes in the GST-CTD fusion protein by using circular dichroism (CD) spectroscopy, a method that is very sensitive to changes in the secondary structure of proteins [141]. We first purified the GST-CTD from the parent and the *rrd1Δ* mutant, as well as GST from the parent to be used as the control. Silver stain analysis of the purified GST-CTD revealed that there was no difference in the size of this protein, whether it was derived from the parent or the *rrd1Δ* mutant (Fig. 4A, lane 3 vs. 5) or when the cells were treated with rapamycin (lane 3 vs. 4 or 5 vs.6). As observed for total extract, the purified GST-CTD showed no alteration in either Ser-5 or Ser-2 phosphorylation (Supple Fig. S1A). To ensure that the observed phosphorylation status of the GST-CTD is similar to Rpb1 CTD phosphorylation, we purified Rpb1 from the TAP tagged strains and monitored this protein for its phosphorylation. Like the GST-CTD, Rpb1-TAP showed no differences in either Ser-5 or Ser-2 phosphorylation following rapamycin treatment (see Supple Fig. S1B). However, this approach may not distinguish between subtle phosphorylation differences that may occur amongst the heptad repeats [150]. Since the GST-CTD is similarly phosphorylated as the endogenous Rpb1, we used it as a tool for further analysis.

CD spectra obtained for the purified GST-CTD derived from either the untreated parent or *rrd1Δ* mutant were indistinguishable, and displayed a minimum at 202 nm (Fig. 4B). In contrast, GST-CTD derived from the parent cells treated with rapamycin exhibited a spectrum with a minimum at 208 nm and shoulder at ~225 nm (Fig. 4B), suggesting that the GST-CTD underwent a detectable change in its secondary structure. Remarkably, rapamycin treatment of the *rrd1Δ* mutant failed to induce this conformational change onto the GST-CTD (Fig. 4B).

Introduction of a single copy plasmid expressing functional Rrd1 in the *rrd1Δ* mutant restored the change in the spectral pattern of the GST-CTD (Fig. 4C)[74]. Additionally, purified GST alone derived from untreated or rapamycin treated parent cells did not exhibit any structural differences, suggesting that it is the CTD portion of the fusion protein that is undergoing the rapamycin-induced changes (Fig. 4D). We further confirmed the structural change of the GST-CTD as observed by CD using limited proteolysis with chymotrypsin, which can distinguish proteins with different secondary structures and exclusively cleaves peptides in the trans-proline conformation [151]. As shown in Fig. 4E, the GST-CTD purified from the rapamycin-treated parent cells was more resistant to limited chymotrypsin digestion, as opposed to the GST-CTD derived from the untreated cells, suggesting that indeed the GST-CTD went through a structural reorganization in response to rapamycin. On the basis of these findings, it would appear that the CTD of Rpb1 changes its structure *in vivo* following exposure to rapamycin, and that Rrd1 is essential for this alteration.

Rrd1 alters the GST-CTD structure in response to 4-NQO, but not MMS

We next checked if isomerization of the CTD is specific for rapamycin. Since the *rrd1Δ* mutant was previously shown to be sensitive to the DNA damaging agent 4-NQO [74], which induces oxidative stress as well as creating bulky lesions onto the DNA [152], we examined for isomerization of the GST- CTD in the parent and the mutant following treatment with this drug. We observed that the structure of the GST-CTD was altered in the parent, but not in the *rrd1Δ* mutant following 4-NQO treatment (Fig. 5A). We also tested another DNA damaging agent, methyl methane sulfonate (MMS), to which the *rrd1Δ* mutant displays parental sensitivity [74]. MMS creates apurinic/apyrimidinic sites in the genome, and for this experiment it was used at a concentration that kills ~70% of the cells. Under this condition, the GST-CTD showed no structural alteration following the MMS treatment (Fig. 5B). On the basis of these findings, it would appear that this phenomenon might occur for other stress conditions besides exposure to rapamycin.

Rrd1 directly alters the structure of the CTD *in vitro*

We next examined whether purified Rrd1 can induce structural changes onto the CTD *in vitro*. To do this, we incubated equimolar amounts of recombinant HIS-Rrd1 purified from *E. coli* with

affinity purified GST-CTD derived from the *rrd1Δ* mutant at 30ºC for 30 min, then recovered the GST-CTD for CD analysis. As shown in Fig. 6, purified HIS-Rrd1 significantly modified the CTD structure under the standard phosphate buffer reaction conditions. Since the Rrd1 isomerase activity has been shown to be stimulated by ATP and Mg^{2+} [133], we examined the effect of these additions to the reaction mixture. Inclusion of ATP and Mg^{2+} in the buffer caused no structural alteration to the CTD in the absence of Rrd1 (Fig. 6). However, addition of purified HIS-Rrd1 to the complete ATP/Mg^{2+} phosphate buffer introduced a more dramatic change to the CTD structure, as compared to the mixture lacking ATP/Mg^{2+} (Fig. 6). Moreover, the purified HIS-Rrd1 did not confer any structural changes onto another purified GST fusion protein, GST-Apn1 (data not shown). These findings suggest that Rrd1 can directly isomerize the CTD.

Comparison of RNA pol II occupancy at rapamycin-responsive genes

Since Rrd1 associates with and isomerizes the CTD, and that *rrd1Δ* mutant did not affect the phosphorylation status of Rpb1, we asked whether it would alter RNA pol II occupancy on rapamycin responsive genes *in vivo*. To do this, we performed chromatin immunoprecipitation (ChIP) analysis of Rpb1 on two known RNA pol II-responsive genes, *RPS26A* and *CPA2* [153]. Since both genes are known to be rapidly downregulated and upregulated, respectively, within 30 min, we treated cells for this time period with rapamycin [113, 128]. In parent cells, the Rpb1-ChIP signal from the *RPS26A* gene was reduced by nearly 8-fold upon rapamycin treatment (Fig. 7A). In contrast, Rpb1 remained associated with *RPS26A* in the *rrd1*Δ mutant (Fig. 7A). In the case of the upregulated gene *CPA2*, we observed an increase in Rpb1-ChIP signal in the parent upon rapamycin, whereas in the mutant there was only a modest increase in the signal (Fig. 7B). The occupancy of RNA pol II on these genes is consistent with the mRNA expression levels [113, 128]. These data raise the possibility that Rrd1 might displace Rpb1 in order to optimize rapid transcriptional changes caused by rapamycin.

Purified Rrd1 stimulates the release of chromatin-bound RNA pol II in vitro

To explore the above possibility, we examined if purified Rrd1 would displace RNA pol II from the chromatin. Briefly, we isolated chromatin containing RNA pol II derived from the *rrd1Δ* mutant, the chromatin was washed and resuspended in the standard phosphate buffer containing ATP and Mg^{2+}. To this reaction, increasing amounts of purified Rrd1 was added and following

incubation the levels of chromatin-bound and soluble Rpb1 were monitored by Western blot. As shown in Fig. 8, increasing concentration of Rrd1 caused a loss of chromatin-bound Rpb1, while there was a correlating gain in the soluble fraction. In contrast, Rrd1 concentration did not affect the level of the control protein Apn1-MYC. Collectively, our data indicate that Rrd1 possesses the ability to isomerize the CTD of Rpb1 thereby promoting its displacement from the chromatin.

DISCUSSION

In the present study, we show that Rrd1 is a chromatin bound protein, which associates with the RNA pol II, presumably through the CTD of Rpb1. We believe that this association allows isomerization of the CTD in response to specific stress such as that caused by rapamycin and 4-NQO but not MMS. In addition, we show that *in vitro* purified Rrd1 (i) can directly alter the structure of the CTD and (ii) dissociate Rpb1 from the chromatin. On the basis of these observations, we propose the following model whereby in response to specific stress conditions the RNA pol II associated Rrd1 isomerizes the CTD of Rpb1 such that the polymerase is dissociated from the chromatin. Once the RNA pol II is released it would be recruited to stress-responsive genes.

There is supporting evidence that elongating RNA pol II is in excess on ribosomal protein genes, surprisingly associated with a low transcriptional rate under glucose grown conditions [154]. However, once these cells are submitted to a metabolic change, e.g., a switch to galactose growth conditions, the level of RNA pol II decreased on these ribosomal genes and the transcriptional rate increased [154]. This shift also simultaneously caused an enrichment of RNA pol II onto mitochondrial genes [154]. This suggests a mechanism where excessive RNA pol II is removed from the ribosomal genes and recruited to mitochondrial genes to increase expression. Therefore, metabolic switches would stimulate re-localization of elongating RNA pol II from one regulon to the other. As it is known that rapamycin mimics starvation conditions and represses ribosomal biogenesis, we suspect a similar mechanism as the glucose-galactose shift is operational to rapidly change transcription. Besides Rrd1, another well characterized peptidyl prolyl isomerase Pin1 can trigger the release of RNA pol II from transcribing genes in human cells [95]. Under normal conditions, Pin1 interacts with the phosphorylated CTD of RNA pol II and this association is retained along the length of transcribed genes [95]. However, when Pin1 is overexpressed it promotes hyperphosphorylation of the CTD during the transition from initiation to elongation, thereby causing RNA pol II to dissociate from active genes and leading to the inhibition of transcription [95, 106]. The dissociated RNA pol II accumulates in enlarged speckle-associated structures enriched for transcription and RNA processing factors [95, 155].

Because Rrd1 intersects with the biological functions of Pin1, it is possible that Rrd1 could modulate the phosphorylation status of the CTD. Recent studies showed that the yeast homologue of Pin1, Ess1, binds and catalyzes the cis/trans isomerization of the CTD such that Ser-5 phosphorylation can be dephosphorylated by the Ssu72 phosphatase [44]. Moreover, a variant of Ess1 (Cys120Arg) caused accumulation of Ser-5 phosphorylation, and not Ser-2 phosphorylation, both of which were monitored using the same set of antibodies (anti-H5, -H14 and -8WG16) as in this study [44]. We found no alteration in the global Ser-2 and Ser-5 phosphorylation status upon rapamycin treatment, as well as between the parent and the *rrd1Δ* mutant using the same set of antibodies (Fig. 3A and Supple Fig. S1). As such, it would seem that Rrd1 uses a novel mechanism independent of phosphorylation to isomerize the CTD, although we cannot exclude the possibility that there are unique Ser-2 and Ser-5 phosphorylation differences which can be masked by neighboring phosphorylations. For example, where one heptad is phosphorylated, but not the adjacent [150]. However, since RNA pol II exists in different phosphorylation forms throughout the transcription cycle, it seems logical to have a mechanism that triggers RNA pol II release independent of its phosphorylation status.

In yeast, the CTD consists of 26 repeats of the heptad sequence YSPTPS. It exists largely in a disordered structure, but adopts a static conformation upon interaction with target proteins such as the mediator complex that regulates transcription initiation and enzymes that modify the 5’ and 3’ends of mRNA [156, 157]. Binding of these proteins to the CTD is modulated by serine phosphorylation and proline isomerization [150]. Thus, a given heptad repeat could give rise to many different conformations with the various combinations of phosphorylated Ser-2, -5 and -7, as well as the *cis*/*trans* isomerization of the two prolines, Pro-3 and Pro-6, to generate a broad range of binding sites to allow precise association with several factors [7, 156, 157]. At least three CTD interacting proteins (Pcf1, Pin1, and Ctg-1 from *C. albicans*) have been shown to bind exclusively the all-*trans* conformation, providing support for the hypothesis that proline isomerization of the CTD plays a critical regulatory role [7]. This strongly suggests that multiple conformations of the CTD exist *in vivo*. Consistent with this notion, we observed by CD analysis two conformations of the CTD that remained stable through out its purification (Lisa Miller, Brookhaven National Laboratories, personal communications) from untreated and rapamycin-treated cells (Fig. 4). These different conformations could be the result of proline isomerization, as prolines are known to be stable in either the *cis* or *trans*

conformation when the protein is in a folded form [158]. Only peptidyl prolyl isomerases such as Pin1/Ess1 are known to trigger a switch between the *cis* and *trans* conformations of the CTD [96], and that in the absence of these enzymes the conformational switch is slow [159]. Because Rrd1 possesses peptidyl prolyl isomerase activity and it associates with RNA pol II, it seems likely that this function is responsible for inducing structural changes to the CTD upon rapamycin exposure. In support of this, Rrd1 directly alters the CTD structure *in vitro* (Fig. 6), and we therefore predict that Rrd1 might act in a similar manner onto the CTD *in vivo*.

In addition to rapamycin, we also observed that the DNA damaging agent 4-NQO, but not MMS, triggered alteration of the CTD structure (Fig. 5). We examined the effect of 4-NQO, as we had previously shown that *rrd1*Δ mutants were sensitive to this agent and not to MMS [74]. The distinct difference between 4-NQO and MMS is that the former agent potently induces the production of reactive oxygen species such as superoxide anions [152]. Both starvation and oxidative stress are known to mediate similar transcriptional programs, also termed as the environmental stress response, for example, where ribosome biogenesis is turned off [71]. This would explain why the *rrd1*Δ mutants are sensitive to 4-NQO, but resistant to rapamycin; (i) genes required for counteracting the 4-NQO-induced oxidative stress are not turned on efficiently and as a result the cells accumulate genotoxic lesions, and (ii) under rapamycin condition nutrients are still available and the failure to alter gene expression allows *rrd1*Δ mutants to grow. Taken together, our data suggest that Rrd1 participates in a novel mechanism that allows redistribution of RNA pol II for transcriptional regulation of genes involved in specific stress conditions.

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FIGURE LEGENDS

Figure 1. *gln3*Δ *rrd1*Δ double mutant is more resistant to rapamycin than either single mutant. Cells were serially diluted and spotted onto YPD plates without and with rapamycin (Rap). Photos were taken after two days of growth at 30ºC.

Figure 2. Rrd1 is associated with the chromatin and interacts with Rpb1. A) Rrd1 is bound to chromatin. Whole cell extract (WCE), soluble (SOL) and chromatin (CHR) fractions were derived from the parent cells expressing either Rrd1-MYC, Swe1-MYC, Rad52-MYC or Apn1-MYC and the distribution of the MYC-tagged proteins was examined by Western blots. The data is representative of two independent analyses. **B)** Rpb1 pull-down of Rrd1. The 8WG16 antibodies were used to immunoprecipitate extracts from untreated (-) and rapamycin-treated (+) (200 ng/ml for 30 min) cells expressing either Rrd1-MYC or Swe1-MYC. The presence of Rrd1 in the immunoprecipitates was determined by Western blotting. **C)** Specificity of Rpb1 pull-down of GFP tagged proteins. The 8WG16 antibodies were used to immunoprecipitate extracts from cells expressing either of the following GFP tagged proteins: Imp2, Rrd1, Rad52 or Sit4. The presence of the GFP-tagged proteins in the immunoprecipitates was detected by Western blotting.

Figure 3. Analysis of the GST-CTD and its interaction with Rrd1. A) Comparison of the expression and phosphorylation status of the GST-CTD between parent and *rrd1*Δ mutant cells following rapamycin exposure. The indicated cells expressing GST-CTD were treated with (+) and without (-) rapamycin (200 ng/ml for 30 min) and total protein extracts were probed for Ser-2 phosphorylation (H5) or Ser-5 phosphorylation (H14). The membranes were stripped and reprobed with anti-GST antibody. **B-D)** Retention of Rrd1-MYC by GST-CTD affinity beads. **B)** The input of parent cells expressing Yap8-MYC and Rrd1-MYC from the endogenous locus. **C)** Empty beads and beads containing either GST-CTD or GST-Apn1. **D)** Total protein extracts derived from the parent or parent expressing either Yap8-MYC or Rrd1-MYC were incubated with the empty beads or beads containing either GST-CTD or GST-Apn1. The beads were then washed and an

aliquot examined for retention of the MYC tagged proteins using anti-MYC antibodies. Results shown are representative of two independent experiments.

Figure 4. ***rrd1*****Δ mutants are unable to induce conformational changes to the GST-CTD in response to rapamycin. A)** Silver stained gel of purified GST and GST-CTD. The indicated strains carrying either the GST (lanes 1 and 2) or GST-CTD expressing plasmid (lanes 3-6) were untreated (-) or treated (+) with rapamycin (RAP) (200 ng/ml for 30 min). **B and C)** Far-UV circular dichroism spectral (CD) analysis of purified GST-CTD. The purified GST-CTD (0.45 μM) was derived from the indicated strains (A) carrying the empty vector or pRRD1 (B) that were untreated or treated with rapamycin. **D)** CD analysis of purified GST (0.76 μM) derived from untreated and rapamycin treated parent cells as above. Results shown are the averages of two independent experiments. **E)** Limited proteolysis of purified GST-CTD derived from parent cells untreated or treated with rapamycin. The purified GST-CTD was subjected to partial chymotrypsin digestion and analyzed by silver staining. Results shown are representative of two independent experiments.

Figure 5. 4-NQO, but not MMS, induces structural changes onto the GST-CTD. A and B, CD analysis of the purified GST-CTD derived from exponentially growing Apn1-MYC tagged parent and *rrd1Δ* mutant were treated with either 4-NQO (2   g/ml 30 min) or MMS (1% for 60 min).

Figure 6. Purified recombinant Rrd1 alters the structure of the GST-CTD *in vitro*. Equimolar amounts (4.5 μM) of purified GST-CTD derived from the *rrd1Δ* mutant and the purified recombinant HIS-Rrd1 were incubated at 30°C in phosphate buffer in the absence and presence of Mg^{2+}/ATP. The resulting GST-CTD was re-purified free of the recombinant HIS-Rrd1 and subjected to CD analysis as in Fig. 4. The result is the average of two independent experiments.

Figure 7. Comparison of RNA pol II occupancy at the indicated target genes in the parent and *rrd1*Δ mutant in response to rapamycin treatment. Cells were untreated

or treated with 200 ng/ml rapamycin for 30 min and Rpb1 localization was analyzed by ChIP assay (see Materials and Methods). Primer locations are indicated below the diagram. The respective input normalized IP amounts were quantified relative to the *ACT1* gene using the ΔΔCT method. Results are shown as the average of three independent experiments. Error bars represent standard deviation and the *P*-values compare untreated vs. treated.

Figure 8. Purified recombinant Rrd1 dissociates Rpb1 from the chromatin *in vitro*. Increasing amounts of purified HIS-Rrd1 were added to the chromatin fraction isolated from *rrd1Δ* mutant strain expressing Apn1-MYC and incubated at 30°C for 1 h in phosphate buffer. Chromatin was recovered from the buffer and both fractions were analyzed by Western blotting probed with 4H8 (against Rpb1) and anti-MYC antibodies. Apn1-MYC was used as loading control. Result shown is representative of at least three experiments.

Figure S1. Analysis of the phosphorylation status of purified GST-CTD and Rpb1-TAP. A) Comparison of the phosphorylation status of the purified GST-CTD derived from the parent and *rrd1*Δ mutant following rapamycin exposure (200 ng/ml for 30 min). The purified GST-CTD was subjected to Western blot analysis and probed for Ser-2 phosphorylation (H5) or Ser-5 phosphorylation (H14). To measure equal loading membranes were stripped and reprobed with anti-GST antibody. **B)** Comparison of the phosphorylation status of purified Rpb1-TAP derived from the parent and *rrd1*Δ mutant cells following rapamycin treatment, as in panel B. Rpb1-TAP was purified by calmodulin affinity column and then probed with the indicated antibodies. To control for equal protein loading the membranes were stripped and reprobed with the anti-PAP antibody. Results shown are representative of three independent experiments.

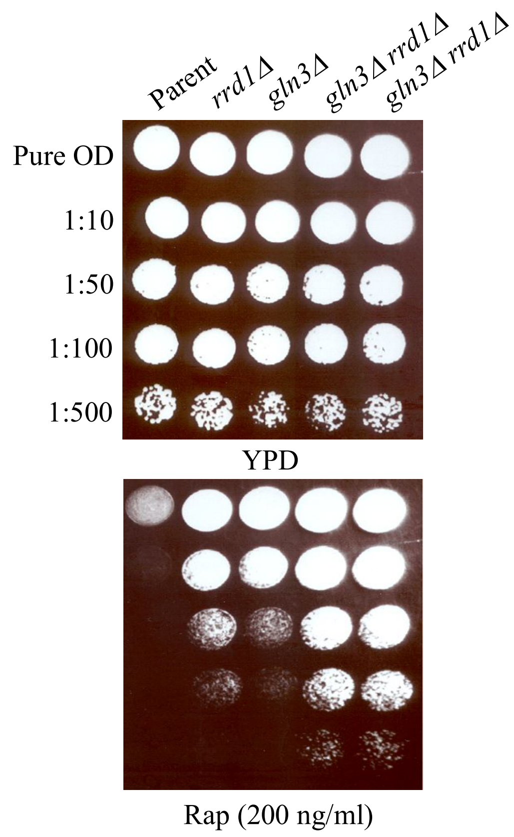
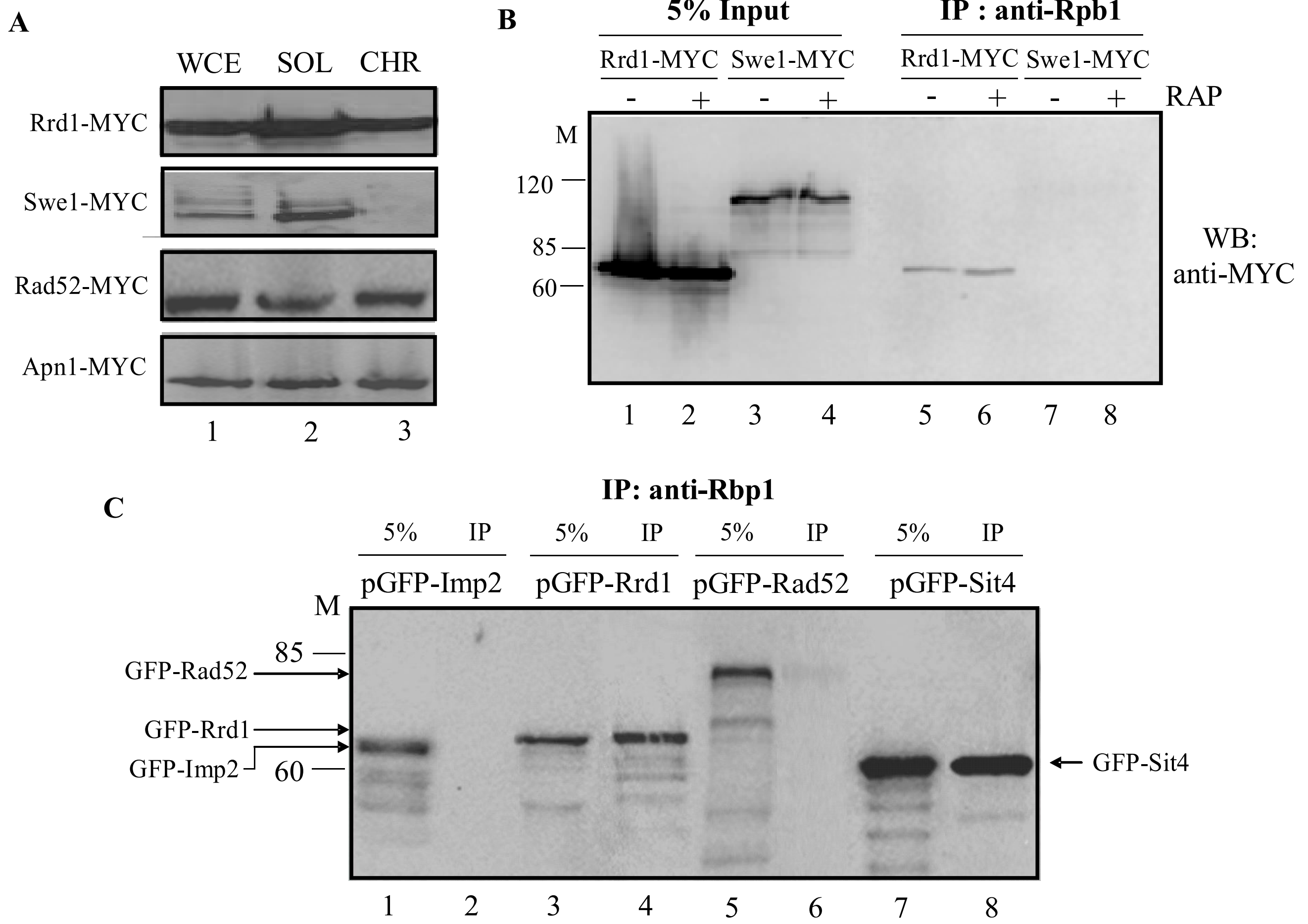


Fig. 1 : Jouvet et al., 2010



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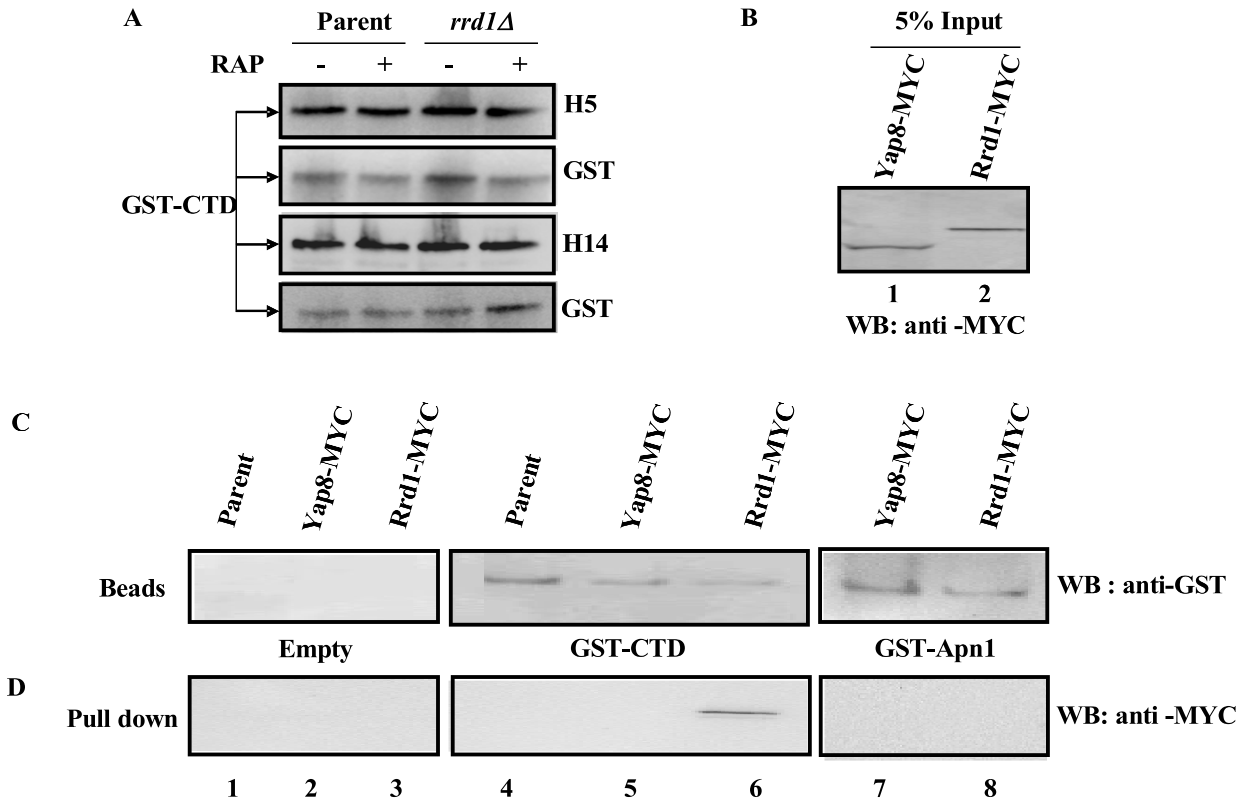

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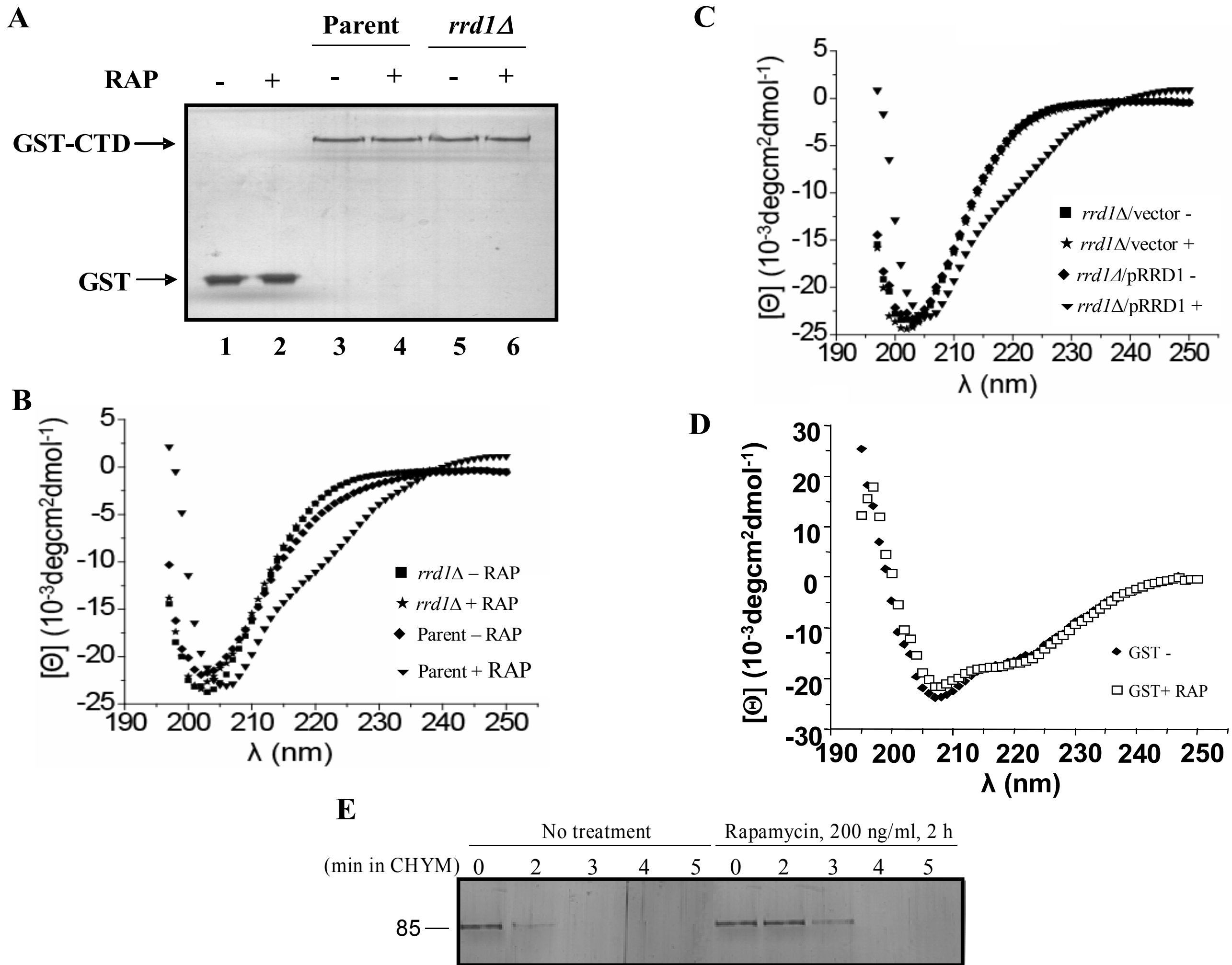

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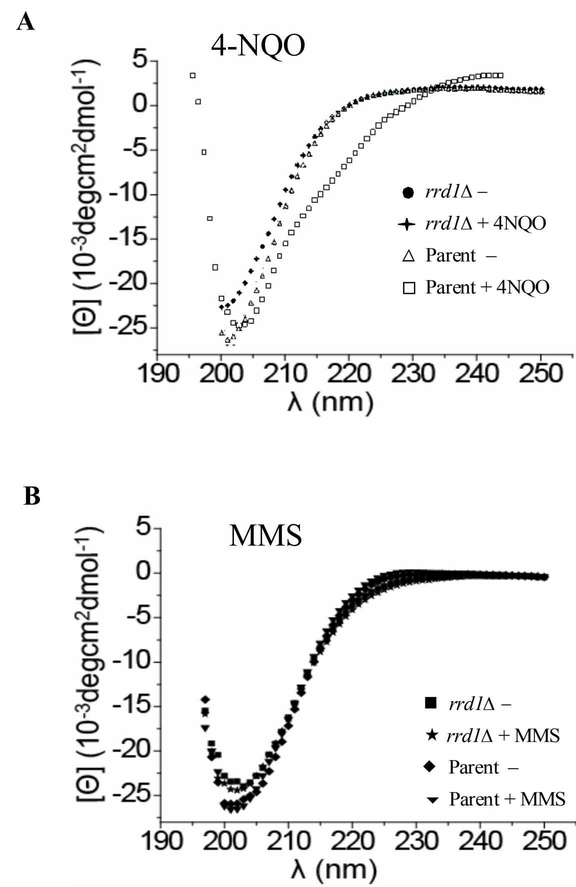

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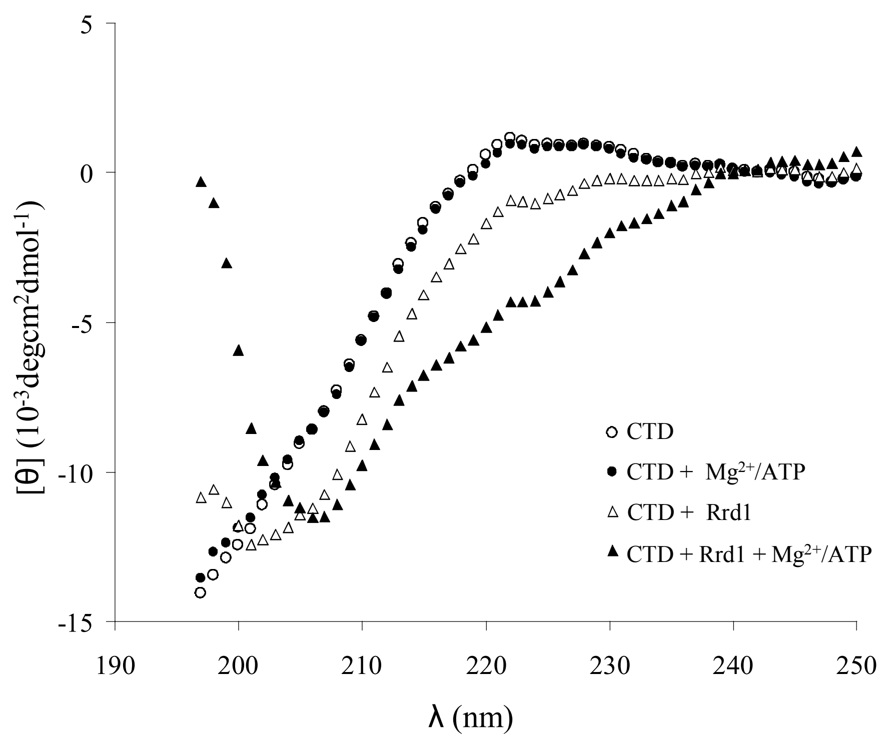

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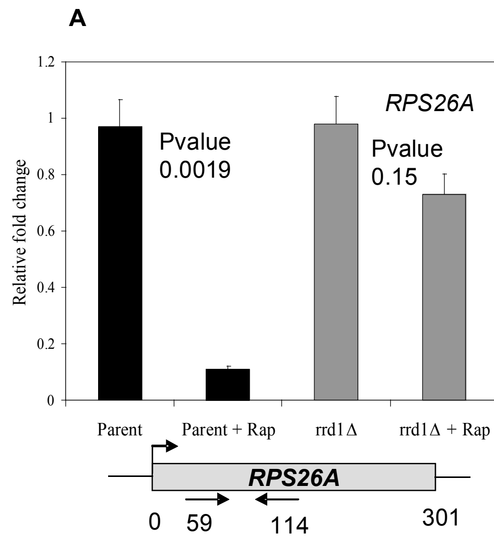

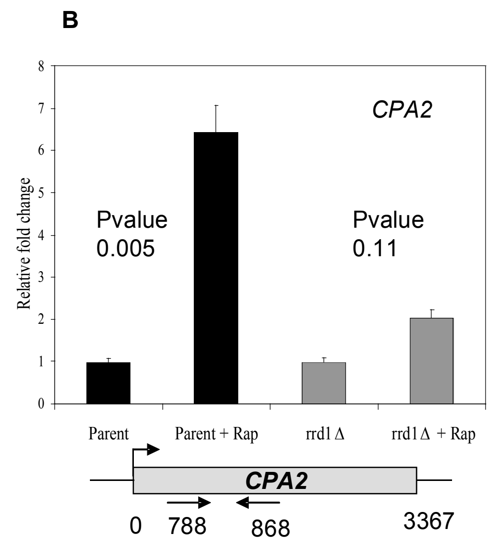

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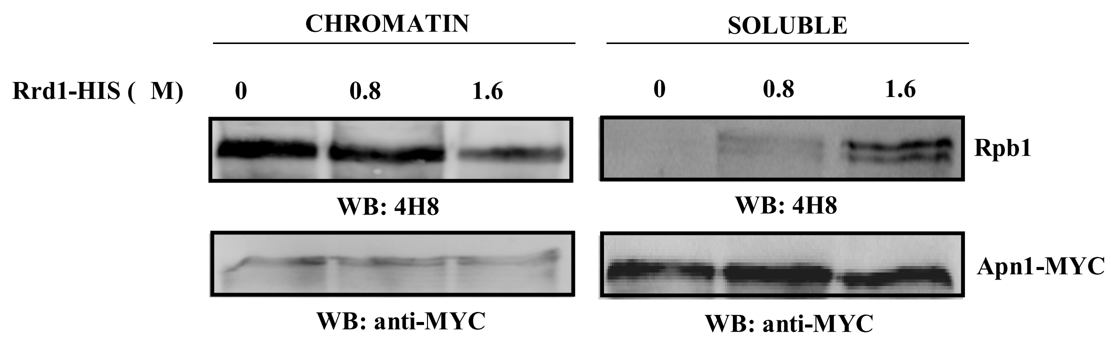

Fig. 8: Jouvet et al., 2010

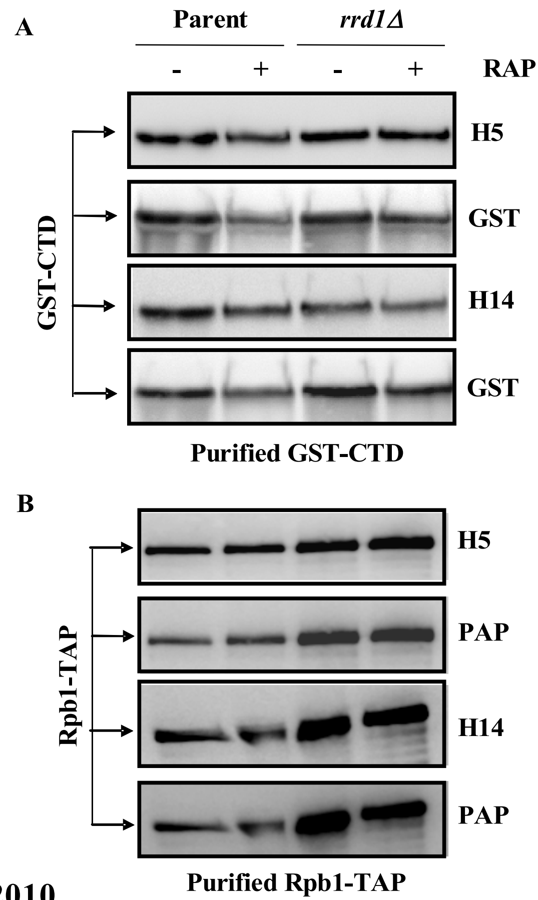

Fig. S1 Jouvet et al., 2010

2.2 The yeast peptidyl prolyl isomerase *Rrd1* is an elongation factor required for transcriptional stress responses

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My contribution to this article:

I would evaluate my contribution to the experimental data to about 90 %. I produced all figures besides figure 6A and B. SD guided me through ChIP on chip experiments and data analysis. PEJ helped for the bioinformatic data analysis. MN performed assays in figure 6A and B under my supervision. KL performed GeXP analysis and data processing.

The entire manuscript was written by myself and then corrected by DR.

The yeast peptidyl prolyl isomerase Rrd1 is an elongation factor required for transcriptional stress responses

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Abstract

Rapamycin is an anticancer molecule and immunosuppressant that acts by inhibiting the TOR signaling pathway. In yeast, rapamycin mediates a profound transcriptional response, for which the *RRD1* gene is required. This gene encodes a peptidyl prolyl isomerase that associates with RNA polymerase II (RNAPII) and isomerizes its C-terminal domain (CTD) in response to rapamycin. To further investigate this biological connection, we performed genome wide association studies of RNAPII and Rrd1 in response to rapamycin to demonstrate that Rrd1 co-localizes with RNAPII on actively transcribed genes and that both are recruited to rapamycin responsive genes. Strikingly, when Rrd1 is lacking, RNAPII fails to dissociate from a large set of ribosomal genes and is recruited to a set of rapamycin responsive genes; this occurs independently of the TATA box binding protein recruitment. We further show that Rrd1 modulates the phosphorylation status of RNAPII CTD, and finally provide evidence that Rrd1 is required for the transcriptional response to various stresses. We propose a model whereby Rrd1 acts as an elongation factor to optimize the transcriptional stress response.

Introduction

Rapamycin is an immunosuppressant and an anticancer molecule that acts through inhibition of the TOR (target of rapamycin) signaling pathway [160, 161]. In the yeast *Saccharomyces cerevisiae, TOR1* and *TOR2* genes encode two serine/threonine kinases whereby each forms the core of the rapamycin sensitive (TORC1) and the rapamycin insensitive TORC2 complex, respectively [58, 162-164]. TORC1 positively regulates anabolic processes, by promoting mRNA translation and the transcription of ribosome biogenesis genes [58, 162-164]. Upon nutrient starvation, or rapamycin treatment, the TORC1 complex becomes inactivated, with the consequence of a severe reduction of anabolic processes, cell cycle progression and growth, as well as the induction of catabolic processes and stress responsive factors [58, 162-164]. This drastic change is mediated by alteration of gene transcription and is regulated through the phosphorylation/dephosphorylation and translocation of transcription factors between the cytoplasm and the nucleus. Ribosomal gene transcription is regulated by the ribosomal gene repressor Crf1, which is sequestered in the cytoplasm when TORC1 is active. Upon TORC1 inactivation, Crf1 is phosphorylated by the Yak1 kinase, translocates into the nucleus and competes with the co-activator Ifh1 for binding to the ribosomal transcription factor Fhl1, thereby repressing transcription [67]. Further, upon TORC1 inactivation, the downstream TORC1 regulator Tap42 activates PP2A and Sit4 phosphatases, which then in turn dephosphorylate the transcription factors Rtg1/2 and Gln3 causing these factors to move into the nucleus and induce the expression of retrograde signaling genes (RTG) and nitrogen discrimination genes (NDG), respectively [60, 118, 162, 163]. In addition, the expression of stress responsive genes is stimulated via activation of Tap42, enhancing nuclear retention of the transcription factors Msn2/4 [58, 60, 163, 164]. Once translocated to the nucleus, these transcription factors bind to specific DNA elements, alter the local chromatin state and recruit the general transcription machinery to mediate assembly of the pre-initiation complex (PIC) and transcription by RNA polymerase II (RNAPII) [16, 165].

The exact mechanisms of these regulatory circuits are not fully understood but genome wide deletion screens in *S.cerevisae* have been a useful tool to identify novel factors that are required to mediate an efficient response to rapamycin [110, 124, 166, 167]. One of these factors is the peptidyl prolyl isomerase Rrd1 (Resistant to rapamycin deletion 1) that was originally identified to play a role in the cellular protection against the carcinogen 4-nitroquinoline-1-oxide, as well as to UVA radiation [74]. *rrd1Δ* mutants exhibit multiple phenotypes, but the most prominent is its extreme resistance to rapamycin [110]. Rrd1 is evolutionally conserved and it shares 35% amino acid sequence identity with the human homologue PTPA [87, 88, 93]. PTPA was first characterized to be an activator of the phospho-tyrosyl phosphatase activity of PP2A phosphatases *in vitro* [85, 86]. However, an *in vivo* role of this activity has not been described yet and subsequent studies revealed that PTPA/Rrd1 is required for PP2A substrate specificity, complex formation and the reactivation of inactive PP2A complexes [89, 90]. It turns out that PTPA/Rrd1 has intrinsic peptidyl prolyl isomerase activity and is able to catalyze proline isomerization on a specific peptide sequence of PP2A [93]. Consistent with this function, we and others found that in yeast Rrd1 interacts with the PP2A like phosphatase Sit4 [111, 112, 114, 117]. Sit4 and Rrd1 form a ternary complex with the Tor signaling mediator Tap42 [117]. As mentioned above, upon TORC1 inactivation Tap42 dissociates from Sit4-Rrd1 which then dephosphorylates and activates the transcription factor Gln3 [113, 118]. Interestingly, *gln3*Δ mutant cells are resistant to rapamycin and therefore it was postulated that *rrd1*Δ mutants are rapamycin resistant since they are involved in the Gln3 pathway [117]. However, we found that the Gln3 target gene *MEP2* was activated independently of *RRD1*, suggesting that *RRD1* has an additional role in the response to rapamycin [113]. Consistent with this, we found that Rrd1 exerts an effect at the transcriptional level: genes known to be upregulated (e.g., the diauxic shift genes *CPA2* and *PYC1*) and down-regulated (e.g., the ribosomal protein genes including *RPS26A*, *RPL30*, and *RPL9*) following rapamycin exposure showed an altered transcription pattern in *rrd1Δ* mutants [113]. Subsequently, we demonstrated that Rrd1 interacts with RNAPII and that it directly isomerizes the CTD of the large subunit (Rpb1) of RNAPII (Jouvet et al., 2010 NAR in revision). In addition, we provided evidence that Rrd1 releases RNAPII from the chromatin, which could be a new mechanism of RNAPII regulation (Jouvet et

al., 2010 NAR in revision). It would appear that Rrd1 exerts its role during transcription elongation as this was similarly shown for another peptidyl prolyl isomerase, Pin1, and its yeast homologue Ess1 [44, 45, 95, 106, 168]. Pin1/Ess1 is thought to isomerize the CTD of RNAPII and regulate elongation [95, 106]. In yeast, the CTD consists of 26 repeats of the $YS_2PTS_5PS_7$ heptad sequence which is differentially phosphorylated on serine 2, serine 5 and serine 7 [7-9, 42, 169, 170]. These different phosphorylation patterns act as a recruitment platform for multiple factors involved in chromatin remodelling, mRNA processing and transcription termination [7-9, 170]. The yeast homologue of Pin1, Ess1 has been shown to stimulate the dephosphorylation of Ser-5 to efficiently terminate transcription of a subset of genes [44].

In this study, we further analyzed how Rrd1 regulates transcription by RNAPII. We mapped the genome wide association of Rrd1 and RNAPII using ChIP-chip analysis under control and rapamycin treated conditions and demonstrate that Rrd1 co-localizes with RNAPII on actively transcribed genes in both conditions. We further show that *rrd1Δ* deletion affects RNAPII occupancy on a large set of rapamycin responsive genes. This happens independently of Spt15 recruitment to the promoter suggesting that Rrd1 acts downstream of PIC formation during transcriptional initiation and elongation. This is further confirmed by the fact that Rrd1 modulates serine 5 phosphorylation of the RNAPII CTD. Finally, we demonstrate that Rrd1 is generally required to regulate gene expression in response to a variety of environmental stresses thus establishing Rrd1 as a new elongation factor required for effective transcriptional responses.

Results

Rrd1 localization correlates with RNAPII along actively transcribed genes.

We recently reported that Rrd1 is associated with chromatin and directly interacts with RNAPII (Jouvet et al., 2010 NAR in revision). To analyze if Rrd1 interacts with RNAPII along transcribed genes and whether this is relevant to the transcriptional response to rapamycin, we used ChIP-chip analysis to address this question. We first examined the genome-wide RNAPII occupancy under exponential growth conditions without and with 30 minutes of rapamycin treatment, where the rapamycin transcriptional response is most prominent based on mRNA expression analysis [128]. In Fig. 1A, we took the median RNAPII occupancy of approximately 5000 ORFs, where RNAPII enrichment was expressed as a log 2 ratio of the immunoprecipitated DNA compared to the corresponding input DNA (enrichment is negative when the IP amount is below the Input amount). Similarly enriched genes were clustered into groups using self organizing maps (SOM) with *cluster 3.0*. SOM creates distinct clusters of similar performing genes within different conditions and allows for the distinction of subgroups [171]. To facilitate the identification of the genes that were affected by rapamycin, we subtracted the rapamycin treated data from the untreated (Fig. 1A row 3). The resulting clusters (W1-W6) were then analyzed for gene ontology (GO) category enrichment with *funcassociate 2.0* [172] (Suppl. Fig. S1). Upon rapamycin treatment RNAPII occupancy was sharply reduced on metabolic genes including ribosome biogenesis (see cluster W1, W6 and Suppl. Fig. S1). In contrast, RNAPII was strongly enriched on genes belonging to nitrogen discrimination, Krebs cycle, stress response and catabolic processes after rapamycin treatment (W3, W4 and Suppl. Fig. S1). These data are consistent with the transcriptional changes reported for rapamycin treatment and the environmental stress response [63, 71, 128].

We next performed ChIP-chip analysis on an endogenous MYC tagged RRD1 strain (Jouvet et al., 2010 NAR in revision) to determine if Rrd1 is associated with actively transcribed genes. We mapped RNAPII and Rrd1-MYC on groups of genes sorted according to their level of RNAPII occupancy (Fig.1B) and found that Rrd1 association correlated with RNAPII on actively transcribed genes (Fig. 1B and C). The

distribution of Rrd1 peaks after the promoter and remained constant throughout the ORF (Fig. 1C). Interestingly, when the same analysis was performed in rapamycin treated cells we observed a similar binding pattern, which suggests that Rrd1, like RNAPII, is recruited to rapamycin induced genes (Suppl. Fig. S2A). In addition, linear regression revealed that Rrd1 localization correlated with that of RNAPII (Suppl. Fig. S2B) [19]. This comparison resulted in a correlation coefficient (R^2) of 0.61 under exponential growth conditions, and which did not alter when the cells were challenged with rapamycin ($R^2 = 0.62$) (Suppl. Fig. S2B).

To further analyze the genes that were bound by Rrd1-MYC, we clustered RNAPII gene occupancy, as well as the difference between untreated and rapamycin-treated gene occupancy for RNAPII and Rrd1-MYC (Fig. 1D). GO analyses performed as above revealed that similar genes as RNAPII were enriched or depleted for Rrd1-MYC upon rapamycin treatment, (Fig. 1D and for GO Suppl. Fig. S3A). We also analyzed single genes which are representative of each of the four clusters from the SOM in Fig. 1D (see Suppl. Fig. S3B). These data suggest that Rrd1 and RNAPII co-localize within the body of most of the actively transcribed genes, even after transcriptional changes such as the one caused by rapamycin treatment, except for Cluster R2 where Rrd1 binding was slightly different from RNAPII binding, which could be because of variability between experiments or that Rrd1 might perform additional roles on the chromatin. However, for most genes these observations are consistent with a model whereby Rrd1 interacts with RNAPII (Jouvet et al., 2010 NAR in revision), and suggests that Rrd1 might act as a transcriptional elongation factor to directly influence the polymerase activity (see discussion).

RRD1 deletion affects RNAPII localization in response to rapamycin

Since Rrd1 presence correlates with RNAPII on actively transcribed genes and it was previously shown to be required to modulate the expression of some genes when cells are challenged with rapamycin (Jouvet et al., 2010 NAR in revision) [113], we mapped the genome-wide location of RNAPII in a *rrd1*Δ mutant using the same conditions as above. First, we plotted RNAPII gene occupancy of the *rrd1*Δ mutant against the WT and calculated the correlation coefficient ($R^2 = 0.87$) under exponential

growth conditions and after rapamycin treatment ($R^2 = 0.75$). This R^2 alteration suggests that RNAPII gene association might diverge between the *rrd1*Δ mutant and WT upon rapamycin treatment (Fig. 2A). The ribosomal biogenesis genes (Ribi) and ribosomal protein genes (RP) were labeled in different colors (Fig. 2A, red and green, respectively). The data revealed that Ribi and RP genes were similarly occupied in WT and *rrd1*Δ mutant cells under normal growth conditions, but when treated with rapamycin these genes were more occupied in an *rrd1*Δ mutant (Fig. 2A), suggesting a defect in transcriptional regulation of these genes. We next compared the RNAPII occupancy difference (i.e., before and after rapamycin treatment) in the WT with that obtained from the *rrd1*Δ mutant (as obtained from Suppl. Fig. S4). We used the untreated RNAPII gene occupancy as a reference (Fig. 2B). This comparison revealed that RNAPII distribution was different in the *rrd1*Δ mutant when compared to the corresponding WT in response to rapamycin for most genes (Fig. 2B). However cluster P2, which was strongly depleted by RNAPII from WT cells in response to rapamycin, was not altered in the *rrd1Δ* mutant, indicating that Rrd1 is required to downregulate this group of genes in response to rapamycin. GO analysis revealed that this cluster was highly enriched for metabolic and ribosomal biogenesis genes as well as for genes with metabolic regulatory functions (Suppl. Fig. S5). Cluster P5 was strongly enriched by RNAPII from WT cells but substantially less in the *rrd1*Δ mutant, suggesting that the recruitment of RNAPII to this group of genes also depends on Rrd1 presence. GO revealed that cluster P5 is highly enriched with catabolic and stress response genes, which are a major part of the transcriptional response to rapamycin and other environmental stresses [63, 71]. The clusters (P1 and P4) that were not dependent upon Rrd1 function were also analyzed by GO: cluster P1, which was depleted for RNAPII after treatment, was highly enriched in ribosome biogenesis genes and cluster P4, which was enriched for RNAPII, contained genes involved in catabolic processes suggesting that not all genes that are regulated by rapamycin treatment are affected by *rrd1Δ* deletion (Suppl. Fig. S5).

Taken together, the data suggest that cells devoid of Rrd1 display altered RNAPII occupancy on specific groups of genes in response to rapamycin. This is the case for genes that are downregulated or upregulated by rapamycin treatment [128]. Rrd1 was only required to dissociate RNAPII from a fraction of ribosomal genes (compare P1 and

P2), notably ribosomal regulatory genes, and similarly, Rrd1 was required to populate RNAPII only on some catabolic genes (P5 but not P4). This suggests that Rrd1 might regulate transcription, not by altering the recruitment of transcription factors, but likely through its association with RNAPII on actively transcribed genes.

To confirm our genome wide findings, we performed independent ChIP analysis followed by Q-PCR on selected genes from our ChIP-chip data. Fig. 3 revealed that genes upregulated by rapamycin, such as *PUT4*, encoding the proline transporter, and *HSP104,* encoding a heat shock protein, were significantly enriched for RNAPII in the WT (panel A) but only slightly enriched for RNAPII in the *rrd1*Δ mutant (Fig. 3B). In the case of genes downregulated by rapamycin, such as *RPL32* and *RPS2* encoding ribosomal proteins, they were depleted for RNAPII in the WT, while the *rrd1*Δ mutant still retained substantial levels (Fig.3A and B). Analysis of a gene that was unaffected by rapamycin treatment, such as the actin coding gene *ACT1*, showed that RNAPII levels were not altered in the WT or the *rrd1*Δ mutant (Fig. 3A and B). The same set of genes was also monitored for Rrd1-MYC occupancy (Fig. 3C). As observed for RNAPII, Rrd1-MYC was enriched on *PUT4* and *HSP104*, but was depleted on *RPL32* and *RPS2* in response to rapamycin whereas Rrd1 levels did not change on the *ACT1* gene (Fig. 3C). It is noteworthy that the amount of Rrd1-MYC immunoprecipitated was low as compared to RNAPII (see discussion). Nonetheless, these data are consistent with the genome-wide distribution of RNAPII and Rrd1, and validate the co-localization of RNAPII and Rrd1-MYC.

Rrd1 regulates RNAPII occupancy independently of TBP binding

To distinguish if Rrd1 influences RNAPII occupancy upstream or downstream of pre-initiation complex (PIC) formation, we examined the genome wide association of the yeast TATA box binding protein (Spt15), using ChIP-chip assay as above. If *rrd1*Δ mutants affect transcription at the level or upstream of PIC formation, we predict that upon rapamycin treatment the occupancy of Spt15-MYC would be similarly altered as RNAPII and Rrd1-MYC. We clustered the binding differences (treated minus untreated) from WT cells for RNAPII, Rrd1-MYC and Spt15-MYC (Fig. 4A). We observed that all three proteins were decreased in cluster S1, whereas in cluster S2, both RNAPII and Rrd1

were decreased, but Spt15-MYC remained unchanged. This suggests that RNAPII occupancy is regulated by two distinct mechanisms, one of which is independent of Spt15 binding. GO analysis of cluster S1 shows that the genes are enriched for ribosomal biogenesis, while cluster S2 is enriched for genes in functions of metabolic regulation (Suppl. Fig. S6 for GO analysis). We note that the occupancy of Rrd1-MYC for clusters S3, S4 and S6 was different from RNAPII and Spt15 suggesting that Rrd1 might perform additional roles besides its association with RNAPII as was also observed in Fig 1D.

We next checked if Spt15 binding correlates with RNAPII occupancy in the absence of Rrd1 by mapping RNAPII and Spt15-MYC in the WT and *rrd1*Δ mutant strains for the clusters S1 and S2 as well as cluster S5 (Fig. 4B). In cluster S1, Spt15-MYC was strongly reduced in the WT and to a lesser extent in the *rrd1Δ* mutant, correlating with the reduction of RNAPII association within these genes. The observation that in the *rrd1*Δ mutant, Spt15 and RNAPII association were less reduced compared to the WT suggests that Rrd1 might influence to a minor extent Spt15 binding and RNAPII recruitment, probably by affecting the signaling cascade upstream of Spt15 binding [117]. In the case of cluster S2, we observed no difference in Spt15 binding between the WT and the *rrd1Δ* mutant. However, there was a substantial difference between WT and mutant in the level of RNAPII in response to rapamycin, suggesting that for these genes Rrd1 is required to regulate RNAPII association downstream of TBP (Fig. 4B). Cluster S5 was similarly enriched for Spt15 binding after rapamycin treatment, but RNAPII occupancy in the *rrd1Δ* mutant failed to reach WT levels, suggesting that a similar mechanism is functioning for these genes.

The above data suggests that Rrd1 regulates some genes independently of Spt15 promoter binding. If this would be true we would expect to see the same pattern of Spt15 binding in the SOM of Fig 2B and therefore mapped Spt15 on the rapamycin regulated clusters (Suppl. Fig. S7). Cluster P1 showed a similar Spt15 reduction following rapamycin treatment as seen in cluster S1 from Fig. 4A, which occurred in both WT and *rrd1Δ* mutant, indicating that these genes are regulated by Spt15 binding (Suppl. Fig. S7). In contrast cluster P2 resembled cluster S2 from Fig. 4A, where Spt15-MYC was not altered, but RNAPII binding was dependent on Rrd1 presence (Suppl. Fig. S7). Cluster P4 was similarly enriched for Spt15 in response to rapamycin as cluster S5, and *rrd1Δ*

mutants failed to efficiently recruit RNAPII (Suppl. Fig. S7). Taken together these data suggest that specific groups of genes are regulated additionally to Spt15 promoter binding and this depends on Rrd1 presence.

***rrd1Δ* mutants exhibit phenotypes associated with a defect in transcriptional elongation**—The above data suggest that Rrd1 regulates transcription independently of TBP and therefore might act on elongating RNAPII. Mutants defective in elongation are known to be sensitive to 6-azauracil (6-AU), an inhibitor of the IMP dehydrogenase (IMDPH), which decreases GTP pools and thereby causing transcriptional arrest [22, 48, 173]. As a positive control we used the TFIIS elongation factor mutant (*dst1*Δ*)*, a well characterized transcription elongation factor [22, 174]. Using this drug, we found that *rrd1*Δ mutants were sensitive to 6-AU, as compared to the WT, but less sensitive than the *dst1*Δ mutant (Fig.5A) suggesting that Rrd1 might indeed play a role in transcription elongation.

To further investigate this possibility, and since we previously have demonstrated that Rrd1 isomerizes the CTD of RNAPII (Jouvet et al., 2010 NAR in revision) we examined whether Rrd1 might influence elongation by altering the phosphorylation status of the RNAPII C-terminal domain (CTD). The CTD consist of 26 repeats of a heptapeptide YSPTSPS in yeast which is differentially phosphorylated on serine 2 (Ser2-P) and serine 5 (Ser5-P). The different phosphorylation states of RNAPII are thought to be hallmarks of elongation, whereby RNAPII is highly phosphorylated on Ser5 at the promoter and the early elongation phase, whereas Ser2-P progressively increases throughout the ORF until it culminates at the 3' end of the gene [7-9, 170]. These two phosphorylations are recruitment platforms for chromatin modifying enzymes, transcription elongation factors, mRNA processing factors and mRNA termination factors [3, 7-9, 14, 170, 175]. These differential phosphorylations can be used as a surrogate for transcription elongation efficiency, as was the case for the peptidyl prolyl isomerase Pin1. It was shown that over-expression of Pin1 leads to increased Ser5-P of RNAPII and its dissociation from the chromatin [95]. In addition, the yeast homologue of Pin1, Ess1, was shown to regulate Ser5-P of RNAPII [44]. We performed ChIP-chip analysis of Ser5-P and Ser2-P in WT and *rrd1*Δ mutant strains under the same conditions

as above. Since RNAPII occupancy was different in WT and *rrd1*Δ mutant cells (see Fig. 2B), we normalized the complete data set of both phosphorylations with the RNAPII occupancy of the WT and the *rrd1*Δ mutant. This allowed for an unbiased representation of the phosphorylations independently of RNAPII occupancy. Next we generated SOM of the differences for Ser5-P and Ser2-P (between *rrd1*Δ mutant and WT) in untreated and rapamycin treated conditions (Fig. 5B). We observed no striking difference in Ser2-P between the WT and *rrd1Δ* mutant in untreated or rapamycin treated conditions (Fig. 5B). However, there were pronounced differences in Ser5-P; all four clusters displayed diminished or increased Ser5-P within both conditions (Fig. 5B), suggesting that in general Ser5-P is altered in *rrd1*Δ mutants. GO analysis revealed that some metabolic genes are decreased in Ser5-P (cluster F2) upon rapamycin treatment but others were increased for Ser5-P (cluster F3) under normal growth conditions (Fig. S8 for GO). Thus, under both growth conditions, Rrd1 may affect Ser5-P of most genes, which could in turn affect RNAPII occupancy. This is consistent with the altered RNAPII occupancy of *rrd1*Δ mutants under rapamycin treated conditions (see Fig. 2B).
Taken together the above data suggests that *rrd1*Δ mutants display phenotypes consistent with a transcriptional elongation defect of RNAPII.

Rrd1 is required to mediate efficient transcriptional stress responses

The above data clearly indicate that Rrd1 is required for an optimal transcriptional response following rapamycin exposure. As such, we predict that this mechanism is implicated in other environmental stress responses. Consistent with this, the *rrd1*Δ mutant exhibits multiple phenotypes including resistance to caffeine, but sensitivity towards vanadate, 4-NQO and calcium [74, 110]. Both, vanadate and 4-NQO are known to cause oxidative stress and thus, we expect the *rrd1*Δ mutant to be sensitive to additional oxidants. To test this, we challenged the mutant cells with the chemical oxidant H_2O_2 as well as sodium arsenite (NaAs) and found that the *rrd1*Δ mutant was indeed sensitive to these agents as compared to the WT (Fig. 6A and B). To ensure that the sensitivity was a result of a defect in gene regulation, we introduced a known arsenite-response reporter that bears the promoter of the *ACR3* gene fused to lacZ [136]. *ACR3* encodes a plasma membrane efflux pump that is upregulated *via* the Yap8

transcriptional activator in response to arsenite [136]. While there was a strong induction of the *ACR3*-lacZ reporter in the WT, it was hardly induced in the *rrd1Δ* mutant (Fig. 6C). This data suggests that in response to NaAs the transcriptional response is also affected in the *rrd1*Δ mutant.

We therefore monitored gene expression of 9 stress responsive genes using the multiplex PCR GeXP expression technique in response to rapamyin, H_2O_2, Na Arsenite and heat shock (see Materials and Methods). We chose genes that are known to be upregulated (*PRX1, ARR3, HSP12, HXK1, TSL1*) or downregulated (*RPL3, RPL32, RPS2, and PRS1*) in response to environmental stresses as well as control genes which are not significantly altered (*ACT1, GAL1*) [71]. First, we compared the untreated and rapamycin treated expression data to the RNAPII median enrichment on these ORFs (Suppl. Fig. S11). This analysis revealed that for the both conditions RNAPII correlated with mRNA expression for most of the genes, except the ribosomal genes. We next compared the gene-expression from WT and the *rrd1*Δ mutant for the different conditions (Fig. 7). Genes that are known to be induced such as (*PRX1, ARR3, HSP12, HXK1, TSL1*) in the WT upon stress were indeed upregulated in the WT, but only slightly in the *rrd1*Δ mutant (Fig. 7A). Genes that are known to be downregulated, such as the ribosomal genes *RPL32, RPS2* and *RPL3,* displayed a similar expression pattern between WT and the *rrd1*Δ mutant (Fig. 7B). It might be possible that for these genes other regulatory mechanisms are active including mRNA stability and translation efficiency (see Discussion). In the case of the *PRS1* gene, it was repressed for some treatments. Finally, the control genes *ACT1* and *GAL1* remained similar between WT and *rrd1*Δ throughout the various treatment conditions (Fig. 7C). Taken together, the expression analysis and the multiple phenotypes of *rrd1*Δ mutants towards environmental stresses suggest that Rrd1 plays a more generally role in transcriptional stress responses.

Discussion

Using ChIP-chip analysis we demonstrated that rapamycin induces a strong reorganization of the genome-wide RNAPII occupancy. RNAPII drastically reduces its association with anabolic genes (e.g. Ribi and RP genes) and is recruited to catabolic and stress response genes. These changes are in a qualitatively and timely relationship with total mRNA expression analyses from rapamycin treated cells [128].

We analyzed the genome-wide association of Rrd1 and found that it co-localizes with RNAPII on actively transcribed genes. Interestingly, Rrd1 remained associated with actively transcribed RNAPII genes independently of rapamycin treatment, suggesting that like RNAPII, Rrd1 is at low levels on repressed genes but highly enriched on newly transcribed genes. The data obtained was comparable to another report where TFIIS occupancy was matched to RNAPII [19]. Using the same assay, we found that the correlation between RNAPII and Rrd1 (0.61) was similar to TFIIS and RNAPII (0.64) (Suppl. Fig. S2B) [19]. This clearly indicates, that like TFIIS, Rrd1 co-localizes with RNAPII. In fact, of all the RNAPII actively transcribed genes (based on ChIP ratio); Rrd1 was present on 75% (2044 genes) of these genes under normal growth conditions. However, when cells were treated with rapamycin Rrd1 was still recruited to 72% of the RNAPII bound genes (2160 genes).

With the help of gene mapping studies we now can precisely monitor Rrd1 within the genes: The Rrd1 gene-association coincides with the reduction of Ser5-P after the promoter, and remains throughout the ORFs. At the end of the gene Rrd1 binding gradually decreases before Ser2-P peaks (see Suppl. Fig. S9). This suggests that Rrd1 might exert its function during transcription elongation events.

Analysis of RNAPII occupancy in the *rrd1Δ* deletion strain revealed that under normal growth conditions most genes are similarly occupied by RNAPII as in WT cells (only a small subset of genes was altered, that contains nucleotide biosynthetic pathway genes (Suppl. Fig. S10)). However, in response to rapamycin, Rrd1 was needed to decrease RNAPII occupancy on a large set of genes including the Ribi and RP genes (Fig. 2A and 2B). In addition, Rrd1 was required to efficiently populate RNAPII on catabolic and stress response genes (Fig. 2B). Interestingly, some anabolic and catabolic

genes were more affected than others, suggesting that Rrd1 does not generally alter transcription, but rather influences genes with variable intensity.

Further analysis revealed that Rrd1 acts independently of the TATA box binding protein Spt15 (TBP). The premise is that upon rapamycin treatment, transcription factors such Gln3, Rtg1/2, Msn2/4 and Crf1 are translocated to the nucleus, recruited to promoters and then stimulate or repress transcription by regulating TBP and PIC assembly [59, 60, 65-67, 165]. If this process is not affected then clearly Rrd1 must act downstream of the PIC. For genes that are upregulated in response to rapamycin, we found that TBP is similarly recruited to the promoter in the WT and the *rrd1Δ* mutant. However, our data showed that RNAPII was less recruited in a rrd1 mutant, suggesting that Rrd1 regulates transcription independently of TBP recruitment. This, and given that Rrd1 is associated within the ORF of most genes we believe that it acts at the level of initiation and/or elongation.

In the case of the rapamycin repressed genes, we found two modes of TBP regulation. First, for a group of genes TBP binding was depleted and in a second group TBP remained bound in response to rapamycin. For the first group, we observed minor differences between WT and *rrd1Δ* mutant for TBP binding (see Fig. 4). This suggests that Rrd1 influences processes upstream of the PIC. At the moment, we do not know how Rrd1 operates upstream of TBP, although it could be through its ability to activate phosphatases [89, 90, 176]. For the second group, although TBP remained the same, RNAPII occupancy decreased in the WT but remained higher in the *rrd1Δ* mutant, suggesting that Rrd1 function is downstream of TBP. This observation is similar as observed for the upregulated genes, again suggesting a role for Rrd1 in initiation/elongation.

We note that our data also reveal a novel observation showing that TBP remained promoter-bound onto a subset of anabolic genes while RNAPII decreased in response to rapamycin and partially dependent on Rrd1 (Fig. 4 and Suppl. Fig. S7). We suspect that this might be a mechanism to temporarily turn down transcription, but rapidly restart gene expression once the stress is over.

Since all the data so far pointed towards a role for Rrd1 in transcription initiation/elongation, we tested and found that *rrd1Δ* mutants displayed hypersensitivity

against the agent 6-AU, which has been widely used to identify elongation mutants [22, 23, 48, 174, 177]. Although this is not a direct proof, this phenotype is in accordance with a potential role of Rrd1 in transcription elongation. Furthermore, we found that *rrd1Δ* mutants display an altered Ser5-P but not Ser2-P form of RNAPII on a lot of genes and this pattern changes upon rapamycin treatment (Fig.5B). We note that for some genes Ser5-P was increased whereas for others it was decreased, suggesting that Rrd1 is required to regulate both states (hyper- and hypophosphorylation status).

It could be that Rrd1 regulates the Ser5-P and thereby influences the rate of transcription of RNAPII. In the case of downregulated genes that are not regulated by TBP, Rrd1 increases Ser5-P and this causes RNAPII to slow down initiation and elongation until the stress is over. Indeed, we observe a cluster where Ser-5P is low on anabolic genes upon rapamycin treatment in the *rrd1Δ* mutant (Fig. 5B cluster F2). In contrast, for genes that are upregulated in response to rapamycin, Rrd1 favours a low Ser5-P form of RNAPII, thereby increasing initiation and elongation efficiency.

It is known that RNAPII occupancy is regulated during transcription elongation, for example, it was previously reported that the Ser5-P form of RNAPII is enriched on ribosomal genes, and this is associated with a slow transcriptional rate [154]. Interestingly, when these cells were transferred from glucose to galactose containing medium, the level of RNAPII decreased on these ribosomal genes and their transcriptional rate increased. Simultaneously, RNAPII was recruited to other genes including mitochondrial genes [154]. This suggests a mechanism where RNAPII can alter its transcriptional rate in order to fine-tune gene expression [154]. Similar to a switch from glucose to galactose, rapamycin induces a transcriptional response which requires some genes to be turned off and others to be induced. We propose that this is regulated through TBP binding, but additionally Rrd1 might influence the Ser5-P of RNAPII thereby fine-tuning the elongation efficiency for up- and downregulated genes.

Although we previously postulated that the phosphorylation status of RNAPII is not altered in *rrd1Δ* mutants when analyzing total cell extract by Western blot (Jouvet et al., 2010 NAR in revision), here we show that Rrd1 influences Ser5-P, but not Ser2-P phosphorylation, using the ChIP-chip assay. Indeed, since some genes contained a higher

amount of Ser5-P RNAPII, while others had less, the Western blot assay might not identify such subtle changes.

Based on the above findings, we expect Rrd1 to have a much broader role in stress response situations, notably the environmental stress responses as they display a similar pattern of gene expression as rapamycin [63, 71]. Indeed, we found *rrd1Δ* mutants to be sensitive towards agents causing oxidative stress which are known to induce a drastic transcriptional response (see Fig. 6) [63, 71]. Although these phenotypes are opposite of the one observed for rapamycin, it is clearly consistent with Rrd1 function. When *rrd1Δ* mutant cells are challenged with rapamycin they do no respond adequately to the starvation signal but the environment is nutrient rich and they are able to continue to grow. However, if that is the case during an increase in reactive oxygen species, their deleterious effects will be amplified and cause cell death. Consistent with this, we show for the first time that Rrd1 is required to adequately induce gene expression on a subset of stress responsive genes upon various stress conditions (Fig. 7). Surprisingly, ribosomal genes were not strongly altered in the WT or in the mutant as was predicted from the ChIP-chip data. We suspect that other mechanisms such as mRNA stability maintain a high amount of mRNA although RNAPII is not transcribing these genes anymore.

Taken together we have shown that Rrd1 regulates transcription elongation of RNAPII in response to stress. Further, the multiple phenotypes of *rrd1Δ* mutants and its inability to adequately respond to transcriptional changes support that Rrd1 is required for transcriptional stress responses. As such, we propose that Rrd1 is a novel transcription elongation factor required to modulate gene expression in response to stresses.

Materials and Methods

Strains, Cell Growth and Crosslinking Conditions

All strains used in this study were from the BY4741 background (*Mat a*, *his3-1*, *leu2-0*, *met15-0*, *ura3-0*). All strains used for ChIP analysis were grown in 50 mL of YPD to an OD_{600} of 0.6-0.8 before crosslinking. For ChIP-chip, strains were crosslinked with 1% formaldehyde for 30 min at room temperature on a rotator.

Chromatin Immunoprecipitation and Antibodies

ChIP experiments were performed as per (Ren et al., 2000) [178], with minor modifications.For myc-tag ChIP, we used 5µg of 9E11 antibody coupled to $2x10^7$ pan-mouse IgG DynaBeads (Invitrogen) per sample. RNAPII ChIPs were done using 2µL of 8WG16 antibody coupled to $2x10_7$ panmouse IgG DynaBeads per sample. For S2P and S5P ChIP, we used 100µL of rat serum (3E8 and 3E10 respectively) coupled to $2x10_7$ protein G DynaBeads per sample [40]. The microarrays used for location analysis were purchased from Agilent Technologies (Palo Alto, California, United States) and contain a total of 44,290 Tm-adjusted 60-mer probes covering the entire genome for an average density of one probe every 275 bp (±100 bp) within the probed regions (catalog # G4486A and G4493A). Myc-tag ChIPs were hybridized against ChIPs from isogenic strains that did not contain the tag as controls. RNAPII ChIPs hybridized against a sample derived from 400ng of input (non-immunoprecipitated) DNA. Q-PCR ChIP experiments were performed as above, only that after phenol chloroform extraction; the DNA was quantified with quantitative real-time PCR analysis, using the ABI 7000 machine (Applied biosciences) and Sybr green PCR mastermix (Applied biosystems). The % IP ORF/ % IP no ORF ratio was determined using the relative efficiency method and calculated as in Lloyd et al. [179]. Briefly, the % IP was calculated with respect to the Input for the target region (ORF) and the control region (no ORF) and then expressed as a ratio [179]. Primers were designed using the primer express software (Applied biosciences) with a total amplification length of maximum 150bp, exclusively matching the ORF of the indicated genes (listed in table S2).

Data Analysis

The data was normalized and replicates were combined using a weighted average method as described previously [178]. The log^2 ratio of each spot of combined datasets was then converted to Z-score, similar to Hogan et al. [180] to circumvent the large differences in the immunoprecipitation efficiencies of the different factors. Visual inspection of the Z-scores was carried out on the UCSC Genome Browser (http://genome.ucsc.edu/). All data analyses described here were done using data from protein-coding genes longer than 500 bp. Median Z-score values for promoter and gene coding sequences were calculated and used in our clustering analyses. Promoters are defined as the shortest of either 250bp or half the intergenic region (half-IG) relative to the reference gene's 5' boundary. Self-organizing map (SOM) clustering was done with the Cluster software [171] and visualized with Java Treeview [181]. Only genes with no missing value were used for clustering. Gene mapping was performed as in Rufiange et al. [182] on selected groups of genes described in the text. Briefly, data were mapped onto the 5' and 3' boundaries in 50 bp windows for each half-gene and adjacent half-IG regions. A sliding window of 300 bp was then applied to the Z-scores to smooth the curve.

GO analyses on clusters were performed with funcassociate *2.0.* For this, an association file for the entire gene set was generated and used for all analyses [172].

Regression analysis was plotted using excel, where the x-axis and y-axis contained the whole data set of the average ORF enrichments of the indicated ChIP. A trend line was plotted and the R2 was calculated. The regression analysis of Suppl. Fig. S2B was plotted as in Ghavi-Helm et al., except that RNAPIII genes were excluded [19].

Phenotype analysis of *rrd1*Δ mutants

The H_2O_2 survival curves were performed as described previously [183], briefly exponentially growing cells were washed once in 50mM KPO4 (p H7.5) and treated with the indicated concentration of H_2O_2 (Bio basic Inc) in 50mM KPO4 (pH7.5) buffer for one hour. Cells were then washed and plated onto YPD agar and scored for colony formation after three days at 30C.

Spottest with Na arsenite (Sigma) analysis was performed as described previously [73]. For spottests with 6-azauracil (Sigma), strains were transformed with and empty vector

bearing the URA3 gene (pTW423) and spotted onto synthetic media agar plates lacking uracil, with the indicated concentrations of 6AU.

β-gal assay

For *lacZ* expression the plasmid bearing the *ACR3* fusion with *LacZ* [136] was transformed into indicated strains and exponentially growing cells were treated with 1mM Na Arsenite (Sigma), aliquots were taken and the β-gal assay was performed as described in [184].

Legends

Figure 1: Rrd1 localization correlates with RNAPII along actively transcribed genes

(A) Self-organizing map (SOM) clustering of RNAPII from WT strain binding at ORFs. Red colour indicates enriched (bound) regions and blue colour represents depleted regions. The difference (diff) was calculated by subtracting the complete untreated (- Rap) RNAPII data set from the treated (+Rap) RNAPII data set. (Rap= rapamycin 100ng/ml for 30 min) The brackets (W1-W6) indicate clusters that were analyzed by gene ontology (GO) (see Suppl. Fig.S1)

(B) RNAPII occupancy mapped on four groups of genes that are distinguished by their amount of bound RNAPII. (red), genes are very highly bound by RNAPII; (orange) genes are highly bound by RNAPII; (light blue) and (dark blue) low and very low RNAPII bound, respectively.

(C) Rrd1-MYC occupancy was mapped on the same groups of genes from panel (B)

(D) SOM clustering of RNAPII WT (-Rap), RNAPII WT difference and Rrd1-MYC difference calculated as in panel (A). Brackets indicate clusters R1-R4 that were analyzed by GO (see Suppl.Fig.3)

Figure 2: ***rrd1Δ*** **deletion affects RNAPII localization in response to rapamycin**

(A) Linear regression of RNAPII enrichment on ORFs from WT (x-axis) and *rrd1Δ* mutant (y-axis). Each circle represents a RNAPII ORF. The trend line and the R^2 is indicated (0.87). The second regression shows the same under rapamycin treated condition; R^2 (0.75). Circles in red represent ribosomal biogenesis genes (Ribi) whereas green circles represent ribosomal protein genes (RP).

(B) Self-organizing map (SOM) clustering of the difference from WT RNAPII (obtained from panel A), the difference from *rrd1Δ* mutant RNAPII (obtained from Suppl. Fig.S4) and the difference from both (subtraction of the *rrd1Δ* mutant diff from WT diff). WT RNAPII (-Rap) was separately added as a reference. The clusters P1- P6 were analyzed by GO (see Suppl. Fig. S5.)

Figure 3: Validation of the genome-wide analysis

(A) ChIP of Rpb1 from WT cells followed by Q-PCR on the indicated genes. Dashed bars indicate rapamycin treatment (100ng/ml for 30 min). The data is represented as a ratio % IP over Input on the ORF compared to % IP over Input in a non transcribed region (noORF).

(B) ChIP of Rpb1 (8WG16 antibodies) from *rrd1Δ* mutant cells performed as in panel A.

(C) ChIP of Rrd1-MYC (Myc antibodies) performed as in panel A.

Results for all panels are shown as an average of at least three independent experiments, error bars indicate the standard deviation and the asterix **(*)** indicates if the Pvalue is below 0.05 between the untreated and treated condition using the Student T test.

Figure 4: Rrd1 is required for RNAPII association independently of TBP recruitment

(A) SOM of RNAPII, Rrd1-MYC and Spt15-MYC difference (+/-) (treated minus untreated) from the ORF, ORF and promoter respectively. RNAPII occupancy of corresponding genes from untreated cells was added separately. Cluster S1-S6 were analyzed by GO (see Suppl. Fig.S6)

(B) Mapping of Spt15-MYC occupancy on genes contained within clusters S1, S2, S4 and S5, from WT (blue) and from *rrd1Δ* mutant (orange). The same clusters were also mapped for RNAPII occupancy from WT (green) and *rrd1Δ* mutant (red). Dashed lines indicate rapamycin treatment and solid lines the untreated.

Figure 5: *rrd1Δ* mutants exhibit phenotypes consistent with a defect in transcriptional elongation

(A) Spottest analysis of WT, *rrd1Δ* and *dst1Δ* mutant strains on agar containing selective media lacking uracil (-URA), containing 6-azauracil (6AU) with the indicated concentration.

(B) SOM clustering of the difference of serine 5 (median enrichment at the promoter) and serine 2 (median enrichment at the ORF) phosphorylated form of RNAPII (*rrd1Δ* mutant minus WT), as well as RNAPII from WT (-Rap) and the difference of

RNAPII (*rrd1Δ* mutant minus WT). RNAPII ORF enrichment was added as a reference (- and + rapamycin). GO analysis data from the genes within cluster SP1-SP4 are found in Suppl. Fig. S8.

(C) Mapping of serine 5 (full line) and serine 2 (dashed line) phosphorylated form of RNAPII on genes contained within clusters F1 to F4, from WT (green) and from *rrd1Δ* mutant (red).

Figure 6 *rrd1*Δ mutants display hypersensitivity to agents causing oxidative stress.

(A) Survival curve of WT (open circle) and *rrd1Δ* mutant strains (closed circle) upon H_2O_2 treatment. H_2O_2 concentrations are indicated below; result shown is an average of three independent experiments and error bars indicate the standard deviation.

(B) Spottest analysis of WT and *rrd1Δ* mutant strain with indicated Na Arsenite treatment (1mM)

(C) LacZ reporter expression analysis from the *ACR3*-lacZ fusion plasmid, expressed in Miller units. WT (open circle) and *rrd1Δ* mutant (closed circle) show the *ACR3*-lacZ expression over time in response to 1mM Na Arsenite treatment.

Figure 7 mRNA expression analyses in response to different stress conditions

GeXP multiplex Q-PCR analysis of 11 genes under untreated, rapamycin treated (100ng/ml for 30 min), 0.6mM H^2O^2 for 30 min, 1mM NaArs for 30 min, heat shock at 37C for 30 min. The data is expressed in log 2 ratio and is calculated relative to an internal PCR control. (A) Five upregulated genes. (B) Four downregulated genes (C) Two control genes.

INVENTORY OF SUPPLEMENTAL INFORMATION

Jeremie Poschmann, Simon Drouin, Karima El Fadili, Pierre-Etienne Jacques, Michael Newmarch, Francois Robert, and Dindial Ramotar

SUPPLEMENTAL EXPERIMENTAL PROCEDURE

Table S1: A list of all primers used for the Q-PCR analysis
Figure S1, S3-S6, S8, S10: GO analysis of figures 1-5

- File S1 contains GO analysis of cluster W1-W6 from figure 1A
- File S3A contains GO analysis of cluster R1-R4 from figure 1D
- from figure 1D
- File S4 contains SOM clustering and GO analysis of *rrd1*Δ mutant (-/+) rapamycin
- File S5 contains GO analysis of cluster P1-P6 of figure 2B
- File S6 contains GO analysis of cluster S1-S6 of figure 4A
- File S8 contains GO analysis of cluster SP1-SP4 of figure 5B

Figure S2: (A) A complement to Figure 1B, same experiment besides that the data was obtained from rapamycin treated cells. (B) Linear regression of all enriched ORFs x-axis Rrd1-MYC and y-axis RNAPII (-) and (+) rapamycin
Figure S3B genome browser analysis of 4 genes representative of each group R1-R4 (WT stands for RNAPII)
Figure S4: contains SOM clustering and GO analysis of *rrd1*Δ mutant (-/+) rapamycin
Figure S7: Spt15 and RNAPII mapping of cluster P1, P2 P3, P4 of fig 2B
Figure S9: Mapping of serine 5, serine 2, RNAPII and Rrd1-MYC on transcribed genes
Figure S10: (A) SOM clustering and GO analysis of WT, *rrd1*Δ mutant as well as the difference (*rrd1*Δ minus WT) without rapamycin treatment.
Figure S11: Comparison of GeXP mRNA expression and RNAPII median average ChIP data of the corresponding gene for untreated and rapamycin treated conditions. Both are expressed in log2 ratio.

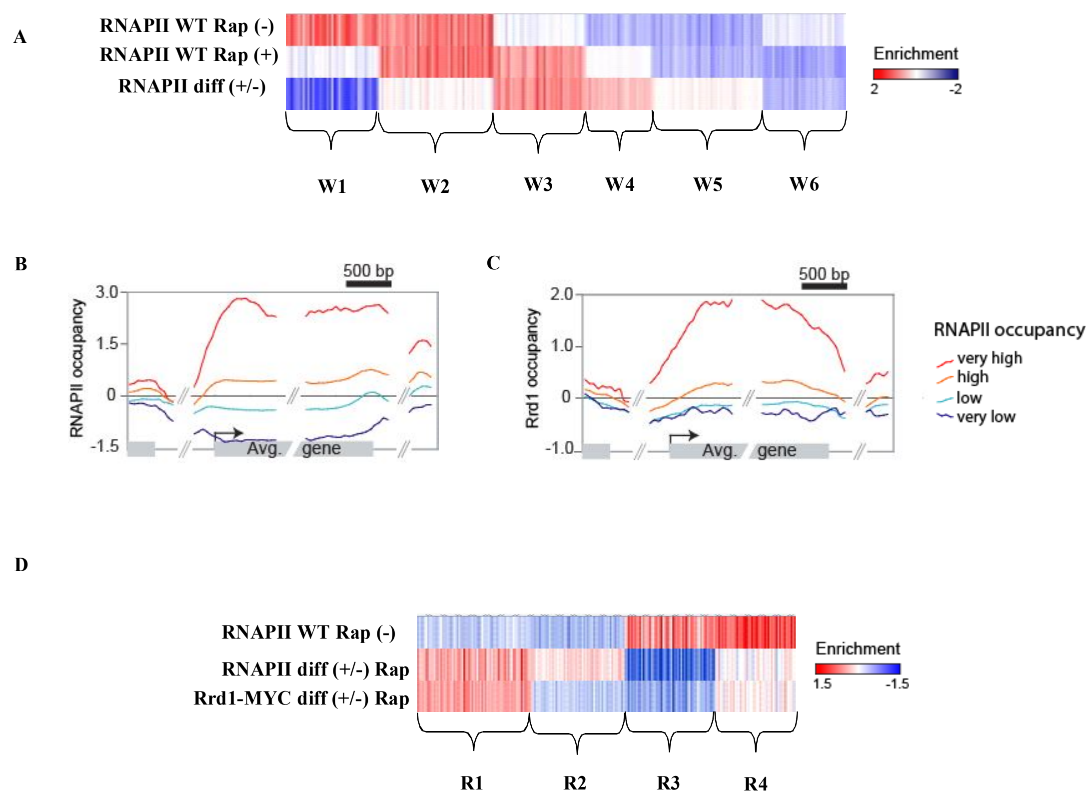


Figure 1 Poschmann J. et al., 2010

A

B

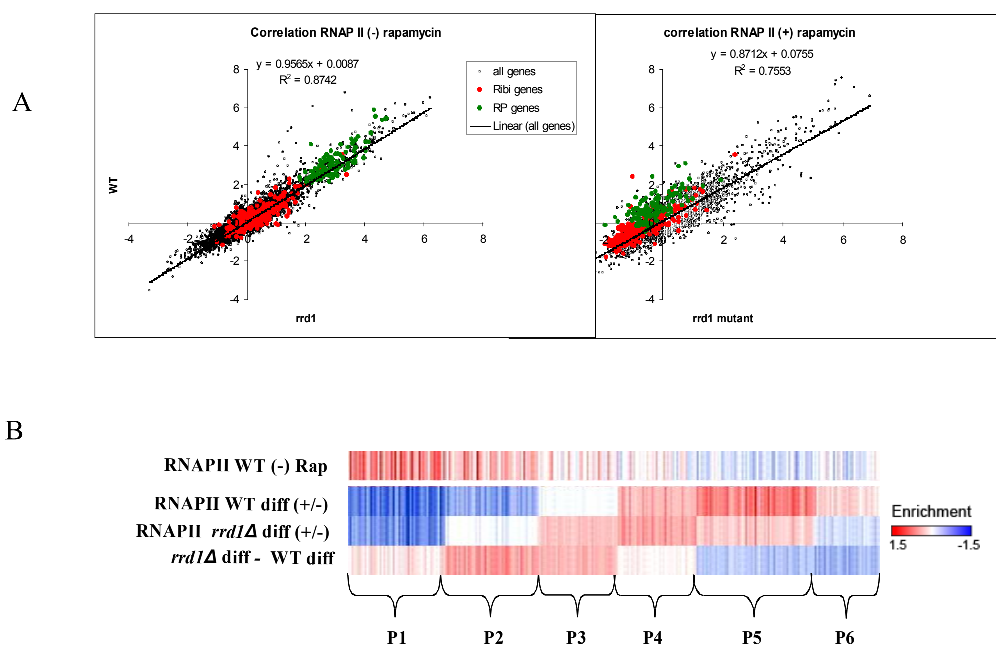


Figure 2 Poschmann J. et al., 2010

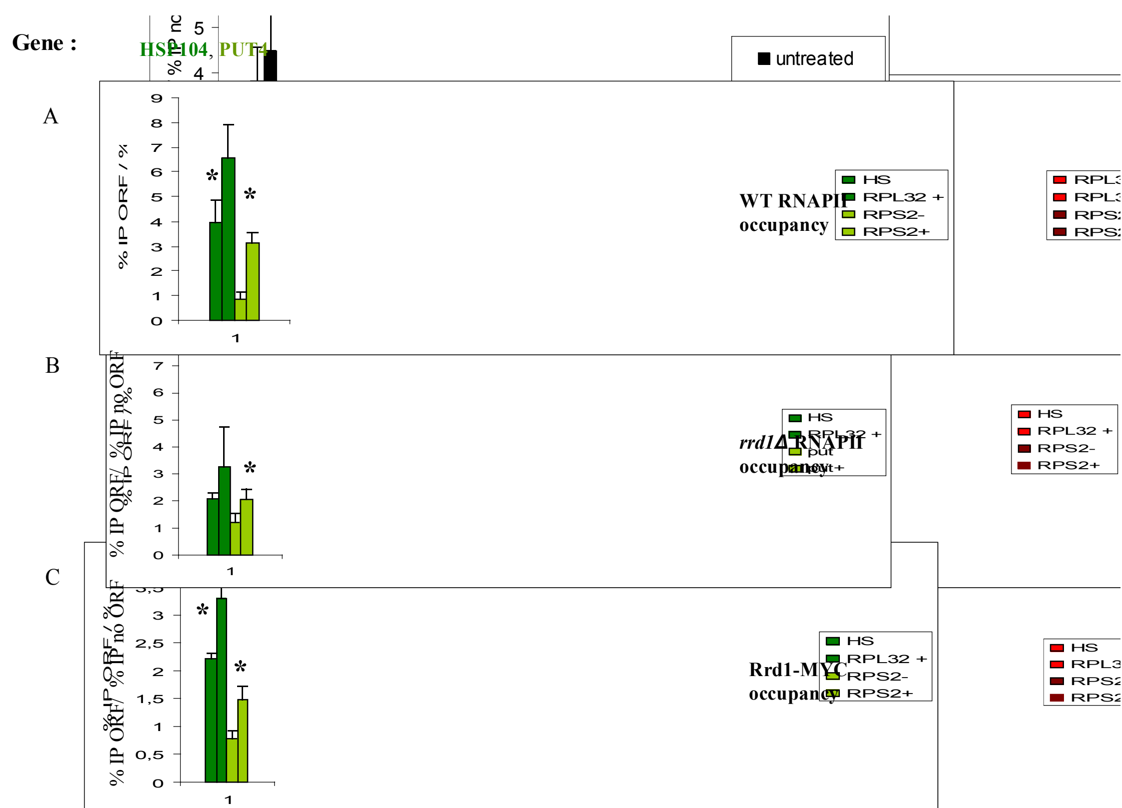


Figure 3 Poschmann J. et al., 2010

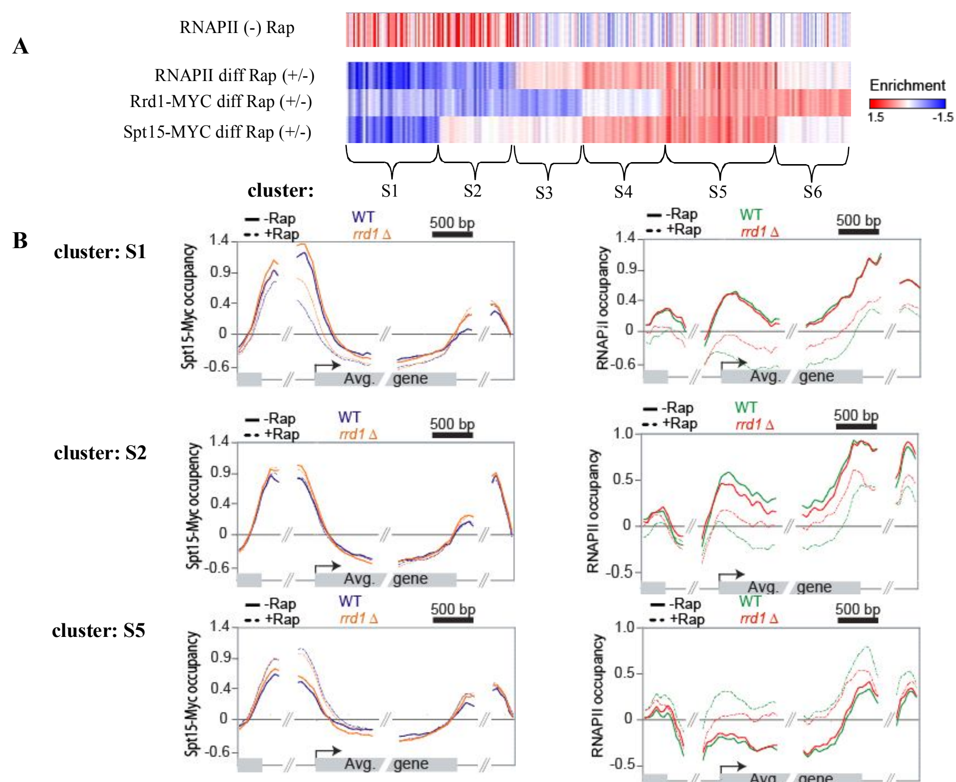


Figure 4 Poschmann J. et al., 2010

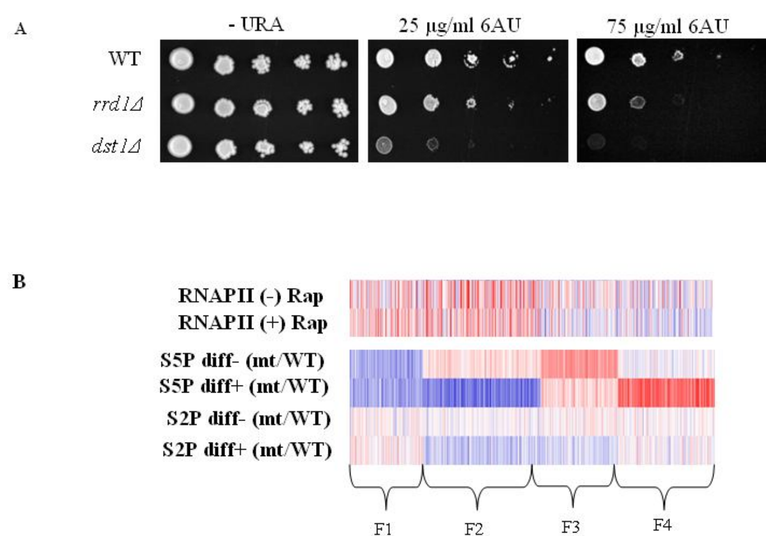


Figure 5 Poschmann J. et al., 2010

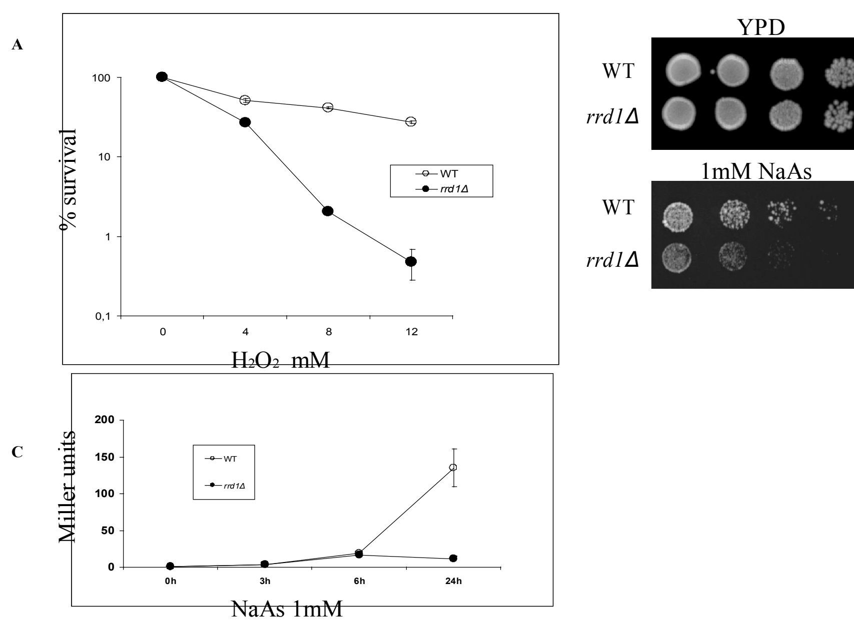


Figure 6 Poschmann J. et al., 2010

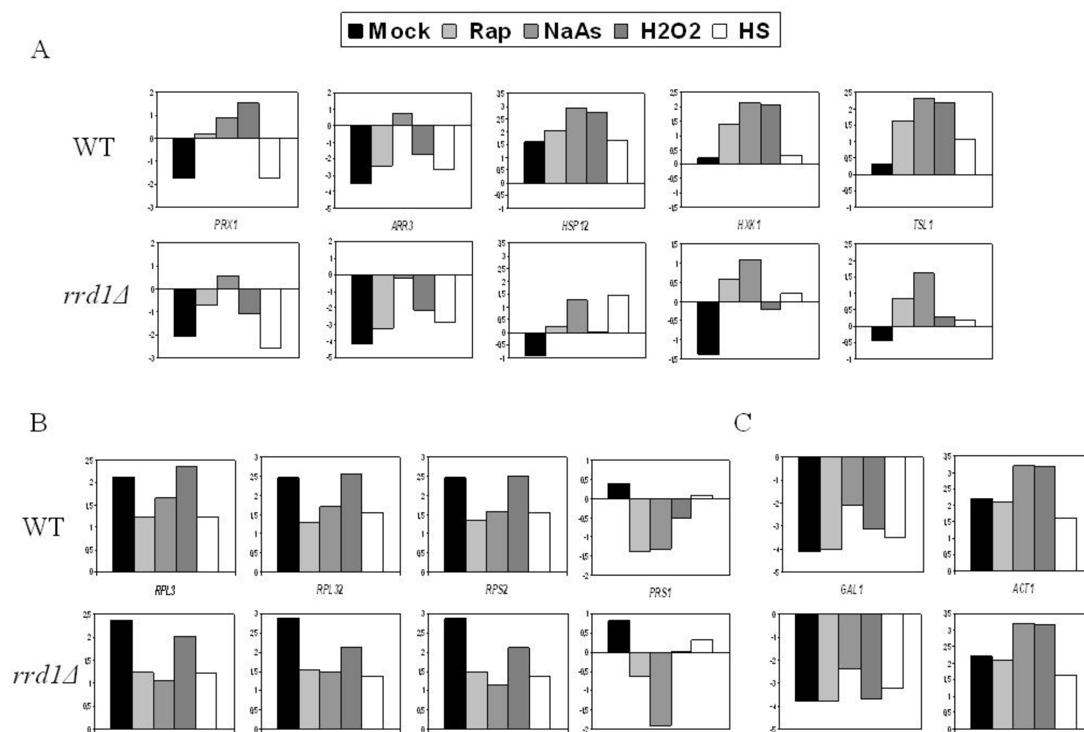


Figure 7 Poschmann J. et al., 2010

W1

N	X	LOD	P	P_adj	Gene-Ontology-ID	Gene-Ontology-Attribute
168	235	1.170	8.557e-82	<0.001	GO:0005840	ribosome
148	192	1.288	1.572e-79	<0.001	GO:0044445	cytosolic part
224	416	0.8585	4.761e-74	<0.001	GO:0006412	translation
149	208	1.164	1.902e-72	<0.001	GO:0033279	ribosomal subunit
147	206	1.158	4.517e-71	<0.001	GO:0003735	structural constituent of ribosome
242	500	0.7680	4.366e-68	<0.001	GO:0030529	ribonucleoprotein complex
77	78	2.448	4.889e-60	<0.001	GO:0022625	cytosolic large ribosomal subunit
171	334	0.7872	2.026e-51	<0.001	GO:0005198	structural molecule activity
264	695	0.5800	3.674e-48	<0.001	GO:0043228	non-membrane-bounded organelle
264	695	0.5800	3.674e-48	<0.001	GO:0043232	intracellular non-membrane-bounded organelle
86	119	1.148	4.233e-42	<0.001	GO:0015934	large ribosomal subunit
364	1216	0.4441	1.198e-39	<0.001	GO:0043284	biopolymer biosynthetic process
366	1227	0.4424	1.604e-39	<0.001	GO:0009059	macromolecule biosynthetic process
54	57	1.917	4.036e-39	<0.001	GO:0022627	cytosolic small ribosomal subunit
340	1123	0.4426	1.224e-37	<0.001	GO:0034961	cellular biopolymer biosynthetic process
341	1133	0.4387	3.772e-37	<0.001	GO:0034645	cellular macromolecule biosynthetic process
450	1687	0.3941	1.954e-36	<0.001	GO:0032991	macromolecular complex

W2

N	X	LOD	P	P_adj	Gene-Ontology-ID	Gene-Ontology-Attribute
35	48	1.030	3.464e-15	<0.001	GO:0015078	hydrogen ion transmembrane transporter activity
35	52	0.9171	1.689e-13	<0.001	GO:0015077	monovalent inorganic cation transmembrane transporter activity
591	2421	0.1876	1.093e-11	<0.001	GO:0044444	cytoplasmic part
46	88	0.6507	1.497e-11	<0.001	GO:0009277	fungal-type cell wall
165	522	0.2938	5.252e-11	<0.001	GO:0044429	mitochondrial part
49	100	0.5947	6.802e-11	<0.001	GO:0005618	cell wall
49	100	0.5947	6.802e-11	<0.001	GO:0030312	external encapsulating structure
27	40	0.9163	9.574e-11	<0.001	GO:0006818	hydrogen transport
47	97	0.5847	2.729e-10	<0.001	GO:0022890	inorganic cation transmembrane transporter activity
22	30	1.029	4.443e-10	<0.001	GO:0015992	proton transport
19	24	1.155	7.790e-10	<0.001	GO:0015985	energy coupled proton transport, down electrochemical gradient
19	24	1.155	7.790e-10	<0.001	GO:0015986	ATP synthesis coupled proton transport
257	931	0.2176	1.291e-9	<0.001	GO:0031090	organelle membrane
23	34	0.9165	2.286e-9	<0.001	GO:0006694	steroid biosynthetic process
23	34	0.9165	2.286e-9	<0.001	GO:0016126	sterol biosynthetic process

W3

N	X	LOD	P	P_adj	Gene-Ontology-ID	Gene-Ontology-Attribute
94	303	0.4127	4.107e-12	<0.001	GO:0009628	response to abiotic stimulus
67	196	0.4692	5.635e-11	<0.001	GO:0009408	response to heat
62	176	0.4881	7.270e-11	<0.001	GO:0034605	cellular response to heat
68	215	0.4180	1.858e-9	<0.001	GO:0009266	response to temperature stimulus
84	290	0.3657	2.765e-9	<0.001	GO:0006807	nitrogen compound metabolic process
14	18	1.246	7.929e-9	<0.001	GO:0006536	glutamate metabolic process
27	57	0.6966	1.525e-8	<0.001	GO:0009064	glutamine family amino acid metabolic process
17	28	0.9209	6.777e-8	<0.001	GO:0009084	glutamine family amino acid biosynthetic process
95	370	0.2931	1.870e-7	<0.001	GO:0006082	organic acid metabolic process
43	128	0.4510	2.970e-7	<0.001	GO:0008652	amino acid biosynthetic process
93	364	0.2896	3.249e-7	<0.001	GO:0019752	carboxylic acid metabolic process

W4

N	X	LOD	P	P_adj	Gene-Ontology-ID	Gene-Ontology-Attribute
152	880	0.2490	2.359e-8	<0.001	GO:0009056	catabolic process
76	356	0.3478	2.912e-8	<0.001	GO:0030163	protein catabolic process
145	841	0.2456	6.220e-8	<0.001	GO:0044248	cellular catabolic process
73	343	0.3449	6.403e-8	<0.001	GO:0044257	cellular protein catabolic process
73	344	0.3432	7.262e-8	<0.001	GO:0034962	cellular biopolymer catabolic process
52	225	0.3856	3.973e-7	<0.001	GO:0019941	modification-dependent protein catabolic process
53	233	0.3767	5.238e-7	<0.001	GO:0051603	proteolysis involved in cellular protein catabolic process
52	228	0.3779	6.171e-7	<0.001	GO:0043632	modification-dependent macromolecule catabolic process

W5

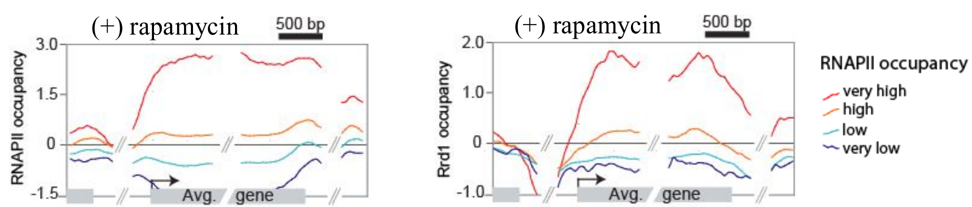
N	X	LOD	P	P_adj	Gene-Ontology-ID	Gene-Ontology-Attribute
149	449	0.3379	1.333e-12	<0.001	GO:0022402	cell cycle process
35	63	0.7169	2.440e-10	<0.001	GO:0043566	structure-specific DNA binding
128	397	0.3138	5.343e-10	<0.001	GO:0006259	DNA metabolic process
94	267	0.3670	8.396e-10	<0.001	GO:0022403	cell cycle phase
148	489	0.2752	3.250e-9	<0.001	GO:0003677	DNA binding
23	35	0.8927	3.394e-9	<0.001	GO:0003690	double-stranded DNA binding
55	134	0.4684	7.454e-9	<0.001	GO:0007126	meiosis
90	271	0.3264	5.033e-8	<0.001	GO:0044427	chromosomal part
79	230	0.3471	6.827e-8	<0.001	GO:0006281	DNA repair
91	281	0.3097	1.596e-7	<0.001	GO:0006974	response to DNA damage stimulus
81	242	0.3300	1.657e-7	<0.001	GO:0034984	cellular response to DNA damage stimulus
46	114	0.4539	2.336e-7	<0.001	GO:0006310	DNA recombination
24	45	0.6749	4.703e-7	<0.001	GO:0010927	cellular component assembly involved in morphogenesis
24	45	0.6749	4.703e-7	<0.001	GO:0030476	ascospore wall assembly
24	45	0.6749	4.703e-7	<0.001	GO:0042244	spore wall assembly

W6

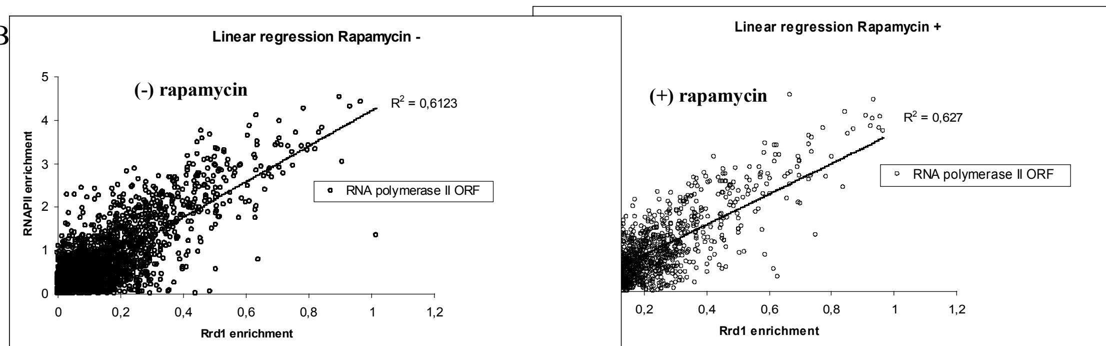
N	X	LOD	P	P_adj	Gene-Ontology-ID	Gene-Ontology-Attribute
297	698	0.7483	2.504e-79	<0.001	GO:0034660	ncRNA metabolic process
255	545	0.8050	1.582e-77	<0.001	GO:0022613	ribonucleoprotein complex biogenesis
257	561	0.7880	1.066e-75	<0.001	GO:0044085	cellular component biogenesis
249	533	0.8005	2.576e-75	<0.001	GO:0042254	ribosome biogenesis
237	512	0.7863	2.693e-70	<0.001	GO:0016072	rRNA metabolic process
372	1094	0.6151	4.053e-69	<0.001	GO:0016070	RNA metabolic process
156	329	0.7672	1.277e-46	<0.001	GO:0034470	ncRNA processing
462	1794	0.4557	2.241e-46	<0.001	GO:0006139	nucleobase, nucleoside, nucleotide and nucleic acid metabolic pro
191	493	0.6222	2.946e-41	<0.001	GO:0006396	RNA processing
112	231	0.7666	2.261e-34	<0.001	GO:0006364	rRNA processing
101	195	0.8202	3.797e-34	<0.001	GO:0005730	nucleolus
438	1900	0.3560	3.308e-29	<0.001	GO:0005634	nucleus
275	1002	0.4118	1.239e-28	<0.001	GO:0044428	nuclear part
100	228	0.6790	2.765e-26	<0.001	GO:0009451	RNA modification
555	2787	0.2920	3.294e-21	<0.001	GO:0034960	cellular biopolymer metabolic process
61	117	0.8089	4.293e-21	<0.001	GO:0030684	preribosome
558	2833	0.2832	4.775e-20	<0.001	GO:0044260	cellular macromolecule metabolic process

Supplemental figure S1 Poschmann J. et al., 2010

A



B



Supplemental figure S2 Poschmann J. et al., 2010

R1

N	X	LOD	P ▲	P_adj	Gene-Ontology-ID	Gene-Ontology-Attribute
355	880	0.2304	2.254e-12	<0.001	GO:0009056	catabolic process
334	841	0.2152	1.244e-10	<0.001	GO:0044248	cellular catabolic process
155	356	0.2691	2.592e-8	<0.001	GO:0030163	protein catabolic process
148	344	0.2588	1.364e-7	<0.001	GO:0034962	cellular biopolymer catabolic process
147	343	0.2556	2.012e-7	<0.001	GO:0044257	cellular protein catabolic process
83	176	0.3259	0.000001121	0.001000	GO:0034605	cellular response to heat
60	117	0.3952	0.000001157	0.001000	GO:0007039	vacuolar protein catabolic process
211	543	0.1856	0.000003599	0.003000	GO:0009057	macromolecule catabolic process
36	62	0.5096	0.000004257	0.003000	GO:0005543	phospholipid binding
15	18	1.014	0.000004529	0.003000	GO:0006536	glutamate metabolic process
105	245	0.2511	0.00001178	0.006000	GO:0006914	autophagy
120	290	0.2254	0.00002109	0.01000	GO:0006807	nitrogen compound metabolic process
186	482	0.1781	0.00002347	0.01100	GO:0051716	cellular response to stimulus
124	303	0.2173	0.00002855	0.01500	GO:0009628	response to abiotic stimulus

R2

N	X	LOD	P ▼	P_adj	Gene-Ontology-ID	Gene-Ontology-Attribute
127	397	0.1929	0.00007151	0.03600	GO:0006259	DNA metabolic process
146	449	0.2062	0.000007578	0.004000	GO:0022402	cell cycle process
17	26	0.7755	0.000007531	0.004000	GO:0007064	mitotic sister chromatid cohesion
20	33	0.6921	0.000006454	0.003000	GO:0007062	sister chromatid cohesion

R3

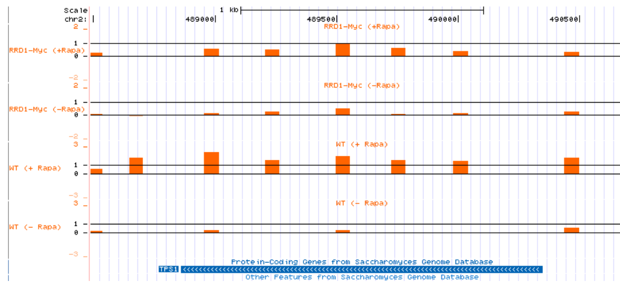
N	X	LOD	P ▲	P_adj	Gene-Ontology-ID	Gene-Ontology-Attribute
354	545	0.8811	1.489e-104	<0.001	GO:0022613	ribonucleoprotein complex biogenesis
357	561	0.8562	1.594e-101	<0.001	GO:0044085	cellular component biogenesis
409	698	0.7770	1.117e-100	<0.001	GO:0034660	ncRNA metabolic process
344	533	0.8695	4.390e-100	<0.001	GO:0042254	ribosome biogenesis
332	512	0.8715	4.156e-97	<0.001	GO:0016072	rRNA metabolic process
528	1094	0.6206	7.328e-89	<0.001	GO:0016070	RNA metabolic process
311	500	0.8131	1.704e-83	<0.001	GO:0030529	ribonucleoprotein complex
234	329	0.9680	3.355e-79	<0.001	GO:0034470	ncRNA processing
184	231	1.154	1.315e-75	<0.001	GO:0006364	rRNA processing
351	695	0.6063	2.782e-61	<0.001	GO:0043228	non-membrane-bounded organelle
351	695	0.6063	2.782e-61	<0.001	GO:0043232	intracellular non-membrane-bounded organelle
109	117	1.654	1.541e-59	<0.001	GO:0030684	preribosome
150	192	1.103	2.632e-59	<0.001	GO:0044445	cytosolic part
273	493	0.6736	3.178e-57	<0.001	GO:0006396	RNA processing
149	195	1.060	1.269e-56	<0.001	GO:0005730	nucleolus
167	235	0.9456	8.286e-56	<0.001	GO:0005840	ribosome
150	208	0.9628	2.136e-51	<0.001	GO:0033279	ribosomal subunit
78	78	2.730	2.265e-50	<0.001	GO:0022625	cytosolic large ribosomal subunit
148	206	0.9564	2.315e-50	<0.001	GO:0003735	structural constituent of ribosome
232	416	0.6692	6.936e-49	<0.001	GO:0006412	translation
649	1794	0.3998	4.684e-48	<0.001	GO:0006139	nucleobase, nucleoside, nucleotide and nucleic acid metabolic process
894	2787	0.3758	2.574e-46	<0.001	GO:0034960	cellular biopolymer metabolic process
899	2833	0.3664	4.048e-44	<0.001	GO:0044260	cellular macromolecule metabolic process
906	2866	0.3654	7.887e-44	<0.001	GO:0043283	biopolymer metabolic process
198	352	0.6685	3.193e-42	<0.001	GO:0003723	RNA binding
910	2907	0.3565	8.728e-42	<0.001	GO:0043170	macromolecule metabolic process
592	1687	0.3557	2.882e-37	<0.001	GO:0032991	macromolecular complex

R4

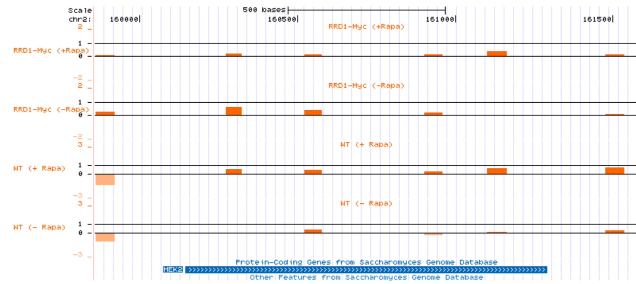
N	X	LOD	P ▲	P_adj	Gene-Ontology-ID	Gene-Ontology-Attribute
178	522	0.2997	7.290e-12	<0.001	GO:0044429	mitochondrial part
47	88	0.6260	6.156e-11	<0.001	GO:0009277	fungal-type cell wall
44	82	0.6295	2.072e-10	<0.001	GO:0001950	PME fraction
50	100	0.5675	3.394e-10	<0.001	GO:0005618	cell wall
50	100	0.5675	3.394e-10	<0.001	GO:0030312	external encapsulating structure
624	2421	0.1655	6.493e-10	<0.001	GO:0044444	cytoplasmic part
18	21	1.284	7.351e-10	<0.001	GO:0031597	cytosolic proteasome complex
18	21	1.284	7.351e-10	<0.001	GO:0034515	proteasome storage granule
30	48	0.7809	1.187e-9	<0.001	GO:0015078	hydrogen ion transmembrane transporter activity
274	931	0.2118	1.457e-9	<0.001	GO:0031090	organelle membrane
77	194	0.3898	7.375e-9	<0.001	GO:0007005	mitochondrion organization
37	71	0.6008	1.655e-8	<0.001	GO:0005576	extracellular region
30	52	0.6955	1.743e-8	<0.001	GO:0015077	monovalent inorganic cation transmembrane transporter activity
13	14	1.514	2.627e-8	<0.001	GO:0004298	threonine-type endopeptidase activity
13	14	1.514	2.627e-8	<0.001	GO:0005839	proteasome core complex
13	14	1.514	2.627e-8	<0.001	GO:0070003	threonine-type peptidase activity
844	3489	0.1447	6.989e-8	<0.001	GO:0043227	membrane-bounded organelle
844	3489	0.1447	6.989e-8	<0.001	GO:0043231	intracellular membrane-bounded organelle
22	34	0.8169	8.203e-8	<0.001	GO:0000502	proteasome complex

Supplemental figure S3 Poschmann J. et al., 2010

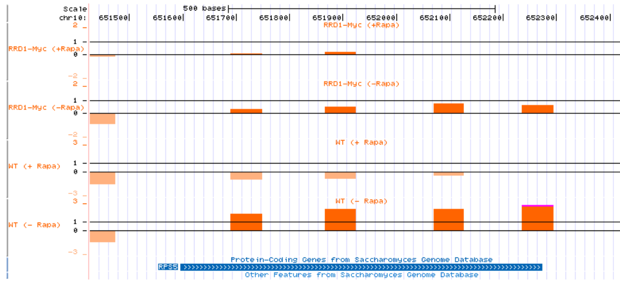
R1



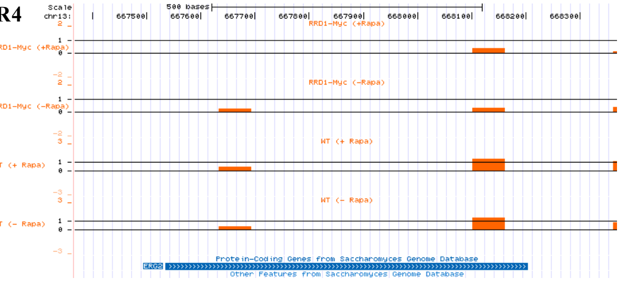
R3



R2

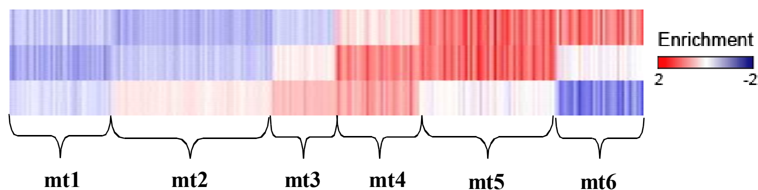


R4



Supplemental figure S3B Poschmann J. et al., 2010

RNAPII *rrd1Δ* Rap (-)
RNAPII *rrd1Δ* Rap (+)
RNAPII diff Rap (+/-)



mt1

N	X	LOD	P	P_adj	Gene-Ontology-ID	Gene-Ontology-Attribute
179	545	0.4627	1.430e-24	<0.001	GO:0022613	ribonucleoprotein complex biogenesis
175	533	0.4611	5.559e-24	<0.001	GO:0042254	ribosome biogenesis
180	561	0.4469	2.234e-23	<0.001	GO:0044085	cellular component biogenesis
168	512	0.4583	5.882e-23	<0.001	GO:0016072	rRNA metabolic process
209	698	0.4093	5.920e-23	<0.001	GO:0034660	ncRNA metabolic process
281	1094	0.3278	6.645e-20	<0.001	GO:0016070	RNA metabolic process
115	329	0.4845	4.251e-18	<0.001	GO:0034470	ncRNA processing
403	1794	0.2712	7.936e-18	<0.001	GO:0006139	nucleobase, nucleoside, nucleotide and nucleic acid metabolic process
146	493	0.3820	1.993e-15	<0.001	GO:0006396	RNA processing
75	195	0.5390	1.375e-14	<0.001	GO:0005730	nucleolus
408	1900	0.2381	2.403e-14	<0.001	GO:0005634	nucleus
245	1002	0.2834	2.947e-14	<0.001	GO:0044428	nuclear part
78	231	0.4494	1.132e-11	<0.001	GO:0006364	rRNA processing
31	69	0.6415	1.246e-8	<0.001	GO:0006400	tRNA modification
42	117	0.4810	1.060e-7	<0.001	GO:0030684	preribosome
67	228	0.3559	1.878e-7	<0.001	GO:0009451	RNA modification

mt2

N	X	LOD	P	P_adj	Gene-Ontology-ID	Gene-Ontology-Attribute
100	245	0.3307	2.269e-8	<0.001	GO:0006914	autophagy
25	40	0.6981	5.508e-7	0.001000	GO:0044438	microbody part
25	40	0.6981	5.508e-7	0.001000	GO:0044439	peroxisomal part

mt3

N	X	LOD	P	P_adj	Gene-Ontology-ID	Gene-Ontology-Attribute
40	176	0.4364	5.973e-7	<0.001	GO:0034605	cellular response to heat
57	303	0.3357	0.000002149	<0.001	GO:0009628	response to abiotic stimulus
44	215	0.3783	0.000003442	0.003000	GO:0009266	response to temperature stimulus
41	196	0.3896	0.000004208	0.004000	GO:0009408	response to heat

mt4

N	X	LOD	P	P_adj	Gene-Ontology-ID	Gene-Ontology-Attribute
74	303	0.3661	1.270e-8	<0.001	GO:0009628	response to abiotic stimulus
81	343	0.3482	1.296e-8	<0.001	GO:0044257	cellular protein catabolic process
81	344	0.3465	1.493e-8	<0.001	GO:0034962	cellular biopolymer catabolic process
57	213	0.4159	2.194e-8	<0.001	GO:0030427	site of polarized growth
82	356	0.3337	3.501e-8	<0.001	GO:0030163	protein catabolic process
53	196	0.4209	4.780e-8	<0.001	GO:0009408	response to heat
20	43	0.7810	6.289e-8	<0.001	GO:0030479	actin cortical patch
23	57	0.6744	1.674e-7	<0.001	GO:0009064	glutamine family amino acid metabolic process
55	215	0.3880	2.043e-7	<0.001	GO:0009266	response to temperature stimulus
36	117	0.4958	2.368e-7	<0.001	GO:0007039	vacuolar protein catabolic process
106	522	0.2662	3.640e-7	<0.001	GO:0044429	mitochondrial part
374	2421	0.1621	7.055e-7	<0.001	GO:0044444	cytoplasmic part

mt5

N	X	LOD	P	P_adj	Gene-Ontology-ID	Gene-Ontology-Attribute
181	497	0.3603	5.041e-16	<0.001	GO:0051246	regulation of protein metabolic process
160	427	0.3767	1.732e-15	<0.001	GO:0006417	regulation of translation
163	438	0.3723	1.885e-15	<0.001	GO:0010608	posttranscriptional regulation of gene expression
166	452	0.3633	4.129e-15	<0.001	GO:0032268	regulation of cellular protein metabolic process
32	48	0.8705	1.493e-11	<0.001	GO:0015078	hydrogen ion transmembrane transporter activity
28	40	0.9330	4.779e-11	<0.001	GO:0006818	hydrogen transport
33	52	0.8111	5.352e-11	<0.001	GO:0015077	monovalent inorganic cation transmembrane transporter activity
26	38	0.9007	5.285e-10	<0.001	GO:0034220	transmembrane ion transport
611	2421	0.1638	1.262e-9	<0.001	GO:0044444	cytoplasmic part
22	30	0.9962	1.455e-9	<0.001	GO:0015992	proton transport
19	24	1.122	2.229e-9	<0.001	GO:0015985	energy coupled proton transport, down electrochemical gradient
19	24	1.122	2.229e-9	<0.001	GO:0015986	ATP synthesis coupled proton transport
66	159	0.4328	4.675e-9	<0.001	GO:0019725	cellular homeostasis
28	50	0.6768	7.970e-8	<0.001	GO:0045454	cell redox homeostasis
12	13	1.492	8.894e-8	<0.001	GO:0033177	proton-transporting two-sector ATPase complex, proton-transporting do
41	88	0.5177	9.333e-8	<0.001	GO:0009277	fungal-type cell wall
316	1166	0.1731	1.023e-7	<0.001	GO:0051234	establishment of localization

mt6

N	X	LOD	P	P_adj	Gene-Ontology-ID	Gene-Ontology-Attribute
215	500	0.7715	1.144e-63	<0.001	GO:0030529	ribonucleoprotein complex
126	192	1.135	1.212e-63	<0.001	GO:0044445	cytosolic part
74	78	2.050	1.796e-59	<0.001	GO:0022625	cytosolic large ribosomal subunit
127	208	1.050	1.071e-58	<0.001	GO:0033279	ribosomal subunit
126	206	1.051	2.378e-58	<0.001	GO:0003735	structural constituent of ribosome
133	235	0.9718	6.377e-56	<0.001	GO:0005840	ribosome
176	416	0.7376	5.260e-50	<0.001	GO:0006412	translation
230	695	0.5828	4.989e-44	<0.001	GO:0043228	non-membrane-bounded organelle
230	695	0.5828	4.989e-44	<0.001	GO:0043232	intracellular non-membrane-bounded organelle
144	334	0.7362	1.150e-41	<0.001	GO:0005198	structural molecule activity
79	119	1.124	2.560e-40	<0.001	GO:0015934	large ribosomal subunit
215	698	0.5253	2.359e-35	<0.001	GO:0034660	ncRNA metabolic process
148	389	0.6432	3.821e-35	<0.001	GO:0005622	intracellular
175	512	0.5789	1.060e-34	<0.001	GO:0016072	rRNA metabolic process
105	231	0.7597	1.196e-32	<0.001	GO:0006364	rRNA processing
177	545	0.5442	7.454e-32	<0.001	GO:0022613	ribonucleoprotein complex biogenesis
179	561	0.5330	3.991e-31	<0.001	GO:0044085	cellular component biogenesis
173	533	0.5420	4.730e-31	<0.001	GO:0042254	ribosome biogenesis
45	57	1.376	1.267e-28	<0.001	GO:0022627	cytosolic small ribosomal subunit
67	117	0.9495	1.390e-28	<0.001	GO:0030684	preribosome
274	1094	0.4111	2.332e-28	<0.001	GO:0016070	RNA metabolic process
123	329	0.6190	3.196e-28	<0.001	GO:0034470	ncRNA processing
123	352	0.5712	4.450e-25	<0.001	GO:0003723	RNA binding
259	1123	0.3487	1.025e-20	<0.001	GO:0034961	cellular biopolymer biosynthetic process

Supplemental figure S4 Poschmann J. et al., 2010

P1

N	X	LOD	P	P_adj	Gene-Ontology-ID	Gene-Ontology-Attribute
263	500	0.7915	6.754e-75	<0.001	GO:0030529	ribonucleoprotein complex
311	698	0.6626	6.115e-67	<0.001	GO:0034660	ncRNA metabolic process
255	512	0.7371	6.642e-66	<0.001	GO:0016072	rRNA metabolic process
265	545	0.7196	7.152e-66	<0.001	GO:0022613	ribonucleoprotein complex biogenesis
268	561	0.7051	1.358e-64	<0.001	GO:0044085	cellular component biogenesis
259	533	0.7166	3.678e-64	<0.001	GO:0042254	ribosome biogenesis
137	192	1.097	1.343e-60	<0.001	GO:0044445	cytosolic part
149	231	0.9644	7.669e-57	<0.001	GO:0006364	rRNA processing
293	695	0.6094	5.295e-56	<0.001	GO:0043228	non-membrane-bounded organelle
293	695	0.6094	5.295e-56	<0.001	GO:0043232	intracellular non-membrane-bounded organelle
392	1094	0.5197	1.638e-54	<0.001	GO:0016070	RNA metabolic process
181	329	0.8020	3.732e-54	<0.001	GO:0034470	ncRNA processing
147	235	0.9266	1.635e-53	<0.001	GO:0005840	ribosome
74	78	1.900	1.794e-50	<0.001	GO:0022625	cytosolic large ribosomal subunit
132	206	0.9495	9.578e-50	<0.001	GO:0003735	structural constituent of ribosome
132	208	0.9378	5.044e-49	<0.001	GO:0033279	ribosomal subunit
92	117	1.246	8.693e-47	<0.001	GO:0030684	preribosome
121	195	0.9070	2.157e-43	<0.001	GO:0005730	nucleolus
193	416	0.6511	5.703e-43	<0.001	GO:0006412	translation
210	493	0.5870	9.781e-40	<0.001	GO:0006396	RNA processing
160	352	0.6224	4.964e-34	<0.001	GO:0003723	RNA binding
152	334	0.6207	2.277e-32	<0.001	GO:0005198	structural molecule activity

P2

N	X	LOD	P	P_adj	Gene-Ontology-ID	Gene-Ontology-Attribute
309	1094	0.3537	3.623e-24	<0.001	GO:0016070	RNA metabolic process
182	545	0.4318	3.767e-22	<0.001	GO:0022613	ribonucleoprotein complex biogenesis
178	533	0.4306	1.187e-21	<0.001	GO:0042254	ribosome biogenesis
216	698	0.3891	1.380e-21	<0.001	GO:0034660	ncRNA metabolic process
183	561	0.4157	5.721e-21	<0.001	GO:0044085	cellular component biogenesis
434	1794	0.2792	9.707e-20	<0.001	GO:0006139	nucleobase, nucleoside, nucleotide and nucleic acid metabolic process
167	512	0.4109	4.274e-19	<0.001	GO:0016072	rRNA metabolic process
90	228	0.5233	3.820e-16	<0.001	GO:0009451	RNA modification
597	2866	0.2048	1.925e-12	<0.001	GO:0043283	biopolymer metabolic process
599	2907	0.1947	2.073e-11	<0.001	GO:0043170	macromolecule metabolic process
575	2787	0.1882	8.766e-11	<0.001	GO:0034960	cellular biopolymer metabolic process
579	2833	0.1802	4.913e-10	<0.001	GO:0044260	cellular macromolecule metabolic process
245	1026	0.2203	1.489e-9	<0.001	GO:0010468	regulation of gene expression
221	914	0.2237	3.912e-9	<0.001	GO:0043412	biopolymer modification
408	1900	0.1790	3.939e-9	<0.001	GO:0005634	nucleus
242	1030	0.2088	1.003e-8	<0.001	GO:0010556	regulation of macromolecule biosynthetic process
236	1002	0.2093	1.298e-8	<0.001	GO:0044428	nuclear part
243	1044	0.2027	2.318e-8	<0.001	GO:0031326	regulation of cellular biosynthetic process
657	3347	0.1625	2.710e-8	<0.001	GO:0044238	primary metabolic process
243	1046	0.2015	2.790e-8	<0.001	GO:0009889	regulation of biosynthetic process
262	1151	0.1913	5.042e-8	<0.001	GO:0060255	regulation of macromolecule metabolic process
116	427	0.2773	6.651e-8	<0.001	GO:0006417	regulation of translation
255	1120	0.1902	8.071e-8	<0.001	GO:0031323	regulation of cellular metabolic process
118	438	0.2724	8.371e-8	<0.001	GO:0010608	posttranscriptional regulation of gene expression
266	1179	0.1860	9.253e-8	<0.001	GO:0019222	regulation of metabolic process
120	452	0.2636	1.553e-7	<0.001	GO:0032268	regulation of cellular protein metabolic process
41	108	0.4816	1.839e-7	<0.001	GO:0016458	gene silencing

P3

N	X	LOD	P	P_adj	Gene-Ontology-ID	Gene-Ontology-Attribute
69	265	0.3416	2.560e-7	<0.001	GO:0051301	cell division
232	1231	0.1813	7.809e-7	<0.001	GO:0043234	protein complex
56	213	0.3442	0.000002583	0.001000	GO:0030427	site of polarized growth
277	1564	0.1488	0.00001253	0.009000	GO:0016043	cellular component organization
298	1704	0.1441	0.00001472	0.01000	GO:0065007	biological regulation
187	996	0.1695	0.00001750	0.01200	GO:0006996	organelle organization
8	11	1.163	0.00001963	0.01300	GO:0001301	progressive alteration of chromatin during cell aging
7	9	1.255	0.00003449	0.01900	GO:0001304	progressive alteration of chromatin during replicative cell aging
9	15	0.9430	0.00005622	0.03300	GO:0010383	cell wall polysaccharide metabolic process

P4

N	X	LOD	P	P_adj	Gene-Ontology-ID	Gene-Ontology-Attribute
50	117	0.6397	4.370e-13	<0.001	GO:0007039	vacuolar protein catabolic process
95	356	0.3342	5.012e-9	<0.001	GO:0030163	protein catabolic process
92	343	0.3366	6.789e-9	<0.001	GO:0044257	cellular protein catabolic process
92	344	0.3348	7.953e-9	<0.001	GO:0034962	cellular biopolymer catabolic process
110	490	0.2320	0.000005332	0.003000	GO:0043285	biopolymer catabolic process
177	880	0.1777	0.00001144	0.008000	GO:0009056	catabolic process
170	841	0.1796	0.00001313	0.01000	GO:0044248	cellular catabolic process

P5

N	X	LOD	P	P_adj	Gene-Ontology-ID	Gene-Ontology-Attribute
82	196	0.4285	1.285e-10	<0.001	GO:0009408	response to heat
262	880	0.2161	1.454e-9	<0.001	GO:0009056	catabolic process
73	176	0.4201	2.180e-9	<0.001	GO:0034605	cellular response to heat
250	841	0.2131	4.572e-9	<0.001	GO:0044248	cellular catabolic process
83	215	0.3690	1.022e-8	<0.001	GO:0009266	response to temperature stimulus
105	303	0.2964	9.931e-8	<0.001	GO:0009628	response to abiotic stimulus

P6: no significant GO

Supplemental figure S5 Poschmann J. et al., 2010

S1

N	X	LOD	P	P_adj	Gene-Ontology-ID	Gene-Ontology-Attribute
199	545	0.8387	3.388e-69	<0.001	GO:0022613	ribonucleoprotein complex biogenesis
226	698	0.7764	2.936e-68	<0.001	GO:0034660	ncRNA metabolic process
195	533	0.8367	8.552e-68	<0.001	GO:0042254	ribosome biogenesis
200	561	0.8222	1.786e-67	<0.001	GO:0044085	cellular component biogenesis
183	512	0.8099	2.973e-61	<0.001	GO:0016072	rRNA metabolic process
276	1094	0.6507	4.014e-59	<0.001	GO:0016070	RNA metabolic process
164	500	0.7348	2.653e-48	<0.001	GO:0030529	ribonucleoprotein complex
129	329	0.8357	3.491e-47	<0.001	GO:0034470	ncRNA processing
185	695	0.6111	4.364e-40	<0.001	GO:0043228	non-membrane-bounded organelle
185	695	0.6111	4.364e-40	<0.001	GO:0043232	intracellular non-membrane-bounded organelle
99	231	0.8815	6.537e-40	<0.001	GO:0006364	rRNA processing
333	1794	0.4924	9.354e-40	<0.001	GO:0006139	nucleobase, nucleoside, nucleotide and nucleic acid metabolic process
145	493	0.6480	5.314e-36	<0.001	GO:0006396	RNA processing
439	2866	0.4642	5.447e-35	<0.001	GO:0043283	biopolymer metabolic process
431	2787	0.4613	6.155e-35	<0.001	GO:0034960	cellular biopolymer metabolic process
434	2833	0.4571	3.387e-34	<0.001	GO:0044260	cellular macromolecule metabolic process
440	2907	0.4547	1.403e-33	<0.001	GO:0043170	macromolecule metabolic process
82	192	0.8671	7.351e-33	<0.001	GO:0044445	cytosolic part
82	195	0.8552	2.872e-32	<0.001	GO:0005730	nucleolus
472	3347	0.4358	5.167e-29	<0.001	GO:0044238	primary metabolic process
48	78	1.176	1.091e-28	<0.001	GO:0022625	cytosolic large ribosomal subunit
86	235	0.7564	1.576e-28	<0.001	GO:0005840	ribosome
79	206	0.7851	8.187e-28	<0.001	GO:0003735	structural constituent of ribosome
78	208	0.7685	1.051e-26	<0.001	GO:0033279	ribosomal subunit
54	117	0.9098	8.160e-24	<0.001	GO:0030684	preribosome
600	5076	0.6424	2.263e-23	<0.001	GO:0044464	cell part
111	416	0.5639	2.948e-23	<0.001	GO:0006412	translation
600	5086	0.6374	5.923e-23	<0.001	GO:0005575	cellular_component

S3: no significant GO

S4

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N	X	LOD	P	P_adj	Gene-Ontology-ID	Gene-Ontology-Attribute
127	841	0.3177	1.362e-10	<0.001	GO:0044248	cellular catabolic process
67	343	0.4332	1.963e-10	<0.001	GO:0044257	cellular protein catabolic process
67	344	0.4315	2.241e-10	<0.001	GO:0034962	cellular biopolymer catabolic process
129	880	0.3014	7.266e-10	<0.001	GO:0009056	catabolic process
67	356	0.4122	1.042e-9	<0.001	GO:0030163	protein catabolic process
495	5076	0.3238	2.354e-8	<0.001	GO:0044464	cell part
495	5086	0.3186	4.025e-8	<0.001	GO:0005575	cellular_component
81	522	0.3110	1.847e-7	<0.001	GO:0044265	cellular macromolecule catabolic process
77	490	0.3162	2.289e-7	<0.001	GO:0043285	biopolymer catabolic process
62	366	0.3511	2.779e-7	<0.001	GO:0006508	proteolysis
82	543	0.2963	5.041e-7	<0.001	GO:0009057	macromolecule catabolic process
43	225	0.4092	8.130e-7	<0.001	GO:0019941	modification-dependent protein catabolic process

S2

N	X	LOD	P	P_adj	Gene-Ontology-ID	Gene-Ontology-Attribute
483	4878	0.4510	1.364e-14	<0.001	GO:0008150	biological_process
325	2866	0.2838	6.650e-13	<0.001	GO:0043283	biopolymer metabolic process
326	2907	0.2748	3.410e-12	<0.001	GO:0043170	macromolecule metabolic process
490	5076	0.4342	4.208e-12	<0.001	GO:0044464	cell part
490	5086	0.4291	7.966e-12	<0.001	GO:0005575	cellular_component
386	3653	0.2855	9.156e-12	<0.001	GO:0044237	cellular metabolic process
80	438	0.4195	1.828e-11	<0.001	GO:0010608	posttranscriptional regulation of gene expression
455	4584	0.3383	2.254e-11	<0.001	GO:0009987	cellular process
395	3785	0.2840	2.386e-11	<0.001	GO:0008152	metabolic process
78	427	0.4184	3.388e-11	<0.001	GO:0006417	regulation of translation
359	3347	0.2671	3.913e-11	<0.001	GO:0044238	primary metabolic process
315	2833	0.2569	6.158e-11	<0.001	GO:0044260	cellular macromolecule metabolic process
311	2787	0.2565	6.272e-11	<0.001	GO:0034960	cellular biopolymer metabolic process
206	1647	0.2668	1.252e-10	<0.001	GO:0009058	biosynthetic process
144	1030	0.3010	1.493e-10	<0.001	GO:0010556	regulation of macromolecule biosynthetic process
79	452	0.3941	2.400e-10	<0.001	GO:0032268	regulation of cellular protein metabolic process
163	1227	0.2805	3.473e-10	<0.001	GO:0009059	macromolecule biosynthetic process
152	1120	0.2875	3.709e-10	<0.001	GO:0031323	regulation of cellular metabolic process
144	1044	0.2929	4.111e-10	<0.001	GO:0031326	regulation of cellular biosynthetic process
155	1151	0.2844	4.170e-10	<0.001	GO:0060255	regulation of macromolecule metabolic process
142	1026	0.2939	4.487e-10	<0.001	GO:0010468	regulation of gene expression
144	1046	0.2918	4.738e-10	<0.001	GO:0009889	regulation of biosynthetic process
161	1216	0.2773	6.201e-10	<0.001	GO:0043284	biopolymer biosynthetic process
83	497	0.3706	8.232e-10	<0.001	GO:0051246	regulation of protein metabolic process
208	1704	0.2516	1.022e-9	<0.001	GO:0065007	biological regulation
156	1179	0.2742	1.396e-9	<0.001	GO:0019222	regulation of metabolic process
183	1486	0.2436	1.080e-8	<0.001	GO:0050794	regulation of cellular process

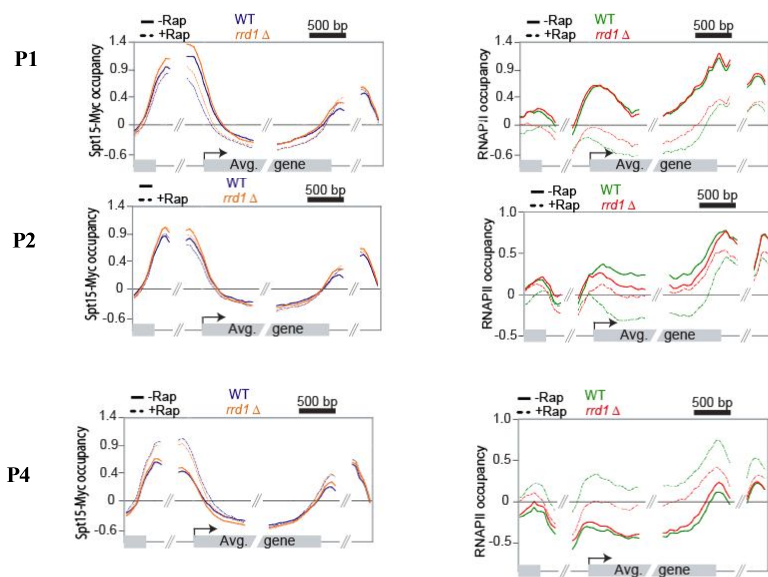
S5

N	X	LOD	P	P_adj	Gene-Ontology-ID	Gene-Ontology-Attribute
72	215	0.5434	9.879e-15	<0.001	GO:0009266	response to temperature stimulus
67	196	0.5552	2.847e-14	<0.001	GO:0009408	response to heat
61	176	0.5622	2.142e-13	<0.001	GO:0034605	cellular response to heat
87	303	0.4490	3.845e-13	<0.001	GO:0009628	response to abiotic stimulus
40	117	0.5454	5.236e-9	<0.001	GO:0007039	vacuolar protein catabolic process
173	880	0.2422	9.117e-9	<0.001	GO:0009056	catabolic process
733	5086	0.2627	1.875e-8	<0.001	GO:0005575	cellular_component
731	5076	0.2564	3.210e-8	<0.001	GO:0044464	cell part
164	841	0.2347	4.553e-8	<0.001	GO:0044248	cellular catabolic process
193	1030	0.2175	5.334e-8	<0.001	GO:0050896	response to stimulus
81	343	0.3271	7.512e-8	<0.001	GO:0044257	cellular protein catabolic process
149	752	0.2405	7.702e-8	<0.001	GO:0006950	response to stress
81	344	0.3253	8.601e-8	<0.001	GO:0034962	cellular biopolymer catabolic process
83	356	0.3200	9.384e-8	<0.001	GO:0030163	protein catabolic process
38	127	0.4588	6.646e-7	<0.001	GO:0010324	membrane invagination

S6

N	X	LOD	P	P_adj	Gene-Ontology-ID	Gene-Ontology-Attribute
126	996	0.2591	1.122e-7	<0.001	GO:0006996	organelle organization
178	1564	0.2203	2.799e-7	<0.001	GO:0016043	cellular component organization
48	281	0.3880	6.352e-7	<0.001	GO:0006974	response to DNA damage stimulus

Figure S6 Poschmann J. et al.,



Supplemental figure S7 Poschmann J. et al., 2010

F1 no significant GO

F2

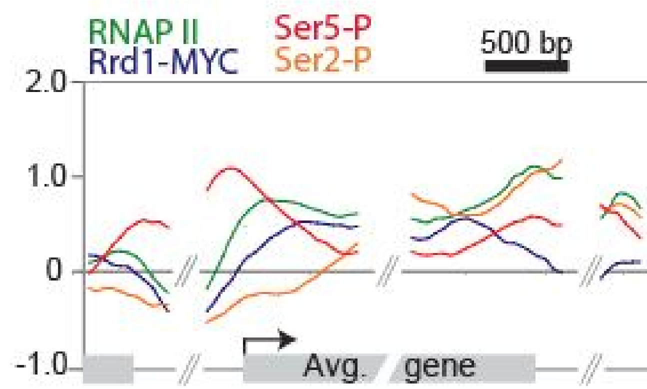
N	X	LOD	P	P_adj	Gene-Ontology-ID	Gene-Ontology-Attribute
671	5036	0.3340	5.437e-11	<0.001	GO:0005575	cellular_component
670	5076	0.3321	5.624e-11	<0.001	GO:0044464	cell part
648	4878	0.2891	3.618e-10	<0.001	GO:0008150	biological_process
266	1734	0.2163	7.344e-10	<0.001	GO:0006139	nucleobase, nucleoside, nucleotide and nucleic acid metabolic process
607	4535	0.2137	1.712e-7	<0.001	GO:0044424	intracellular part
398	2737	0.1379	4.920e-7	<0.001	GO:0034960	cellular biopolymer metabolic process
185	1120	0.2037	5.052e-7	<0.001	GO:0031323	regulation of cellular metabolic process
403	2030	0.1304	6.170e-7	<0.001	GO:0044260	cellular macromolecule metabolic process
103	545	0.2611	7.495e-7	<0.001	GO:0022613	ribonucleoprotein complex biogenesis
101	533	0.2520	8.570e-7	<0.001	GO:0042254	ribosome biogenesis
171	1026	0.2054	8.852e-7	<0.001	GO:0010468	regulation of gene expression
499	3653	0.1702	9.221e-7	<0.001	GO:0044237	cellular metabolic process

F3

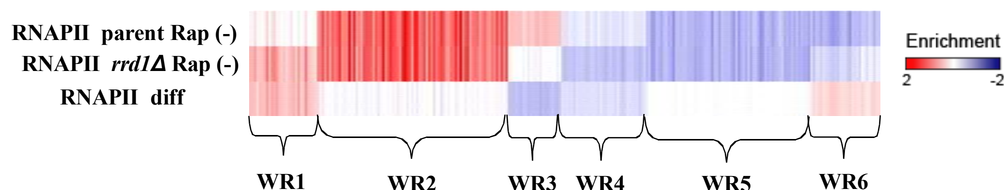
N	X	LOD	P	P_adj	Gene-Ontology-ID	Gene-Ontology-Attribute
1062	5076	0.5661	7.607e-34	<0.001	GO:0044464	cell part
1063	5036	0.5673	1.081e-33	<0.001	GO:0005575	cellular_component
1010	4878	0.3660	3.657e-22	<0.001	GO:0008150	biological_process
958	4535	0.3252	8.296e-20	<0.001	GO:0044424	intracellular part
948	4531	0.2896	1.946e-16	<0.001	GO:0009987	cellular process
804	3735	0.2235	9.899e-14	<0.001	GO:0008152	metabolic process
552	2421	0.2153	6.667e-13	<0.001	GO:0044444	cytoplasmic part
720	3317	0.2014	2.664e-12	<0.001	GO:0044238	primary metabolic process
774	3653	0.2055	3.184e-12	<0.001	GO:0044237	cellular metabolic process
785	3742	0.2111	1.252e-11	<0.001	GO:0043229	intracellular organelle
372	1533	0.2113	1.414e-11	<0.001	GO:0044267	cellular protein metabolic process
785	3734	0.2003	1.470e-11	<0.001	GO:0043226	organelle
372	1539	0.2087	2.562e-11	<0.001	GO:0019538	protein metabolic process
831	4020	0.1991	8.504e-11	<0.001	GO:0003674	molecular_function
624	2936	0.1623	1.130e-10	<0.001	GO:0043283	biopolymer metabolic process
630	2907	0.1793	2.204e-10	<0.001	GO:0043170	macromolecule metabolic process
612	2833	0.1711	1.308e-9	<0.001	GO:0044260	cellular macromolecule metabolic process
601	2737	0.1665	3.494e-9	<0.001	GO:0034960	cellular biopolymer metabolic process
426	1907	0.1592	7.682e-8	<0.001	GO:0005737	cytoplasm
374	1647	0.1624	1.247e-7	<0.001	GO:0009058	biosynthetic process
290	1227	0.1760	1.421e-7	<0.001	GO:0009059	macromolecule biosynthetic process
69	205	0.3594	1.506e-7	<0.001	GO:0033279	ribosomal subunit
719	3439	0.1502	1.531e-7	<0.001	GO:0043227	membrane-bounded organelle
719	3439	0.1502	1.531e-7	<0.001	GO:0043231	intracellular membrane-bounded organelle
287	1216	0.1753	1.963e-7	<0.001	GO:0043284	biopolymer biosynthetic process

F4 no significant GO

Supplemental figure S8 Poschmann J. et al., 2010



Supplemental figure S9 Poschmann J. et al., 2010



WR1

N	X	LOD	P	P_adj	Gene-Ontology-ID	Gene-Ontology-Attribute
8	9	1.693	1.089e-7	<0.001	GO:0006189	'de novo' IMP biosynthetic process
12	21	1.061	1.792e-7	<0.001	GO:0009127	purine nucleoside monophosphate biosynthetic process
12	22	1.017	3.571e-7	<0.001	GO:0009126	purine nucleoside monophosphate metabolic process
8	10	1.471	4.947e-7	<0.001	GO:0006188	IMP biosynthetic process
8	10	1.471	4.947e-7	<0.001	GO:0046040	IMP metabolic process
11	20	1.024	0.000001004	<0.001	GO:0009168	purine ribonucleoside monophosphate biosynthetic process
11	21	0.9803	0.000001911	<0.001	GO:0009167	purine ribonucleoside monophosphate metabolic process

WR2

N	X	LOD	P	P_adj	Gene-Ontology-ID	Gene-Ontology-Attribute
167	215	0.9735	3.776e-52	<0.001	GO:0044445	cytosolic part
186	253	0.8797	1.046e-51	<0.001	GO:0005840	ribosome
167	233	0.8347	6.709e-44	<0.001	GO:0033279	ribosomal subunit
230	370	0.6579	8.049e-44	<0.001	GO:0006412	translation
165	233	0.8159	3.023e-42	<0.001	GO:0003735	structural constituent of ribosome
147	203	0.8461	1.611e-39	<0.001	GO:0010608	posttranscriptional regulation of gene expression
140	190	0.8725	4.382e-39	<0.001	GO:0006417	regulation of translation
146	208	0.7985	1.116e-36	<0.001	GO:0032268	regulation of cellular protein metabolic process
212	372	0.5581	2.464e-32	<0.001	GO:0005198	structural molecule activity
166	271	0.6277	2.012e-30	<0.001	GO:0051246	regulation of protein metabolic process
77	93	1.087	8.337e-28	<0.001	GO:0022625	cytosolic large ribosomal subunit
97	136	0.8108	1.798e-25	<0.001	GO:0015934	large ribosomal subunit
288	614	0.3867	2.105e-24	<0.001	GO:0030529	ribonucleoprotein complex
56	64	1.234	5.861e-23	<0.001	GO:0022627	cytosolic small ribosomal subunit
201	401	0.4316	3.535e-21	<0.001	GO:0005622	intracellular
70	97	0.8218	3.760e-19	<0.001	GO:0015935	small ribosomal subunit
357	873	0.2807	2.032e-17	<0.001	GO:0043228	non-membrane-bounded organelle
357	873	0.2807	2.032e-17	<0.001	GO:0043232	intracellular non-membrane-bounded organelle
499	1313	0.2388	4.847e-17	<0.001	GO:0044267	cellular protein metabolic process
665	1873	0.2017	3.121e-15	<0.001	GO:0032991	macromolecular complex
518	1429	0.2012	3.842e-13	<0.001	GO:0019538	protein metabolic process
703	2086	0.1585	1.838e-10	<0.001	GO:0005737	cytoplasm
321	856	0.2066	6.681e-10	<0.001	GO:0010468	regulation of gene expression
326	873	0.2038	8.346e-10	<0.001	GO:0010556	regulation of macromolecule biosynthetic process
330	888	0.2004	1.224e-9	<0.001	GO:0031326	regulation of cellular biosynthetic process
596	1750	0.1563	1.844e-9	<0.001	GO:0065007	biological regulation
330	892	0.1969	2.224e-9	<0.001	GO:0009889	regulation of biosynthetic process

WR3

N	X	LOD	P	P_adj	Gene-Ontology-ID	Gene-Ontology-Attribute
27	123	0.5307	9.728e-7	<0.001	GO:0030684	preribosome
13	36	0.8282	0.000002002	0.002000	GO:0030687	preribosome, large subunit precursor
104	861	0.2381	0.000004841	0.003000	GO:0016070	RNA metabolic process
340	3683	0.1771	0.00001337	0.007000	GO:0043227	membrane-bounded organelle
340	3683	0.1771	0.00001337	0.007000	GO:0043231	intracellular membrane-bounded organelle
30	165	0.4280	0.00001475	0.008000	GO:0042254	ribosome biogenesis
32	189	0.3904	0.00003443	0.02400	GO:0022613	ribonucleoprotein complex biogenesis
33	201	0.3743	0.00004934	0.04000	GO:0044085	cellular component biogenesis
141	1316	0.1832	0.00005341	0.04200	GO:0043234	protein complex

WR4 **no significant GO**

WR5

N	X	LOD	P	P_adj	Gene-Ontology-ID	Gene-Ontology-Attribute
28	45	0.6984	1.109e-7	<0.001	GO:0030476	ascospore wall assembly
28	45	0.6984	1.109e-7	<0.001	GO:0042244	spore wall assembly
28	46	0.6742	2.165e-7	<0.001	GO:0070726	cell wall assembly

WR6 **no significant GO**

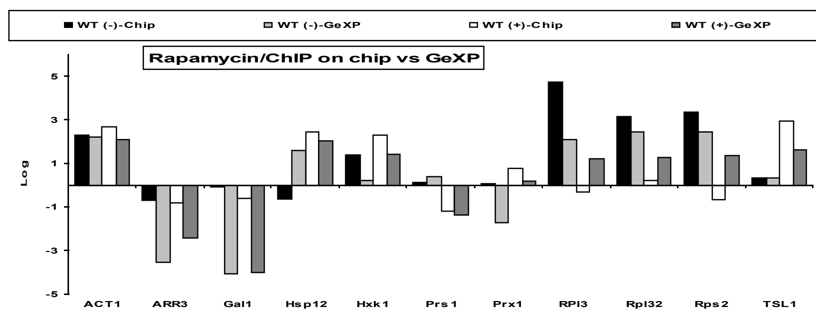
Supplemental figure S10 Poschmann J. et al., 2010

Table S1 Supplementary information Poschmann J. et al.,

Q-PCR primers used in this study

rpl32F	GCCGCTGAAATTGCTCACA
rpl32R	CTTAGCTCTAGCCAAAATGACAACTC
put4F	AGCGAGCCGCACAAACTAA
put4R	AGCGCGATCAGTTGCACAT
hsp104F	TTGAGGCCATCAAGCAACAA
hsp104R	AGCGCCACGAGAGTCAATTC
rps2F	AACAGAGGCCGTCCAAACAG
rps2R	GGAACCCATCCCTTTTCTTCA
Tel1F (noORF)	TTGTAGAAAAAACGTGGACGGTAA
Tel1R (noORF)	GAAGCCGCACATTTCCAATT
Act1F	AAACTATGTTACGTCGCCTTGGA
Act1R	ACCATCTGGAAGTTCGTAGGATTT

Supplemental tabe S1 Poschmann J. et al., 2010



Supplemental figure S11 Poschmann J. et al., 2010

3 DISCUSSION

From its discovery in 1998 until today, our laboratory has studied the role of Rrd1 to identify its cellular function [74]. Since *rrd1Δ* deletion was found to contribute to oxidative stress and DNA damage induced by the carcinogen 4NQO, we were interested in identifying the role of Rrd1 in DNA response pathways [74, 114]. A breakthrough was made when we found that Rrd1 is required for the transcriptional response towards rapamycin and its PPIase function was published in 2006 [93, 113]. This project started with the hypothesis that Rrd1 is involved in the transcriptional regulation of RNAPII. From here we were able to make several discoveries that led to a model for Rrd1 regulating RNAPII in response to various stresses. These discoveries are listed and discussed below, followed by a proposed model of regulation.

3.1 Rrd1 acts independently of the Sit4-Tap42 complex

As known from the literature, *rrd1Δ* mutants are highly resistant to rapamycin and the Rrd1 protein interacts with the PP2A-like phosphatase Sit4 [111-113]. Sit4 and Rrd1 interact with Tap42 and are thought to dephosphorylate and activate the transcription factor Gln3 in response to rapamycin or nutrient starvation. This results in the activation of nitrogen discrimination genes, including the *MEP2* gene which is required for ammonium intake [113]. Therefore, it was postulated that Rrd1 acts in conjunction with Sit4-Tap42 in response to rapamycin and that this function accounts for the high resistance of *rrd1Δ* mutants [117]. However, our lab found that the Gln3 target gene *MEP2* is induced independently of Rrd1 [113]. This suggests that the Gln3 signaling pathway might not require the function of Rrd1 and that Rrd1 might be functioning elsewhere in the response to rapamycin. To further investigate the possibility this possibility, we created a double deletion *rrd1Δ-gln3Δ* strain. This strain displayed an additive phenotype respective to the single mutants in response to rapamycin (article 1 figure 1). This genetic evidence supports that Rrd1 has

additional roles in the response to rapamycin. However, we cannot exclude that this new role of Rrd1 is independent of its association with Sit4 (see below).

3.2 Rrd1 interacts with RNAPII

This novel function for Rrd1 was confirmed when we showed that Rrd1 is associated with chromatin. This suggests a role in DNA metabolism and indeed, using different assays we identified that the major subunit of RNAPII interacts with Rrd1. In addition to co-immunopreciptiation and pull-down assays, ChIP analysis showed that both proteins co-localize on actively transcribed genes, and that this association remains during transcriptional changes induced by rapamycin. Actually, we found that Rrd1 was bound to 75 % of genes (approx. 2000) that were occupied by RNAPII. More precisely, Rrd1 localizes within the body of the gene, suggesting a role during transcription elongation (article 2 Supplemental figure S9). Indeed, multiple elongation factors associate with RNAPII once it dissociates from the promoter and initiates elongation. For example, using the same type of assay, TFIIS was shown to have a similar association with RNAPII as did Rrd1 [19] (article 2 supplemental figure S2B). Similar to Rrd1, another peptidyl prolyl isomerase, Pin1/Ess1, is known to interact with RNAPII during elongation [44, 95, 106].

When Rrd1 was immunoprecipitated with RNAPII, we were also able to immunoprecipitate the PP2A-like phosphatase Sit4. It is very likely that Rrd1 and Sit4 interact together when associated with RNAPII. Our data did not allow us to exclude a role for Sit4 in Rrd1 function; therefore, this would be interesting to investigate further.

Interestingly, we found that Rrd1 did associate with the CTD of Rpb1. This association points towards a regulatory role of Rrd1, since the CTD is a recruitment platform for multiple factors during elongation and is strongly modified throughout these steps. More interestingly, the CTD has been previously shown to be isomerized by the PPIase Pin1/Ess1 [95, 106, 159]. Taken together our data reveal for the first time that Rrd1 interacts directly with the transcriptional machinery on actively transcribed genes. The interaction is at least partially mediated via the CTD of RNAPII and this interaction takes place when RNAPII is moving along the ORFs.

3.3 Rrd1 isomerizes the CTD of RNAPII

Since Rrd1 is a peptidyl prolyl isomerase and it interacts with RNAPII, we addressed the question of whether RNAPII would be a substrate of Rrd1. Using circular dichroism, we showed that Rrd1 isomerizes the CTD of RNAPII *in vivo* in response to rapamycin and also directly *in vitro* when both are purified. In addition, we confirmed a structural change of the CTD in response to rapamycin using a limited proteolysis assay.

To analyze proline isomerization, one can make use of four different assays, namely circular dichroism, limited proteolysis, limited proteolysis coupled to fluorescence and NMR analysis. The first two allow the use of the complete protein with the disadvantage that one will not determine the precise proline that is isomerized. The latter two will allow for identification of the exact proline, but requires that the protein be segmented into peptides. We used CD analysis since it allowed us to purify two different stable conformations of the CTD from cells (see chapter 2.1 figure 4). Instead of purifying the whole RNAPII complex, which contains at least 12 large proteins, we generated a GST-CTD fusion protein that is easily purified from cells and was also shown to be functional *in vivo* (e.g. phosphorylation status) (see chapter 2.1 supplemental figure S1). The advantage of the fusion protein (GST-CTD) is that it retained its structural conformation throughout the purification steps. This is not the case with peptides as they are known to spontaneously and slowly alter their proline configuration over time [159]. In addition, using a CTD that can be phosphorylated (see chapter 2.1 figure 3A and supplemental figure S1) was helpful since the exact substrate specificity of Rrd1 is not known. Using peptides, we would first have to determine the exact phosphorylation status of the substrate peptide. However, the disadvantage of using the CD technique is that we could not determine exactly where Rrd1 isomerizes the CTD or in which configuration it needs to be (e.g. phosphorylation status). Also, albeit that we demonstrated that purified Rrd1 can alter purified GST-CTD structure, we have no direct proof that this is through proline isomerization, although this is the most probable explanation. Future work is required to directly demonstrate that Rrd1 isomerizes the CTD of Rpb1. Experiments should

include a catalytically inactive mutant of Rrd1 and the use of peptides coupled to NMR or limited proteolysis with fluorescence in order to determine the exact substrate configuration of the CTD. Another interesting alternative would be to co-crystallize Rrd1 with the CTD of Rpb1 as this would provide structural cues of its interaction and substrate binding.

It is noteworthy that we showed for the first time that a PPIase isomerizes the CTD of Rpb1 *in vivo* using CD analysis. Although it is widely accepted in the literature that Pin1/Ess1 is a CTD isomerase, a direct demonstration that the CTD isomerization happens *in vivo* is not available yet. The only direct proof that Ess1 isomerizes the CTD of Rpb1 was shown *in vitro* using small peptides as substrates for the protease assay and NMR [159].

3.4 Rrd1 modulates transcription in response to rapamycin

The consequences of CTD isomerization can be multiple. For example, it may interfere with recruitment of CTD binding proteins or alter the phosphorylation status of the CTD as was shown for Ess1 [44]. However, our previous studies revealed that Rrd1 is required to modulate the expression of a subset of genes in response to rapamycin. In addition, we found that although RNAPII is degraded in response to rapamycin after a long period of treatment, this degradation is strongly reduced in the *rrd1Δ* mutant [113].

The observation that RNAPII is degraded in response to rapamycin might be due to a mechanism that occurs after a long period of hunger. We observed an inhibition of this degradation in the *rrd1Δ* mutant but this was not specific to Rrd1. In fact, this degradation was also diminished when other rapamycin resistant mutant were tested [113] (unpublished data). One could imagine that when nutrient limitation (or rapamycin treatment) persists, cells catabolise excessive protein in order to survive longer. It is noteworthy that the degradation only starts after 45 min to 1 hour, whereas the most drastic transcriptional changes occur at 30 min, which then decrease within the next hour [128]. This suggests a timely response where gene expression of stress response genes are turned on before and while excessive RNAPII is degraded

and explains why genes are enriched for RNAPII, but the overall RNAPII levels eventually decrease.

Since Rrd1 interacts with RNAPII and modulates its expression, we used ChIP on chip analysis to determine how many genes are affected by the deletion of *RRD1*. Although this question could have been addressed by performing expression analysis and quantifying the mRNA using microarrays, we chose to use ChIP on chip technology as this gives additional information on the localization of RNAPII and its association with Rrd1. We found that Rrd1 plays a major role in transcriptional regulation of genes that are repressed or induced in response to rapamycin. Our gene ontology analysis revealed that Rrd1 is especially required for the repression of regulatory metabolic genes, including genes involved in regulatory functions of transcription, translation and ribosome biogenesis (see chapter 2.2 figure 2B and supplemental figure S5). We suspect that Rrd1 might be part of a specific regulatory mechanism. For example, the expression of regulatory genes needs to be modulated rapidly in order to alter the expression of other metabolic genes (e.g. ribosomal structural genes). Results obtained until now don't allow us to prove this hypothesis, which would require additional expression analysis after shorter periods of drug treatment of the regulatory metabolic genes and compare them with structural ribosomal genes. However, using our assay, we have demonstrated that Rrd1 regulates transcription independently of its Tap42- Sit4-Gln3 signaling activity since this branch does not regulate ribosome biogenesis [60]. In addition, we found catabolic and stress genes to be regulated by Rrd1, which are part of the Msn2/Msn4 signalling branch thought to be regulated only partially through Tor signaling (see section 1.1.8.1.1) [63].

Taken together, results indicate that Rrd1 regulates a large set of genes in response to rapamycin in an unknown fashion. The next question to be addressed was how Rrd1 regulates transcription.

3.5 Rrd1 is a transcription elongation factor

We have obtained several data that point towards a role of Rrd1 in transcription elongation, where it regulates RNAPII association with the gene. First,

as already discussed above, Rrd1 isomerizes the CTD of RNAPII, the CTD occupying a central role during transcriptional regulation [7-9, 14, 26]. Second, using a new assay developed by our team, we found that purified Rrd1 was able to release RNAPII from the chromatin *in vitro* (see chapter 2.1 figure 8). We conclude from this experiment that Rrd1 enables RNAPII dissociation from genes, presumably through its interaction with and isomerization of Rpb1. Third, with our *in vivo* data from ChIP on chip analyses, we showed an opposite effect when *RRD1* was absent: RNAPII remained on ribosomal genes as well as regulatory metabolic genes involved in the response to rapamycin. Thus, *in vivo* Rrd1 also promotes the release of RNAPII from specific genes.

A similar observation was found with Pin1. When Pin1 is overexpressed it promotes RNAPII dissociation from active genes, followed by the accumulation of the dissociated RNAPII in enlarged structures [95, 155]. Although these assays are different, it seems that PPiases are capable of releasing RNAPII from genes. Consistent with this data, it was previously published that a slow transcribing and highly serine 5 phosphorylated form of elongating RNAPII is present on a group of ribosomal genes under glucose grown conditions[154]. When these cells were stressed by a switch from glucose to galactose containing medium, this form of RNAPII disappeared on the ribosomal genes and RNAPII was recruited to newly activated genes [154]. The authors concluded that under normal growth conditions, RNAPII is enriched on ribosomal genes [154], which could be a 'storage' mechanism. Thus, in response to metabolic changes, this excessive RNAPII would be rapidly recruited to other genes. As demonstrated for rapamycin treatment, a similar situation occurs where RNAPII needs to be released from genes that are downregulated and be recruited to newly activated genes.

Fourth, from our ChIP on chip data, we find that *rrd1*Δ mutants display in general an altered serine-5-phosphorylated form of RNAPII on most genes under normal growth or rapamycin treated conditions. This suggests that Rrd1 is required is required to modulate serine 5 phosphorylation of RNAPII population, for example during a transcriptional stress where RNAPII needs to be redistributed.

3.6 Rrd1 is required for a broad range of stresses

The litterature confirms that *rrd1Δ* mutants display multiple phenotypes in response to a broad range of drugs (see section 1.2.2). One common aspect shared by many of these drugs is that they all cause oxidative stress. Our study also identified drugs that generate ROS and cause *rrd1Δ* mutant sensitivity (see chapter 2.2 figure 6). We further note that Rrd1 might be required for the osmotic stress response. Our unpublished data shows that *rrd1Δ* mutants display sensitivity towards osmotic stress induced by NaCl in a specific yeast background (W303). Another study has shown that this response occurs in response to Ca^{2+} [110]. These findings bring up the question of what is common between oxidative stress, osmotic stress and rapamycin. The answer to this question is that they all induce similar transcriptional responses [71]. To equivalent extents all induce stress, causing the induction of the expression of catabolic genes and the inhibition of ribosome biogenesis; these responses all being mediated by the same transcription factors [63]. Since *rrd1Δ* mutants are sensitive to all three conditions we believe that Rrd1 is required for the same mechanism of transcriptional regulation that has been demonstrated for rapamycin. We have already obtained initial data, that Rrd1 is required for the transcription of the *ACR3*-LacZ reporter gene in response to Na Arsenite treatment (see chapter 2.2 figure 6C). *ACR3* encodes an efflux pump that is highly upregulated in response to Na Arsenite and is crucial for its cellular detoxification [136].

To confirm the lacZ reporter assay and to test if *rrd1Δ* mutants are also required for other stresses, we used the GeXP multiplex PCR system to simultaneously quantify several mRNAs (see chapter 2.2 fig. 7). The GeXP system was developed by Beckman and the principles are similar to those in quantitative real-time PCR. To distinguish between different genes within the same sample, LM-PCR adds a different fragment to each target gene, so that each can be distinguished by a different length. This analysis is performed by a fluorescence sensitive capillary system, simultaneously measuring the intensity (quantity) and size (gene specificity). The Kanamycin resistance marker is added as an internal control and as a control for normalization in between different conditions.

The results presented clearly indicate that Rrd1 is not only required for the expression of rapamycin regulated genes, but also, in response to other environmental stresses such as oxidative stress and heat shock (see chapter 2.2 figure 7). It is noteworthy that all chosen genes do not respond to the various treatments in a similar manner. A striking example is the *ACR3* gene which is only upregulated in response to Na arsenite but not to the other stresses. This is also true for the oxidative stress gene *PRX1* which is not induced in response to rapamycin or heat shock. Independent of this, Rrd1 is clearly required to regulate expression of these genes as most of them fail to reach WT expression levels in response to the various stresses.

Interestingly, the ribosomal genes were not strikingly downregulated as would been suggested by the ChIP on chip data (see chapter 2.2 figure 7) and was shown in [71]. This might be due to different strain backgrounds used (By4741 as opposed to DBY7286). In the By4741 background it might be the case that the ribosomal mRNAs are additionally regulated by their stability and that they have a longer half-life then the other genes analyzed and therefore do not show a big difference in response to the various stresses. However, this was not the case for all downregulated genes since Prs1 also showed a repression in response to various stresses (see chapter 2.2 fig. 7). Here one can also observe that *rrd1Δ* mutants failed to adequately inhibit transcription for most conditions.

Taken together, our data suggests that Rrd1 modulates the environmental stress response (ESR) by regulating transcription of RNAPII.

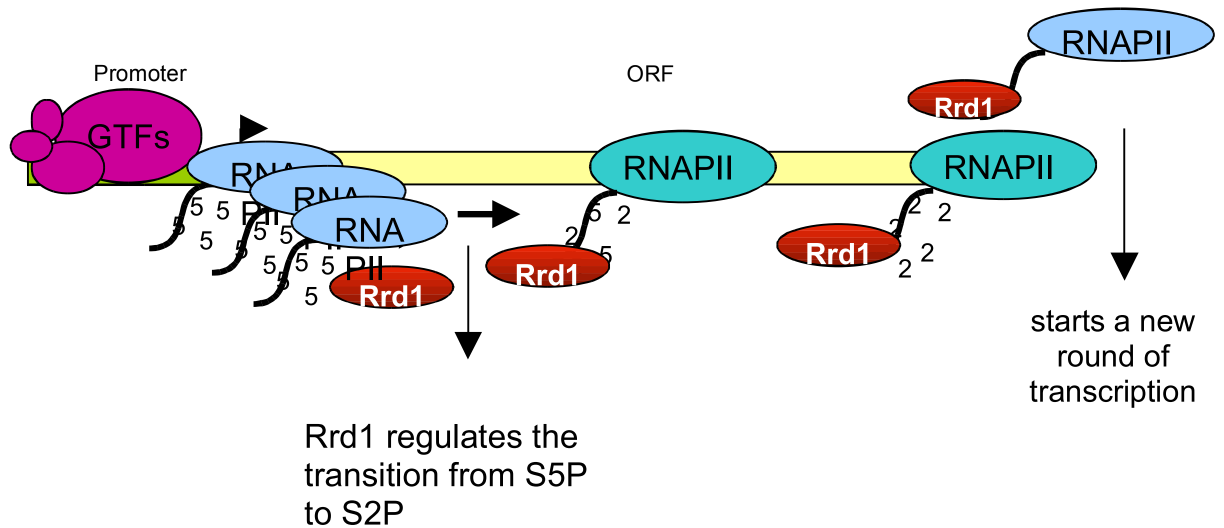
3.7 Model of RNAPII regulation

From the above considerations, we propose the following model: under normal growth conditions, Rrd1 is associated with RNAPII complex on actively transcribed genes (figure 8A). While associated to these genes, Rrd1 may promote the transition from the serine 5 phosphorylated form to the serine 2 phosphorylated form of RNAPII. This would occur on specific genes including the ribosomal genes and could be done by isomerizing the CTD of Rpb1, thereby recruiting a phosphatase that would dephosphorylate serine 5 and increase the RNAPII transcription rate, thus releasing it from the gene. These genes contain a relatively high amount of RNAPII,

which is associated with a low transcription rate. Upon stress, two mechanisms of transcriptional inhibition are activated. First, genes that are required to regulate metabolic processes are downregulated rapidly via Rrd1 using the above mechanism (figure 8B). In this group of genes, the PIC remains assembled, allowing RNAPII to be rapidly recruited to restart transcription once the stress is resolved. Second, genes encoding structural factors involved in metabolism, such as ribosome biogenesis genes, are downregulated due to a rapid release of RNAPII via Rrd1 in addition to the PIC being disassembled at the promoter (figure 8B). RNAPII remains associated with Rrd1 and this complex along with the GTFs, can then be recruited to newly activated genes such as those needed for catabolism and stress responses. The role of Rrd1 in these situations would be similar to what was described above, which would be to assist RNAPII elongation and, if necessary, to stimulate the release of RNAPII (figure 8A).

Our model is based on our experimental data presented in the two articles. However, two statements in this model are based on an assumption that has not been tested: (i) although it was shown that ribosomal genes that contain a high amount of the serine 5 phosphorylated form of RNAPII are associated with a low transcriptional rate [154] we can only assume that Rrd1 is required to stimulate the transcriptional rate by decreasing serine 5 phosphorylation. This assumption needs to be tested and involves a complicated assay that to date, has only been published by the same group [185]. A possible alternative is that in response to rapamycin Rrd1 increases serine 5 phosphorylation of RNAPII on these genes, thereby slowing down even more transcription. This would happen on the set of genes where TBP remains bound and once the stress is over these genes can then be rapidly re-activated. (ii) Second, we postulated that this might be the mechanism by which Rrd1 releases RNAPII from genes. Although this is a logical interpretation of the data, we cannot exclude an alternative mechanism of RNAPII release by Rrd1. For example, in response to stress, Rrd1 can be activated by post-translational modifications and thus may alter its activity causing it to isomerize RNAPII in such a way that it is released in a more drastic fashion. We have analyzed the post-translation modifications of Rrd1 using mass spectrometry and found a putative ubiquitylation site (K236) (unpublished

data). Therefore, it could very well be that under certain circumstances, Rrd1 is regulated by a signaling pathway that leads to the ubiquitylation and modification of Rrd1 activity. Further investigation of this putative ubiquitylation site is required, such as the generation of site directed mutants of this specific lysine (R236K).



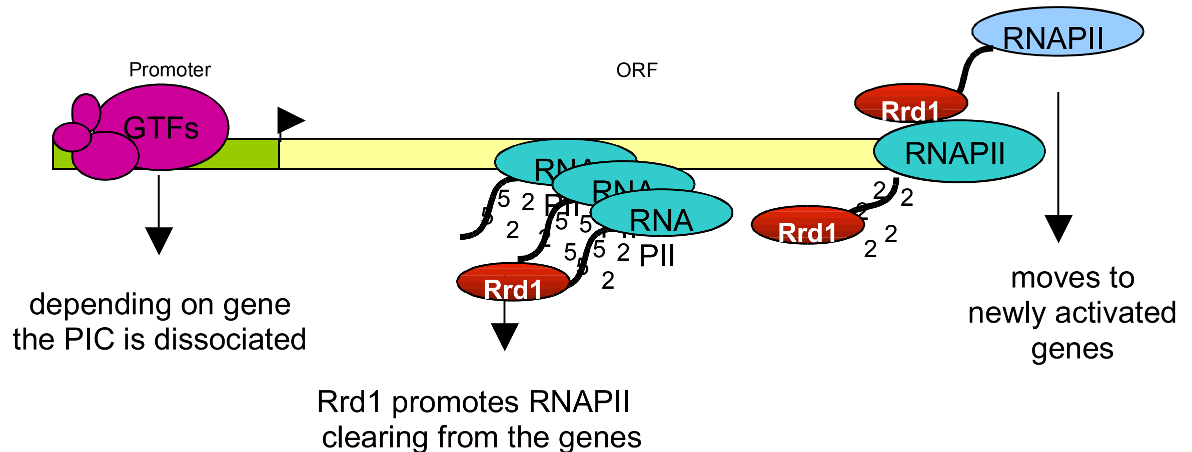


Figure 8 Model of RNAPII regulation

GTFs represent the PIC localized on the promoter. RNAPII (light blue) is the initiating RNAPII which is highly phosphorylated on serine 5 of its CTD, as indicated by the black line and the number 5. The darker blue RNAPII is the elongating RNAPII that has increased serine 2 phosphorylation.

3.8 The catalytic activity of Rrd1

Rrd1 has been extensively characterized as a peptidyl prolyl isomerase [87, 88, 93]. We, so far, have analyzed the *rrd1Δ* mutant phenotypes, the Rrd1 associations with RNAPII, its genome wide localization and shown from a biochemical point of view that Rrd1 is able to release RNAPII from the chromatin *in vitro*. Another critical experiment would be to demonstrate that the catalytic PPIase activity of Rrd1 is really involved in the isomerization of the CTD. To do this, site directed mutagenesis can be used to alter critical residues in the catalytic center of the PPIase domain. Such residues have already been characterized, including a mutation which results in the conversion of the glycine into an aspartate at site 205 (G200D in *S.cerevisiae*) which was shown to impair the isomerase activity of Rrd1 (see figure 7) [87, 88]. We now have designed a plasmid that expresses the *RRD1* gene containing the G200D mutation to purify sufficient amounts of the protein for use in *in vitro* assays. We would like to address two critical questions with this mutant: first, is this mutant able to isomerize the CTD-GST fusion protein using the CD analysis? Second, does this mutant release RNAPII from the chromatin?

The first question is still in the process as it requires several purification steps and it is currently being performed by Nathalie Jouvet. However, for the second question we can now show that the catalytic D200G mutant is not able to release RNAPII from the chromatin (see figure 9). This clearly indicates that the isomerase activity of Rrd1 is required for this step. This experiment does not directly support the hypothesis that Rrd1 isomerizes the CTD of RNAPII, but it does confirm that Rrd1 is able to release RNAPII form the chromatin *in vitro*, and that this activity requires the PPIase domain of Rrd1. It is still possible that besides the CTD of RNAPII, other PPIase substrates

of Rrd1 are required for the release of RNAPII from the chromatin (discussed in the next section).

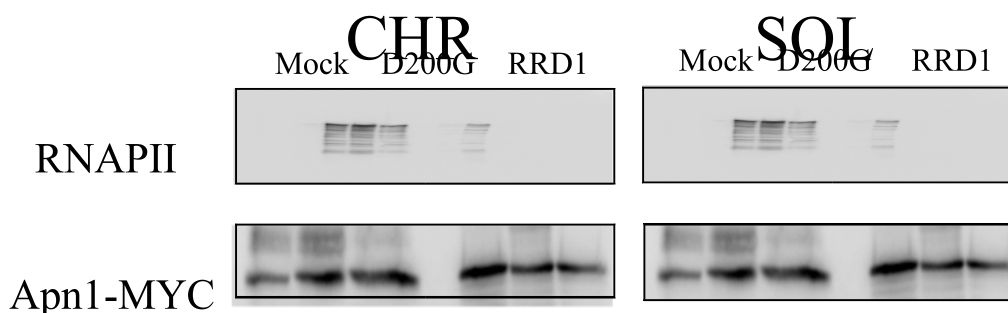


Figure 9 The Rrd1 catalytic mutant (D200G) does not release RNAPII from the chromatin. Experiments were performed as in chapter 1 figure 8, only that Mock (no protein added), D200G (Rrd1 catalytic mutant protein added) RRD1 (native protein added).

3.9 Alternative targets of Rrd1

To date, only two substrates of Rrd1 isomerase activity have been discovered. The first substrate to be identified was a peptide matching a specific sequence of PP2A (186LQEV**P**HEGAMCDL198) [93]. Rrd1 does not share the same substrate specificity as Pin1/Ess1, which targets phosphorylated threonine or serine residues that precede the proline residue [93]. This suggests a precise substrate specificity of Rrd1 but it does not exclude that Rrd1 might isomerize other targets of the transcriptional machinery besides the CTD of RNAPII. One can imagine that Rrd1 might isomerize a phosphatase that would then dephosphorylate serine 5 of the CTD of RNAPII. Also, depending on whether a cell is under 'normal growth conditions' or 'stress conditions', Rrd1 substrate specificity could be altered by post-translational modifications, therefore drastically alter RNAPII gene association.

As it is known, Pin1 has multiple cellular targets involving a pleiotropy of cellular events [94-96, 186]. The same could be true for Rrd1, since we have already identified a novel function of Rrd1. In addition, our genome-wide Rrd1 association

study showed that Rrd1 associates with a group of genes independently of RNAPII (article 2 figure 1D and 4A), suggesting that it might perform additional roles to the one described here.

Very recently, a new role of Rrd1 was found in the regulation of telomere healing [187]. It is thought that Rrd1 regulates the Pph3 phosphatase, which in turn is recruited to double strand breaks (DSB) where it promotes the recruitment of Cdc13 by dephosphorylating its residue S306. Rrd1 seems to play a role here similar to its previously described function as a phosphatase activator [90]. If Rrd1 is lacking Pph3 does not adequately dephosphorylate Cdc13 and its recruitment to DSB sites is prevented by its phosphorylation [187]. This new mechanism supports a model where Rrd1 as a peptidyl prolyl isomerase has multiple functions within various cellular conditions.

3.10 Involvement of Rrd1 in regulatory pathways

As stated above, Rrd1 is required for the regulation of some (though not all) ribosomal and metabolic regulatory genes. In addition, it is required for some stress responsive genes (See chapter 2 Figure 2B). This is quite surprising since the ribosomal genes and the stress responsive genes are orchestrated as regulons which are in turn controlled by the recruitment of transcription factors [63]. The ribosomal genes are controlled by Fhl1 and Crf1 and the stress responsive genes are controlled by Gln3, Msn2/4 and Rtg1/2 [59-61, 63, 65-67]. If Rrd1 is somehow involved in the transition of these transcription factors from the cytoplasm to the nucleus, one would expect that all genes would be similarly regulated by Rrd1. This, however, is not the case, so we expect Rrd1 to function elsewhere.

It is largely accepted that ribosome biogenesis is regulated in such a way that all components are transcribed in a coordinated manner (from RNAPI, II and III) so that the right amount of structural parts are produced [121]. So why does Rrd1 only regulate a fraction of ribosomal genes expressed by RNAPII?

First of all, the above described model proposes that Rrd1 regulates RNAPII during transcription elongation, thus eliminating any upstream regulatory mechanisms.

Second, several hypotheses could explain this phenomenon:

Rrd1 functions as a molecular timer to regulate specific subclasses of ribosomal genes in response to stress. For example, the mechanism of transcriptional regulation of Rrd1 is required to rapidly remove RNAPII from a specific type of ribosomal genes that are not of structural but rather regulatory nature (see chapter 2.2 Suppl. Fig. S5). Since we only look at a specific time point (30 min after treatment) we do not know if these genes are eventually repressed in an *rrd1*Δ mutant. Indeed ribosomal genes have been separated in ribosomal protein (RP) genes which are the structural proteins of the ribosome as well as ribosomal biogenesis (Ribi) genes, which are necessary for proper transcription, assembly and maintenance of the ribosomes [121]. In chapter 2.2 Figure 2A we have already observed that most Ribi and RP genes are affected in an *rrd1Δ* mutant in response to rapamycin. To further test whether Rrd1 affects RP and/or Ribi genes, RP genes and Ribi genes were added to figure 2D of chapter 2.2 (see figure 10, below).

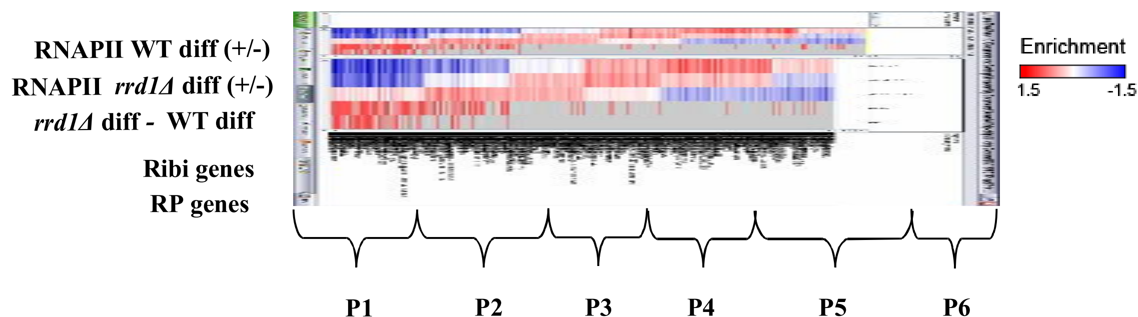


Figure 10 Rrd1 regulates Ribi genes and RP genes. Each line (red) acounts for a Ribi or RP genes depending on the row. This figure is modified from chapter 2.2 figure 2B.

It turns out that *rrd1*Δ deletion does affect Ribi genes much more strongly then RP genes (compare cluster P1, P2 and P6). Rrd1 affects about 37 % of the rapamycin downregulated regulated Ribi genes (cluster P2). Most of the other Ribi genes (59 %) are found in cluster P1, which is only mildly affected by *rrd1*Δ deletion. Also, 90% of the RP genes are found in cluster P1 and only 5% are found in cluster P2. This suggests that Rrd1 does not specifically regulate the RP or the Ribi genes but rather affects both groups of genes with variable intensity. It is noteworthy that

that some factors affect gene expression of Ribi genes but not RP genes. For example, Dot6 and Tod6 have been shown to be specifically required to inhibit the expression of Ribi genes but not RP genes [188]. Another example is that Med20 (Srb2), a component of the mediator complex, is required to exclusively repress RP genes in response to rapamycin treatment [189]. It is known that RP genes and Ribi genes contain different transcription factor binding sites within their promoters and therefore, the different activities of Med20, Dot6 and Tod6 on Rp and Ribi gene expression can be explained [188, 189]. However, for Rrd1, this is not the case since it affects both the Ribi and the RP genes.

So far the only common feature of the Rrd1 regulated genes is that TBP (Spt15) did not alter its promoter association (See chapter 2.2 figure 4). In response to rapamycin TBP remained similarly associated to the promoter but RNAPII decreased on these genes. In the *rrd1Δ* mutants, TBP also similarly remained at the promoter but RNAPII occupancy was higher as compared to WT cells.

How and why does this group retain TBP (and probably the rest of the PIC) at these promoters is not clear. However, TBP binding at these genes resembles a phenomenon that has been discovered thanks to genome wide association studies and is called a poised state. It is postulated that RNAPII can remain poised at the promoter within the PIC, for example in yeast during stationary phase [190]. In higher eukaryotes, this poised complex is often found within developmental genes or heat shock protein genes, and then once the signal is given, RNAPII starts transcribing [190]. We could observe a similar mechanism here, where the PIC remains assembled at these promoters, and once the starvation period is over, it is likely that transcription can rapidly resume by recruiting RNAPII. We did not observe an accumulation of RNAPII at these promoters, suggesting that only the PIC remains poised. RNAPII might be recruited to other genes for transcription of stress response genes, or for degradation of excess proteins. Taken together, we propose a new mechanism of RNAPII transcriptional regulation that does not fit with any known regulatory mechanism, but resembles a poised form of RNAPII.

This might explain why only some ribosomal genes are downregulated by Rrd1. However, this mechanism does not explain why only some genes are

upregulated by Rrd1 and this again, is independent of TBP recruitment (see chapter 2.2 figure 4). One common link between the upregulation and downregulation by Rrd1 is that both are independent of TBP binding and (probably) PIC assembly. Instead, the transcription changes occur during transcription elongation, which is further indicated by the fact that serine 5 phosphorylation of the RNAPII CTD is strongly altered in *rrd1*Δ mutants. Therefore, we believe that whether it is upregulation or downregulation, Rrd1 influences the transcription elongation process through a so far unknown mechanism which involves serine 5 phosphorylation. The big question remains, why are some genes affected strongly by Rrd1 but not others? Here again one can propose the hypothesis, that Rrd1 as a peptidyl prolyl isomerase acts as a molecular timer to timely optimize the transcriptional response.

3.11 *S.cerevisiae* as a model

Throughout the entire project, *S.cerevisiae* was used as a model. Although it is largely accepted that yeast is a good model to study complex molecular biology processes such as transcription, DNA replication or cell cycle, herein, we demonstrate again that *S.cerevisiae* is a powerful research tool. Had genome-wide deletion arrays not been available, the role of Rrd1 in response to rapamycin would probably not yet have been elucidated. Our unpublished data (Nathalie Jouvet) from experiments with mammalian cells using either siRNA or shRNA directed against PTPA has not yet allowed us to determine whether PTPA is required for the cellular response to rapamycin. Several variables might explain the discrepancy, such as the longer rapamycin treatment used in mammalian cells compared to yeast, the % knockdown as well as the variability obtained in each experiment and in the different cell lines. Therefore, the generation of a PTPA-knockout mouse would be a necessary step to further analyze the role of PTPA in mammalian cells. However, the generation of transgenic mice is known to be a costly and lengthy procedure.

Our study has used multiple ChIP on chip analyses on a genome-wide scale in *S.cerevisiae*. In order to perform such an analysis in mammalian cells, one would need to use a sequencing technique since the human genome only contains approximately 2% of coding regions, compared to *S.cerevisiae* which has a much

higher % of coding regions [191]. Although ChiP-Seq is more precise, the problem lies in the cost of performing such experiments. Another major advantage to working with yeast is that genes can be endogenously tagged (e.g. *RRD1*-MYC and *SPT15*-MYC), allowing the expression to be driven by their endogenous promoter, giving rise to the expression levels that aren't artificial as would be the case for over-expression plasmids. This can be very useful for ChIP analyses, in cases where the antibody directed against the protein of interest is not available. However, the distance of evolution between yeasts and humans is significant; therefore, not all mechanisms are conserved. Thus, yeast is a great model to be used in research, but its use is limited to initial discoveries and analysis of basic to highly complex mechanisms, which then need to be confirmed in higher eukaryotes. Especially, if one takes into account the fact that yeast is a unicellular organism: It does not require the integration of multiple signalling pathways-- such as those from neighbouring cells, multiple (growth) hormonal signaling pathways, developmental processes and cellular differentiation into specialized tissues. Furthermore, cancer preventing mechanisms such as apoptosis, although known to be present in yeast, are not required to be highly controlled and regulated [192]. All these mechanisms add levels of complexity for the integration of multiple signalling pathways in multicellular eukaryotes which cannot be studied in yeast.

3.12 Is the TORC1 complex regulated by superoxide anions?

During the progress of our work, a paper was published by another group proposing that the binding of Tor to the rapamycin-Fpr1 complex is inhibited by ROS [124]. In this publication, the authors performed a genome wide mutant sensitivity screen in response to rapamycin [124]. Similarly to other published screens, they identified mutants highly resistant to rapamycin, including *rrd1Δ* and *gln3Δ* [166, 167]. Since 2 of the 15 highly resistant mutants they identified were involved in the oxidative stress response, they investigated whether all mutants were rapamycin-resistant due to elevated levels of ROS. Their results showed indeed, all rapamycin-resistant mutants displayed elevated ROS levels, when compared to the WT. Furthermore, they showed that Tor binding to the Fpr1-rapamycin complex was

inhibited when cells had elevated levels of ROS. Therefore, they concluded that the mutants were resistant to rapamycin not due to their function, but rather because they displayed elevated levels of ROS. We disagree with their conclusion for several reasons. First, their experimental data is not conclusive. For example, they compared total mRNA expression in *gln3Δ* and *sod1Δ* mutants to the expression in the WT and concluded that expression levels were different. However, if this difference was caused by the elevated levels of ROS, one would expect that *gln3Δ* and *sod1Δ* mutants would display the same expression levels; however, their expression levels were strikingly different. In addition, although the authors showed that all mutants displayed elevated levels of ROS, they did not show a conclusive experiment where elevated ROS levels (e.g. induced by H_2O_2) cause rapamycin-resistance in the WT strain.

Also, besides showing that Tor does not seem to bind to the Fpr1 kinase, they never demonstrated whether crucial downstream targets of the Tor signaling pathway are affected by elevated ROS levels (e.g. gene expression of Tor target genes).

Second, if their hypothesis is true and strains bearing elevated levels of ROS are rapamycin-resistant, theirs and other genome wide drug screens would have identified multiple resistant mutants bearing elevated ROS levels [166, 167], which could include every factor involved in ROS catabolism. Surprisingly, only two factors, a superoxide dismutase and its chaperone, render cells resistant to rapamycin, when deleted.

Third, our data clearly indicates that Rrd1 has a function in transcription elongation, which does not exclude a role for Rrd1 in ROS metabolism. In contrast, since ROS is a form of stress, transcription of genes would be affected by Rrd1. However, our data clearly reject the hypothesis that the *rrd1Δ* mutant phenotype is only due to elevated ROS levels. In the *rrd1Δ* mutant, only certain groups of rapamycin-responsive genes are affected, which means that despite the absence of Rrd1, Tor was inactivated by rapamycin. Furthermore, TBP recruitment to gene promoters was similarly affected in the WT and the *rrd1Δ* mutant, suggesting that Tor signaling is functional in the *rrd1Δ* mutant.

As such, we only partially agree with the conclusions presented in Neklesa and Davis (PNAS, 2008) and still believe that most of the rapamycin-resistant mutants are so because their gene products are somehow functionally involved in the Tor signaling pathway.

4 CONCLUSION

Transcriptional regulation in response to environmental changes is essential for survival of cells and allows them to live within most conditions. Yeast cells constantly sense the environment and alter their transcription profile and metabolism in response to the current condition. These pathways are conserved during evolution and they allow us to adapt to multiple environmental conditions, including living in hot or cold environments, under strong sun exposure, under exposure to pollutants such as cigarette smoke, food restriction or excess. Whether these pathways are perturbed in yeast cells or in humans they both cause an inability to adapt to stress conditions and which will eventually lead to disease and cell death. Therefore, understanding and knowing how these pathways function is crucial for disease treatment and prevention. We have used yeast as a model to identify a new factor that is involved in the response to stresses and from this study we have made several conclusions which will be listed in a point form below:

4.1 Rrd1 is a transcription elongation factor required for the ESR in yeast

We have analyzed the role of the peptidyl prolyl isomerase Rrd1 in response to rapamycin and to other environmental stresses. Our results demonstrate a completely new role for Rrd1: Rrd1 is a transcription elongation factor that is required to regulate RNAPII gene transcription in response to environmental stresses including, starvation, oxidative stress, and temperature stress. The precise mechanism of transcriptional regulation in response to stresses is as followed. Rrd1 is a transcription elongation factor that isomerizes the CTD of RNAPII and this interaction regulates RNAPII association with the gene. So far this mechanism only becomes prominent when cells are challenged with stresses that mediate a profound transcriptional response. In this case, Rrd1 is required to redistribute RNAPII. This mechanism is additional to general transcription regulation and it is required to optimize the transcriptional response. It is likely that this mechanism acts as a molecular timer for an optimal response to the environmental changes. It is not part

of the fundamental regulatory mechanism of transcriptional regulation which involves the transcription factors and recruitment of the RNAPII machinery, but rather, an additional mechanism of regulation that allows the fine-tuning of the transcriptional response. This fine-tuning seems to be required for the ESR in particular since although the ESR is activated in response to various stresses, it is specific for every stress [63]. Notably for oxidative stresses, most, but not all genes are activated as in the heat shock or starvation stress response. Therefore, multiple steps of transcriptional regulatory circuits are necessary to precisely express the right genes for the right condition.

Herein we have discovered a new regulatory mechanism, which regulates transcription during the elongation phase after assembly of the pre-initiation complex and recruitment of RNAPII.

4.2 The reason why *rrd1*Δ mutants have pleiotropic phenotypes

*rrd1*Δ mutants have been found by multiple labs to have a variety of phenotypes. These mutants are resistant to rapamycin and caffeine but hypersensitive to 4NQO, H_2O_2, NaAs, UVA, Vanadate, Ca^{2+}, ketokonazole and cycloheximide. This always begged the question why *rrd1*Δ mutants are resistant to some agents but sensitive to others. We are the first to be able to provide a logical answer. Rrd is required for an optimal transcriptional response to environmental stresses. Since, most of these drugs stimulate the ESR response (besides cycloheximide and ketokonazole), it is likely that *rrd1*Δ mutants are hypersensitive to these agents because the transcriptional response is not adequate and cells will eventually die out because of the cellular toxicity. However, for rapamycin and caffeine (which only mimic starvation and oxidative stress respectively (see 1.1.8.1 and 1.2.2)), but do not actually cause a nutrient depletion or ROS, *rrd1*Δ mutants become resistant as no adequate stress response is activated and there is no cellular toxicity and sufficient nutrients are available for continued growth. WT cells will activate the ESR in response to both agents with the consequence of growth inhibition.

We note that the drug ketokonazole, which is known to inhibit ergosterol synthesis, might also cause a sort of ESR. This is probably because of a lack of ergosterol. This

lack could be regarded as a form of starvation that activates an ESR to increase uptake of components required for ergosterol synthesis. Finally, the sensitivity to cycloheximide could be explained by the fact that since translation is inhibited, a transcriptional stress response is activated to increase ribosome gene expression, and *rrd1Δ* mutants might not be able to adequately mediate this response.

4.3 Rrd1 is the second PPIase that isomerizes the CTD of RNAPII

We demonstrate that a second peptidyl prolyl isomerase is required to isomerize the CTD of RNAPII besides Pin1/Ess1. Ess1 is thought to isomerize the CTD of RNAPII to alter its phosphorylation status, thus allowing the recruitment of transcription termination factors (Nrd complex) in order to arrest transcription of snoRNA [44]. Rrd1 also isomerizes the CTD of RNAPII and alters the phosphorylation status of the CTD. In both cases, when *RRD1* or *ESS1* genes are mutated, serine 5 phosphorylation is altered. However, the outcome is different for *rrd1*Δ mutants as one only observes a transcriptional defect under stress conditions. Furthermore, we exclude the possibility that Rrd1 may be involved in transcription termination as we would have observed an accumulation of RNAPII on the 3' end of the gene in an *rrd1*Δ mutant as was observed for the *ess1-TS* mutation [44]. Therefore, we suspect that Rrd1 is likely to act on serine 5 phosphorylation within the 5' region of the gene, regulating the elongation of RNAPII. This is also consistent with the fact that under stress conditions, the Rrd1 regulated genes are less well transcribed in an *rrd1*Δ mutant. However it would be interesting to determine the phenotypes of an *ESS1-RRD1* double deletion strain. Ess1 is essential, but there are thermosensitive mutants which could be used for that.

It is not surprising that another PPIase that isomerizes the CTD has been discovered, since the highly conserved structure of the CTD $(YSPTSPS)^n$, contains 2 prolines per heptapeptide. The CTD is seen as a recruitment platform for multiple elongation factors and needs to be highly modifiable so that different factors can be ejected and recruited during the transcription cycle [7-9, 193]. This can be done through phosphorylation of serine 2, serine 5 and serine 7, as well as through isomerisation by Ess1 and Rrd1. Thus, one can only imagine the possibilities of

different conformations that may arise when one takes into account that in yeast there are only 26 repeats of the heptapeptide as compared to up to 52 repeats in mammals [193].

4.4 The new role of Rrd1 might be conserved during evolution

Pin1 is evolutionally conserved and in humans it plays multiple roles including immunological responses, cancer and diseases such as Alzheimer's [186]. Since Rrd1 is also evolutionary conserved, its human homologue PTPA might perform a similar function as does Rrd1 in yeast. Preliminary data already suggests that this might be the case since PTPA was also capable of isomerising the CTD *in vitro* (unpublished data). In addition we and others have shown that the overexpression of PTPA can complement for the lack of *RRD1* gene in yeast [74]. We predict here that if its role in transcription elongation is conserved it would play a role in many biological processes and thus might be important to prevent disease and cancer. Mammalian cells also need to respond to environmental stresses such as heat shock, starvation and oxidative stresses. Therefore, it would not be surprising that similar mechanisms of transcriptional regulation are present as the one we found in yeast.

A simple strategy would be to use rapamycin, since it is used in clinics as an immunosuppressant as well as for kidney carcinoma [160, 161]. So far, knockdown strategies have not been supportive, which could be because we are yet to obtain a strong knockdown of the protein. The other alternative to studying PTPA is to produce knockout cell lines and we are currently generating a PTPA knockout mouse which will allow us to better study the function of this protein. Besides these efforts, it would also be interesting to investigate the role of Rrd1 in other yeast species such as *S.pombe* or *C. albicans*, in order to know if this function is conserved in these yeast species as well. So far no publications are available in this regards.

4.5 Rrd1 as a potential drug target

Pin1/Ess1 was found to be inhibited by the molecule juglone, and this could be used in clinics for disease treatment [194]. It turns out that the unique structure and the high substrate specificity of these PPIases makes them interesting drug targets. Similarly, the unique structure of PTPA and its substrate specificity might also allow for the generation of specific inhibitors of this protein [93]. This would be another reason to continue the investigation of the function of Rrd1/PTPA in other organisms.

4.6 Rrd1 has multiple cellular roles

Rrd1 was first described to be a phosphatase activator required to stimulate the *in vitro* phospho-tyrosyl phosphatise activity of serine/threonine PP2A phosphatases. Subsequently, it was shown that it is a peptidyl prolyl isomerase that is required for phosphatase assembly and substrate specificity *in vivo*. Rrd1 associates with at least three phosphatases and multiple roles have now been discovered: Rrd1 associates with Sit4 and Tap42 to dephosphorylate Gln3 upon Tor signaling, although this function is controversial (see 1.2.8). We have now shown that Rrd1 interacts with RNAPII on actively transcribed genes. We also have the first indication that Rrd1 is indeed acting through isomerisation as the catalytic inactive Rrd1 (D200G) protein was unable to release RNAPII from the chromatin *in vitro* (see section 3.8). Finally, during the writing of the thesis, new research was published showing that Rrd1 is involved in telomere healing as it was found to activate the Pph3 phosphatase [187]f. Thus, Rrd1 is involved in different cellular mechanisms and more roles may yet be discovered for it. It is noteworthy that Pin1 has over 30 cellular targets and it is involved in various cellular pathways (see 1.2.5).
This clearly shows that contrary to the previously held belief, PPIases have additional roles besides their function in protein folding. As was shown for FKBPs and cyclophilins, the parvulins and the PTPA family, PPIases are likely to continue to surprise investigators.

With the help of modern molecular biology techniques we were able to describe a so far uncharacterized regulatory mechanism of transcriptional regulation which involves the PPIase Rrd1. However, this was only the ground work description of a novel mechanism. There are still a lot of open questions that need to be answered: How is Rrd1 regulated by itself? What are the other factors required to mediate RNAPII release? What is the exact function of Rrd1 under normal growth conditions? But the main question we would like to address is if this function is evolutionary conserved. Future work should shed light on these questions.

5 PERSPECTIVES

As mentioned above this study has presented the groundwork of a new mechanism of transcriptional regulation. Although we now have a model which is supported by extensive experimental data, we do not know all the answers and further experiments are required in order to fully elucidate this mechanism. In addition, many questions arise from our proposed model, which will be addressed in this section in a point form:

- How does Rrd1 choose which gene to regulate?

The data from our genome-wide analyses allowed us to make the observation that Rrd1 is associated with RNAPII on a large subset of genes (see chapter 2.2 figure 1). However, in response to rapamycin, Rrd1 did not have an effect on every gene, but rather on a specific group of genes that are downregulated and a group of genes that are upregulated. Therefore, it would be interesting to investigate how Rrd1 is active on these specific genes but not on the others, although they are regulated by the same transcription factors.

Proteins with regulatory functions are often regulated by post-translational modifications and as a starting point it would be interesting to analyze the post-translational modification of Rrd1 found using mass spectrometry. A close example is Pin1, which has been shown to be phosphorylated and also oxidised, two post-translation modifications which alter the isomerase activity of Pin1 [109]. As previously stated, we have found a potential ubiquitylation site of Rrd1 on the K236. Ubiquitylation is not only known to target degradation of the ubiquitylated protein but also to regulate localization, protein-protein interactions and protein activity. Therefore this ubiquitylation site might provide cues on how Rrd1 is regulated and how it chooses specific genes on which to regulate RNAPII. To test this, a K236R mutation could be introduced into the *RRD1* gene in order to express an ubiquitylation deficient mutant protein. With this, we then can test the isomerisation of the CTD using CD, or test whether this mutant is able to release RNAPII from the chromatin as we have shown for the catalytic mutant (see figure 9). There are two further questions: does Rrd1 become ubiquitylated upon rapamycin treatment, and does it interact with RNAPII when it is in its ubiquitylated form. These could be

tested by co-immunoprecipitation experiments with RNAPII and then with an ubiquitylation specific antibody to see if Rrd1 is ubiquitylated when pulled down by RNAPII. Initial experiments have already been conducted but the ubiquitylation specific antibody was of poor quality and therefore, the results were inconclusive (data not shown). The hypothesis of ubiquitylation of Rrd1 could also be addressed in a genetic way. For example by testing whether the deletion of ubiquitin ligases affect the phenotypes of *rrd1*Δ mutants. There are multiple ubiquitin ligases, but one (Rsp5) would be of special interest as it has been shown to ubiquitylate RNAPII and its vicinity within the elongation machinery makes it a possible ubiquitin ligase of Rrd1 [195]. Nathalie Jouvet has performed complementation assays showing that overexpression of *RRD1* from a plasmid abolishes the hypersensitivity of an *rsp5-TS* mutant towards rapamycin thereby supporting a potential genetic interaction between Rsp5 and Rrd1.

A second way to investigate how Rrd1 regulates gene expression is to analyze whether recruitment of proteins to the CTD of RNAPII are affected by the action of Rrd1. We have shown that the isomerization of RNAP's CTD by Rrd1 engenders a profound alteration of the secondary structure. In addition, we know that the phosphorylation status of RNAPII is also altered in an *rrd1*Δ mutant, especially for serine 5 phosphorylation. As it is widely accepted that the CTD of RNAPII is a recruitment platform for several elongation factors during the transcription cycle, and that the phosphorylation status and the conformation of the CTD are crucial for the timely coordinated recruitment and exchange of these factors, Rrd1 could use this mechanism to recruit other factors that then regulate transcription. To test this, one could develop an *in vitro* CTD binding assay where total cell extracts are added either before or after isomerization of the CTD by Rrd1 (similar to [196]). We would expect then that depending of the CTD configuration, different factors might associate with it. The identity of the factors associated would then be determined by silver staining and subsequent mass spectrometry analysis. An alternative experiment would be to monitor the recruitment of known transcription elongation factors including Paf1, the CTD phosphatases, FACT or Spt4/5 to a model gene (e.g. *PUT4* or *RPL32*) in response to rapamycin. This can be done in a similar manner as our demonstration of

the recruitment of RNAPII and Rrd1-MYC to these genes, using ChIP-QPCR (see chapter 2.2 Figure 3).

- How is RNAPII released from transcribed genes?

rrd1Δ mutants display an elevated level of the serine 5 phosphorylated form of RNAPII on certain genes. This suggests that RNAPII might be slowly transcribing on these genes, meaning that it maintains a slow transcriptional rate [154]. Therefore, it would be interesting to test whether Rrd1 influences transcription elongation by regulating the transcriptional rate of RNAPII. As mentioned previously (see section 3.7) [185], another group has developed a transcriptional run-on assay that measures the transcriptional rate of RNAPII. It would be of significant interest to run this assay in an *rrd1Δ* mutant as our model of transcriptional regulation by Rrd1 is based on this principle.

An alternative would be to test factors that associate with the Rrd1-isomerized CTD in an *in vitro* RNAPII transcription assay. For example, does the transcription change when Rrd1 is added to the *in vitro* assay? If this is the case, which factors are required to mediate the effects of Rrd1? We have already performed intitial experiments where addition of Rrd1 increases the transcription of RNAPII (unpublished data, collaboration with Luc Gudreau USherbrooke). Now to find which factor is required to mediate the effects of Rrd1 we could use cell extracts that lack specific factors (e.g. the above mentioned transcription elongation factors) and monitor if Rrd1 still enhances transcription. This would determine which factors mediate the regulatory effects of Rrd1 *in vitro*. This could then be further tested *in vivo* using genetics and ChIP assays.

- Identification of additional Rrd1 substrates

As mentioned previously, it would be interesting to determine whether Rrd1 isomerizes other factors than PP2A and the CTD of RNAPII. This is not an easy task since it is not easy to measure proline isomerization as it is to monitor phosphorylation or ubiquitylation. A more complex strategy is necessary to study proline isomerization. The first step would be to exactly identify the CTD configuration (phosphorylation status) that is necessary for it to be isomerized by Rrd1. Next, the exact sequence surrounding the proline isomerized by Rrd1 needs to

be identified. From this information, one can use sequence similarity to identify potential other substrates of Rrd1.

An alternative way to identify potential Rrd1 substrates would be to use a co-purification assay. Although protein-protein interactions have been performed on virtually every protein in yeast, none of these studies have identified RNAPII as a partner of Rrd1 [197, 198]. Therefore, one would need to optimize the conditions necessary to identify novel partners of Rrd1. Our unpublished data from tandem affinity purification assays of Rrd1 suggests that Rrd1 interacts with components of the mediator complex as well as the Swi/Snf chromatin remodeling complex. These complexes, which are also involved in transcription, might be potential targets of the isomerases activity of Rrd1.

Furthermore, the fact that Rrd1 influences serine 5 phosphorylation of RNAP's CTD suggests that a phosphatase or kinase might be a target of Rrd1. Since only a limited set of kinases (Kin28) and phosphatases (Ssu72 and Rtr1) are known to influence serine 5 phosporylation, it would be worthwhile to test whether these enzymes are actually isomerized by Rrd1 [199, 200]. This could be done similarly to what was shown for the CTD of RNAPII *in vitro*.

- Is the role of Rrd1 conserved throughout evolution?

This is the most exciting question to investigate since it could be of medical importance. This question should be addressed not only in mammalian systems but also in other yeast species such as *S.pombe* and *C.albicans*. For investigations in other yeast species, the strategy would be to first analyze if the phenotypes of *rrd1*Δ mutants that were observed in *S.cerevisiae* also occur in these other yeasts. Furthermore, it would be of interest to test whether Rrd1 interacts with and regulates RNAPII in these yeast species.

To test the role of PTPA in mammalian cells, multiple strategies can be used. First a knockdown of PTPA expression using siRNA or shRNA might allow us to study its response to rapamycin (in progress). Second, the generation of a PTPA knockout mouse to better analyze the multiple roles of PTPA *in vivo,* such as development would most definitely provide some answers.

Third, the analysis of protein-interactions in mammalian cell extracts using different immunoprecipitation techniques would be required for the validation of our yeast model. We need to confirm whether PTPA interacts with RNAPII and whether PTPA is recruited to chromatin.

Fourth, it would be interesting to verify if PTPA is required for the rapamycin and oxidative stress response: Since rapamycin inhibits B-cell and T-cell proliferation, it would be interesting to investigate whether PTPA is required for this process [160].

Our group has recently published that the overexpression of PTPA stimulates the apoptosis of mammalian cells, clearly indicating that PTPA plays an important role in cell viability [201]. Furthermore, *in vitro* analyses have shown that purified PTPA is capable of isomerizing the CTD of RNAPII (unpublished data). These data encourage further investigation into the cellular functions of PTPA in mammalian cells.

Taken together, the work that led to the model of Rrd1 transcriptional regulation in response to environmental stresses opens up a lot of questions that are of interest to the scientific community and of likely medical interest as well. First, the model needs to be further tested in the yeast *S.cerevisiae*, in order to establish Rrd1 as a new transcription elongation factor. Then, it needs to be determined whether this mechanism occurs in other yeast species as well. Finally, the main focus should be to analyze the evolutionary conservation of this mechanism in higher eukaryotes such as mammals, with relevance to development, cancer and various other diseases.

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