Université de Montréal

# Investigating the role of the isomerase Rrd1/PTPA: from yeast to human

par

Nathalie Jouvet

Programmes de biologie moléculaire

Faculté de médecine

Thèse présentée à la Faculté de médecine
en vue de l'obtention du grade de Ph.D.
en biologie moléculaire

Décembre 2011

Université de Montréal
Faculté de médecine

Cette thèse intitulée:

# Investigating the role of the isomerase Rrd1/PTPA: from yeast to human

Présentée par:

Nathalie Jouvet

A été évaluée par un jury composé des personnes suivantes:

Martine Raymond
président rapporteur

Dindial Ramotar
directeur de recherche

Janos Filep
membre du jury

Jacques Côté
examinateur externe

Pierre Belhumeur
représentant du doyen de la faculté de médecine

## RÉSUMÉ

Chez *Saccharomyces cerevisiae*, les souches mutantes pour Rrd1, une protéine qui possède une activité de peptidyl prolyl *cis/trans* isomérase, montrent une résistance marquée à la rapamycine et sont sensibles au 4-nitroquinoline 1-oxide, un agent causant des dommages à l'ADN. PTPA, l'homologue de Rrd1 chez les mammifères, est reconnu en tant qu'activateur de protéine phosphatase 2A. Notre laboratoire a précédemment démontré que la surexpression de PTPA mène à l'apoptose de façon indépendante des protéines phosphatase 2A. La fonction moléculaire de Rrd1/PTPA était encore largement inconnue au départ de mon projet de doctorat.

Mes recherches ont d'abord montré que Rrd1 est associé à la chromatine ainsi qu'à l'ARN polymérase II. L'analyse *in vitro* et *in vivo* par dichroïsme circulaire a révélé que Rrd1 est responsable de changements au niveau de la structure du domaine C-terminal de la grande sous-unité de l'ARN polymérase II, Rpb1, en réponse à la rapamycine et au 4-nitroquinoline 1-oxide. Nous avons également démontré que Rrd1 est requis pour modifier l'occupation de l'ARN polymérase II sur des gènes répondant à un traitement à la rapamycine. Finalement, nous avons montré que suite à un traitement avec la rapamycine, Rrd1 médie la dégradation de l'ARN polymérase II et que ce mécanisme est indépendant de l'ubiquitine.

La dernière partie de mon projet était d'acquérir une meilleure connaissance de la fonction de PTPA, l'homologue de Rrd1 chez les mammifères. Nos résultats montrent que le «knockdown» de PTPA n'affecte pas la sensibilité des cellules à

différentes drogues telles que la rapamycine, le 4-nitroquinoline 1-oxide ou le peroxyde d'hydrogène ($H_2O_2$). Nous avons également tenté d'identifier des partenaires protéiques pour PTPA grâce à la méthode TAP, mais nous ne sommes pas parvenus à identifier de partenaires stables. Nous avons démontré que la surexpression de la protéine PTPA catalytiquement inactive n'induisait pas l'apoptose indiquant que l'activité de PTPA est requise pour produire cet effet. Finalement, nous avons tenté d'étudier PTPA dans un modèle de souris. Dans un premier lieu, nous avons déterminé que PTPA était exprimé surtout au niveau des tissus suivants : la moelle osseuse, le thymus et le cerveau. Nous avons également généré avec succès plusieurs souris chimères dans le but de créer une souris «knockout» pour PTPA, mais l'allèle mutante ne s'est pas transférée au niveau des cellules germinales.

Mes résultats ainsi que ceux obtenus par mon laboratoire sur la levure suggèrent un rôle général pour Rrd1 au niveau de la régulation des gènes. La question demeure toujours toutefois à savoir si PTPA peut effectuer un rôle similaire chez les mammifères et une vision différente pour déterminer la fonction de cette protéine sera requise pour adresser adéquatement cette question dans le futur.

**Mots clés** : transcription, RNA polymérase II, Rrd1, PTPA, PP2A, isomérisation

## ABSTRACT

In *Saccharomyces cerevisiae*, mutants devoid of Rrd1, a protein possessing *in vitro* peptidyl prolyl *cis/trans* isomerase activity, display striking resistance to rapamycin and show sensitivity to the DNA damaging agent 4-nitroquinoline 1-oxide. PTPA, the mammalian homolog of Rrd1, has been shown to activate protein phosphatase 2A. Our laboratory previously found that overexpression of PTPA leads to apoptosis independently of PP2A. At the outset of my thesis work, the molecular function of Rrd1/PTPA was largely unknown.

My work has shown that Rrd1 is associated with the chromatin and interacts with RNA polymerase II. *In vitro* and *in vivo* analysis with circular dichroism revealed that Rrd1 mediates structural changes of the C-terminal domain of the large subunit of RNA pol II, Rpb1, in response to rapamycin and 4-nitroquinoline 1-oxide. Consistent with this, we demonstrated that Rrd1 is required to alter RNA pol II occupancy on rapamycin responsive genes. We also showed that upon rapamycin exposure Rrd1 mediates the degradation of RNA polymerase II and that this mechanism is ubiquitin-independent.

Another part of my work was to gain insight into the function of PTPA, the mammalian counterpart of Rrd1. PTPA knockdown did not affect sensitivity to rapamycin, 4-nitroquinoline 1-oxide or $H_2O_2$. We also attempted to find protein interaction partners for PTPA using tandem affinity purification, but no stable partners for PTPA were found. We also demonstrated that overexpression of a catalytically inactive PTPA mutant did not induce apoptosis, indicating that PTPA activity is required to produce this effect. Finally, we attempted to study PTPA in a

mouse model. We first determined that PTPA was expressed in a tissue-specific manner and was most abundant in bone marrow, thymus and brain. We pursued creation of a knockout mouse and successfully generated chimeras, but the mutated allele was not transmitted to the germline.

My data and other data from our laboratory regarding the yeast work suggest a general role for Rrd1 in regulation of gene transcription. Whether PTPA has a similar function in mammalian cells remains unknown, and a different vision of what the protein does in mammalian cells will be required to adequately address this question in the future.

**Keywords:** transcription, RNA pol II, Rrd1, PTPA, PP2A, isomerisation

# TABLE OF CONTENTS

**RÉSUMÉ** ..... **iii**

**ABSTRACT** ..... **v**

**TABLE OF CONTENTS** ..... **vii**

**LIST OF FIGURES** ..... **xi**

**LIST OF ABBREVIATIONS** ..... **xiii**

**ACKNOWLEDGMENTS** ..... **xvii**

**1 INTRODUCTION** ..... **2**

**1.1 Rapamycin and TOR** ..... **5**
1.1.1 Rapamycin ..... 5
1.1.2 TOR ..... 6
1.1.3 Yeast *TOR* ..... 6
1.1.4 Mammalian TORC composition ..... 8
1.1.4.1 mTORC1 ..... 9
1.1.4.2 mTORC2 ..... 10
1.1.5 Rrd1 and TOR ..... 10

**1.2 Rrd1/PTPA overview** ..... **11**
1.2.1 PTPA ..... 13
1.2.2 Rrd1/PTPA structure ..... 13
1.2.3 PP2A ..... 14
1.2.3.1 Post-translational modifications ..... 16
1.2.4 Rrd1/PTPA and PP2A ..... 17
1.2.5 PTPA and apoptosis ..... 17
1.2.6 Rrd1/PTPA as a peptidyl prolyl isomerase (PPIase) ..... 18

**1.3 Transcription** ..... **21**
1.3.1 Overview ..... 21
1.3.2 Transcription initiation ..... 22
1.3.3 Transcription elongation ..... 23
1.3.4 Transcription termination ..... 25
1.3.5 RNA polymerase II structure ..... 26
1.3.5.1 RNA pol II C-terminal domain ..... 27

1.3.6 RNA pol II arrest ..... 29
1.3.6.1 Transcription-coupled nucleotide excision repair (TC-NER) ..... 30
1.3.6.2 RNA pol II ubiquitylation ..... 31
1.3.6.3 RNA pol II sumoylation ..... 32

**2 ARTICLE #1 ..... 36**

**2.1 Authors' Contributions ..... 36**

**2.2 Abstract ..... 37**

**2.3 Background ..... 38**

**2.4 Methods ..... 41**
2.4.1 Strains, media and plasmids ..... 41
2.4.2 Spot test analysis ..... 41
2.4.3 Extraction of chromatin-associated proteins ..... 41
2.4.4 Co-Immunoprecipitation experiments ..... 42
2.4.5 GST and GST-CTD purification ..... 42
2.4.6 Purification of Rpb1-TAP ..... 43
2.4.7 Western blot analysis of GST, GST-CTD and Rpb1-TAP ..... 43
2.4.8 Interaction between Rrd1-MYC and GST-CTD ..... 44
2.4.9 Circular dichroism spectroscopy ..... 44
2.4.10 Limited chymotrypsin digestion assay ..... 45
2.4.11 *In vitro* isomerase assay ..... 45
2.4.12 *In Vitro* Rpb1 release assay ..... 45
2.4.13 ChIP assay ..... 46

**2.5 Results ..... 47**
2.5.1 Rrd1 is associated with the chromatin and interacts with Rpb1 ..... 47
2.5.2 Rrd1 associates with the CTD of Rpb1 and alters its structure in response to rapamycin ..... 50
2.5.3 Rrd1 alters the GST-CTD structure in response to 4-NQO, but not MMS ..... 56
2.5.4 Rrd1 directly alters the structure of the CTD in vitro ..... 57
2.5.5 Comparison of RNA pol II occupancy at rapamycin-responsive genes ..... 59
2.5.6 Purified Rrd1 stimulates the release of chromatin-bound RNA pol II in vitro ..... 60

**2.6 Discussion ..... 62**

**2.7 Conclusion ..... 66**

**2.8 Acknowledgments ..... 67**

**2.9 References ..... 68**

**3 ARTICLE #2 ..... 73**

**3.1 Authors' contributions ..... 73**

**3.2 Abstract ..... 74**

**3.3 Introduction ..... 75**

**3.4 Materials and methods ..... 77**
3.4.1 Strains, media, plasmids and antibodies ..... 77
3.4.2 Analysis of degradation of Rpb1 from whole cell extracts ..... 77
3.4.3 Co-immunoprecipitation ..... 78
3.4.4 Spot test analysis ..... 78
3.4.5 Extraction of chromatin-associated proteins ..... 78
3.4.6 In vitro chromatin assay ..... 79

**3.5 Results and discussion ..... 80**
3.5.1 Rrd1 is required for efficient RNAPII degradation in response to rapamycin ..... 80
3.5.2 Rapamycin induces degradation of Rpb1 independent of ubiquitylation ..... 85

**3.6 Acknowledgment ..... 90**

**3.7 References ..... 91**

**4 ARTICLE #3 ..... 95**

**4.1 Authors' contribution ..... 95**

**4.2 Abstract ..... 96**

**4.3 Introduction ..... 97**

**4.4 Material and methods ..... 99**
4.4.1 Cells, culture conditions and siRNA transfection ..... 99
4.4.2 Western blotting ..... 99
4.4.3 Colony formation assays. ..... 100
4.4.4 Chromatin assay ..... 100
4.4.5 Isolation of RNA and RT-PCR ..... 101
4.4.6 Mitochondrial fractionation ..... 102
4.4.7 Cell cycle analysis ..... 102
4.4.8 Purification of PTPA-associated proteins and co-immunoprecipitation ..... 102
4.4.9 Plasmids ..... 103
4.4.10 Generation of knockout mice and genotyping ..... 103

**4.5 Results ..... 105**
4.5.1 Reduction of PTPA by siRNA knockdown in HCT116 cells ..... 105

4.5.2 PTPA siRNA diminishes colony formation ..... 106
4.5.3 RNA pol II accumulates on chromatin after PTPA knockdown ..... 107
4.5.4 The growth defect in PTPA knockdown cells is p53-independent ..... 109
4.5.5 PTPA knockdown cells accumulate in G1 ..... 111
4.5.6 PTPA shRNA knockdown cells do not exhibit an *rrd1*Δ-like phenotype ..... 112
4.5.7 PTPA does not stably interact with other proteins and shows no evidence of post-translational modifications ..... 115
4.5.8 Distribution of PTPA in the mouse organs ..... 120
4.5.9 Attempt to generate a PTPA knockout mouse ..... 120

**4.6 Discussion ..... 123**

**4.7 References ..... 127**

**5 DISCUSSION ..... 130**

**5.1 Yeast Rrd1 ..... 130**
5.1.1 Rrd1 and RNA pol II ..... 130
5.1.2 RNA pol II loss ..... 134
5.1.3 Rrd1 and TOR ..... 135
5.1.4 Model ..... 135

**5.2 PTPA in mammals ..... 138**
5.2.1 PTPA knockdown ..... 138
5.2.2 PTPA substrates ..... 139
5.2.3 PTPA in mice ..... 140

**5.3 Conclusion ..... 141**

**REFERENCES ..... 143**

## LIST OF FIGURES

### Chapter 1

Figure 1.1: Multiple sequence alignment between PTPA proteins ..... 12
Figure 1.2: PP2A holoenzyme composition ..... 15
Figure 1.3: Peptide bond preceding a proline adopts the *cis* or *trans* conformation ... 19
Figure 1.4: Possible modifications of the CTD ..... 27

### Chapter 2

Figure 2.1: Rrd1 is associated with the chromatin and interacts with Rpb1. ..... 48
Figure 2.2: Analysis of the GST-CTD and its interaction with Rrd1 ..... 51
Figure 2.3: *rrd1*Δ mutants are unable to induce conformational changes to the GST-CTD in response to rapamycin ..... 55
Figure 2.4: 4-NQO, but not MMS, induces structural changes onto the GST-CTD ... 57
Figure 2.5: Purified recombinant Rrd1 alters the structure of purified GST-CTD *in vitro* ..... 58
Figure 2.6: Comparison of RNA pol II occupancy at the indicated target genes in the parent and *rrd1*Δ mutant strain in response to rapamycin treatment. ..... 60
Figure 2.7: Purified recombinant Rrd1 dissociates Rpb1 from the chromatin *in vitro* ..... 61

### Chapter 3

Figure 3.1: *rrd1*Δ or *tor1-1* mutants are unable to efficiently degrade RNAPII in response to rapamycin ..... 81
Figure 3.2: Rrd1 is required for the release of RNAPII from chromatin. ..... 84
Figure 3.3: Ubiquitylation of RNAPII is not required for its response to rapamycin. ..... 87

**Chapter 4**

Figure 4.1: Reduction of PTPA by siRNA knockdown in HCT116 cells. ..... 106
Figure 4.2: PTPA siRNA diminishes colony formation. ..... 107
Figure 4.3: RNA pol II accumulation in PTPA knockdown HCT116 cells. ..... 108
Figure 4.4: p53-independent cell death in PTPA knockdown cells. ..... 110
Figure 4.5: PTPA knockdown cells accumulate in G1 phase. ..... 112
Figure 4.6: PTPA knockdown cells by shRNA does not lead to sensitivity to 4-NQO, MMS or $H_2O_2$ or resistance to rapamycin. ..... 113
Figure 4.7: PTPA knockdown in various cell lines. ..... 114
Figure 4.8: FLAG-HA-hPTPA overexpression in HeLa cells. ..... 116
Figure 4.9: PTPA protein does not stably interact with any protein. ..... 118
Figure 4.10: PTPA do not interact with the β subunit of the CCT protein. ..... 119
Figure 4.11: PTPA protein distribution in mouse organs. ..... 120
Figure 4.12: No germline transmission after mouse genotyping. ..... 122

**Chapter 5**

Figure 5.1: Proposed model of Rrd1 activity on RNA pol II ..... 137

## LIST OF ABBREVIATIONS

**4EBP1:** 4E binding protein 1

**4-NQO:** 4-nitroquinoline 1-oxide

**6-AU:** 6-azauracil

**6-4PP:** 6-4 photoproduct

**AP:** Apurinic /apyrimidinic

**ATP:** Adenosine triphosphate

**Avo1/2/3:** Adheres voraciously 1/2/3

**BER:** Base excision repair

**$Ca^{2+}$:** Calcium

**CD:** Circular dichroism

**CDK:** Cyclin-dependent kinase

**ChIP:** Chromatin immunoprecipitation

**CPD:** Cyclobutane pyrimidine dimers

**CPSF:** Cleavage and polyadenylation specific factor

**CSA/B:** Cocayne syndrome factor A/B

**CTD:** C-terminal domain

**Cyps:** Clyclophilins

**DNA:** desoxyribonucleic acid

**DSB:** double-strand break

**DSIF:** DRB sensitivity-inducing factor

**ELL:** eleven–nineteen lysine-rich leukemia

**EGF:** Epidermal growth factor

**FDA:** Food and drug administration

**FKBP:** FK-506 binding protein

**GEF:** General elongation factor

**GFP:** Green fluorescent protein

**GG-NER:** Global genome NER

**GST:** Glutathione S-transferase

**GTF:** General transcription factor

**GTP:** Guanine triphosphate

**HAT:** Histone acetyltransferase

**HDAC:** Histone deacetylase

**HR:** Homologous recombination

**Inr:** Initiator element

**KDa:** Kilodalton

**Kog1:** Kontroller of growth 1

**LCMT1:** Leucine carboxyl methytransferase 1

**MAPK:** Mitogen-activated protein kinase

**$Mg^{2+}$:** Magnesium

**miRNA:** micro RNA

**MMS:** Methyl-methane sulfonate

**MMR:** Mismatch repair

**mRNA:** messenger RNA

**Msn2/4:** multicopy suppressor of SNF1 mutation 2/4

**NaAs:** Sodium arsenite

**NELF:** Negative elongation factor

**NER:** Nucleotide excision repair

**NHEJ:** Non-homologous end-joining

**NMR:** Nuclear magnetic resonance

**Npr1:** Nitrogen permease reactivator 1

**Pcf1:** Polyadenylation cleavage factor 1

**PDK1:** Phosphoinositide-dependent kinase 1

**PI3K:** Phosphatidylinositol 3-kinase

**PIC:** Pre-initiation complex

**PKC:** Protein kinase C

**PME-1:** PP2A-specific methylesterase 1

**PP2A:** Protein phosphatase 2A

**$PP2A_A$:** PP2A scaffolding subunit

**$PP2A_B$:** PP2A regulatory subunit

**$PP2A_C$:** PP2A catalytic subunit

**$PP2A_D$:** PP2A core dimer

**PPIase:** Peptidyl prolyl isomerase

**PSF:** Protein-associated splicing factor

**P-TEFb:** Positive transcription-elongation factor b

**PTPA:** PP2A phosphatase activator

**Rheb:** Ras homologous enriched in brain

**Rictor:** Rapamycin-insensitive companion of TOR

**Rtg1/2/3:** Retrograde regulation 1/2/3

**RNA:** ribonucleic acid

**RNAi:** RNA interference

**RNA pol II:** RNA polymerase II

**ROS:** Reactive oxygen species

**Rrd1/2:** Rapamycin resistant deletion 1/2

**rRNA**: Ribosomal RNA

**Ser/Thr:** Serine/Threonine

**SSD:** Single-strand damage

**shRNA:** Small hairpin RNA

**siRNA:** Small interfering RNA

**snRNA:** Small nuclear RNA

**snoRNA:** Small nucleolar RNA

**SUMO:** Small ubiquitin-like modifier

**Swi/SNF:** Switch/Sucrose nonfermentable

**Tap42:** Type 2A associated protein-42kDa

**Tip:** Tap42-interacting protein

**TBP:** TATA box binding protein

**TC-NER:** Transcription-coupled NER

**TOR:** Target of rapamycin

**tRNA:** Transfer RNA

**TSC:** Tuberous sclerosis

**UV:** ultraviolet

**YY1:** Yin yang 1

## ACKNOWLEDGMENTS

First and foremost, I would like to thank my director; Dr Dindial Ramotar for giving me an opportunity to be a part of his dynamic team and also for providing me with the resources and encouragement throughout my project. I learned more than I expected and this knowledge will always follow me.

I am especially grateful to Dr Jim Daley for his support throughout my Ph.D. program and great help with this thesis. Thank you for transmitting me your passion for science; your advices will always be an immense inspiration in my life. Your silliness made me feel more ″comfortable″ in the lab. All those memorable moments will always remain in my memory. A heartfelt ‘’Thank you’’ to the international Dr Jeremie Poschmann for those stimulating discussions as well as for your ideas, legendary smell and precious friendship.

I would like to thank Dr Julie Douville and Anick Leduc for teaching me the basics of science and enlightening me with the first glance of research. I also want to express my thank you to all the present and past members of the lab for creating a dynamic work atmosphere. Also, I wish to thank my lab-neighbours Dr Jasmine Lefebvre and Dr Marie-France Lusignan for those great discussions over the lunches and their friendly support. Thank you to Dr Julie Ross and Charles-Étienne Lebert-Ghali for the productive exchange of dialogues and great help with the mice work.

My sincere thanks to Dr Elliot Drobetsky for his support during my stay at the research centre. Thank you for those animated discussions and for taking time off your busy schedule to write me all those reference letters. Thank you to Dr Bachir El

Affar and Helen Yu for their expertise and help with the protein purification project. I would like to express my special thanks to the members of the animal facility department for their patience while teaching me all the basics of working with mice.

Thank you to my jury members: Dr Martine Raymond, Dr Janos Filep, Dr Jacques Côté and Dr Pierre Belhumeur for reading and analyzing this thesis and for their valuable comments and constructive criticism.

In a more personal way, thank you to my mother Lorraine and her boyfriend Yves for their patience, support and great dinners. Thank you to my father Denis for moral support as well as my brother David for being so silly. A note of thanks to the rest of my family especially my grandfather who has taught me to always look ahead in life.

Finally, a special thank you to Sonu for sharing my ups and downs by being a part of my life every single day. I also want to thank all my close friends for being supportive and loyal. A coffee, a dinner or a discussion was always helpful in those difficult moments. I would like to say that I am very fortunate to know that I can always rely on you and that your friendship means a lot to me.

Last but not the least my gratitude is also extended to the molecular biology program (University of Montreal) for supporting my work through several scholarships.

# CHAPTER 1

## Litterature review: Transcription, cell growth and Rrd1/PTPA

# 1 INTRODUCTION

According to the Canadian Cancer Society, cancer represents the leading cause of death in Canada since 2007. Studying cell metabolism is crucial both for a better understanding of the disease and for generating possible treatments. Cancer is a very complex disease and many factors can contribute to its development. Lifestyle, environmental events and heredity are all possible causes for cancer although it is often a combination of multiple factors. In order to circumvent these events, cells have developed a plethora of mechanisms to ensure regulated proliferation and growth and to maintain genomic integrity.

Deregulation of two classes of genes, named oncogenes and tumor suppressor genes, can lead to the onset of cancer. Inappropriate upregulation of oncogenes induces unregulated cell proliferation, leading to cancer. For example, activating mutations of the proto-oncogene Ras, a gene normally quiescent, are found in about 20% of all tumours and lead to uncontrolled growth of the cells [1, 2]. Current drugs used in cancer treatment target these genes or their products [3, 4]. Tumor suppressor genes function in pathways that protect against mutation or unregulated growth. Inactivating mutations in these genes can lead to tumorigenesis. Examples of tumor suppressors include genes involved in DNA repair, apoptosis, and transcription factors activated by cellular stress that induce cell cycle arrest in order to ensure DNA integrity [5]. Half of all cancers involve alteration of the important and best described tumor suppressor gene p53 [6]. Mutations in oncogenes and tumor suppressor genes arise when DNA damage is not repaired correctly, leading to irreversible mutations that alter protein function and regulation. Two categories of sources can cause DNA

damage: exogenous and endogenous. Exogenous DNA damaging agents include ultraviolet (UV) light from the sun, radiation such as x-rays or γ-rays, viruses, toxins or chemicals, and the main endogenous source is reactive oxygen species (ROS) [7, 8]. Cells have developed specific mechanisms to repair each type of DNA lesion in order to prevent carcinogenesis. Single-strand damage (SSD) is characterized by damage on only one strand of the DNA double helix [9]. Excision repair mechanisms use the intact strand as a template to repair the defective one. In base excision repair (BER), a damaged base is removed by a DNA glycosylase, resulting in an apurinic/apyrimidinic (AP)-site that is cleaved by an AP-endonuclease. Synthesis of the DNA is performed by a DNA polymerase and a DNA ligase seals the nick to complete repair [10]. Nucleotide excision repair (NER) repairs lesions caused by ultraviolet light and can be divided into global genome NER (GG-NER) or transcription-coupled repair (TC-NER). These two pathways differ in how the lesion is recognized, but share the later steps in which the lesion is excised and the resulting gap is filled [11]. Finally, mismatch repair (MMR) recognizes erroneous insertion, deletion or mis-incorporation of bases and repairs the wrong nucleotides with the correct ones [12].

Double-strand breaks (DSBs) are lesions of both strands of the DNA and can be repaired using either non-homologous end-joining (NHEJ) or homologous recombination (HR) [13]. In NHEJ, cells directly rejoin both ends of the break. This pathway is mostly used before DNA replication, when a sister chromatid is not available to serve as a template [14, 15]. On the other hand, HR uses the identical sequence from replication as a template to accurately repair the break.

Better understanding of proteins involved in the oxidative stress response is important for prevention of cancer and other diseases. Oxidative stress is caused by an imbalance between antioxidants and ROS such as free radicals or peroxides. Free radicals are unstable molecules with an unpaired electron that will rapidly react with proteins, lipids or DNA. Several types of DNA damage result from ROS including oxidized bases and strand breaks [7]. A main objective of our lab was to discover new genes important in the oxidative stress response. This was investigated in the budding yeast *Saccharomyces cerevisiae*, since this eukaryotic cell is genetically easy to manipulate. Homology between key proteins from yeast and mammalian cells also strengthens the notion of using yeast as model [16].

A yeast screen revealed that cells with a mutation in the *RRD1* gene were hypersensitive to 4-nitroquinoline 1-oxide (4-NQO) but resistant to ultraviolet C (UVC). 4-NQO causes a variety of DNA damage such as bulky adducts and oxidative stress. The metabolic activated form 4-hydroxyaminoquinoline interacts with DNA to form stable quinoline-purine monoadducts repaired by NER [17-19]. UVC (280-100 nm) engender bulky adducts on DNA [20] also repaired by NER [21]. Further characterization of the *rrd1* deletion in different genetic backgrounds showed the same phenotype [22]. *rrd1Δ* mutants are sensitive to 4-NQO [22, 23], vanadate, cycloheximide, ketoconazole and high concentration of $Ca^{2+}$, they are resistant to caffeine [24] and show defect in cell cycle progression and morphology [25]. Surprisingly, *rrd1Δ* mutant cells were later characterized to be resistant to rapamycin, a drug that inhibits the Target of rapamycin (TOR) signalling pathway (discussed in detail below) [24]. Our lab became interested in understanding *RRD1* function in the

cell since it seemed important for both genomic integrity and cell growth. The first chapter of my thesis will review literature on *RRD1* and related topics important for understanding its function in the cell.

## 1.1 RAPAMYCIN AND TOR

In *Saccharomyces cerevisiae*, *rrd1Δ* mutants are resistant to rapamycin, hence the gene name **r**apamycin **r**esistant **d**eletion **1** [24]. Rapamycin treatment is known to mimic starvation conditions in yeast by inhibiting the master kinase Target of Rapamycin (TOR). Genome array studies have revealed that expression of multiple genes is altered by rapamycin treatment, including repression of ribosomal genes and activation of nitrogen source utilization genes [26].

### 1.1.1 Rapamycin

Rapamycin is a bacterial product found on Easter Island that interacts with the isomerase FKPB12 (Fpr1 in *S. cerevisiae*) to inhibit TOR [27]. Rapamycin is widely used in transplant therapy since it inhibits the proliferation of T cells. Rapamycin has several effects on the immune system such as inhibiting type I interferon production in plasmacytoid dentritic cells [28], modulation of T cell trafficking [29] and regulating Foxp3 expression in regulatory T cells [30]. On the other hand, recent studies have snown that rapamycin can increase the generation of $CD8^+$ memory T cells [31-33]. Late administration of rapamycin can increase lifespan in mice [34]. Understanding the exact mechanism of TOR inactivation in T cells represents the next challenge of future work.

Rapamycin has also been used as an anticancer treatment acting as a cytostatic agent on several cancer cell lines. It can also sensitize cells to apoptosis when treated in combination with other chemotherapeutics agents [35-37]. In past years, analogs of rapamycin have been synthesized to circumvent the poor water solubility and low bioavailability of rapamycin [38]. New molecules such as temsirolimus and everolimus have recently been approved by the Food and Drug Administration (FDA) to treat advanced/metastatic renal cell carcinoma [39, 40].

### 1.1.2 TOR

The target of rapamycin (TOR) is a conserved Ser/Thr protein kinase which represents the catalytic activity of two complexes in the cell, TOR complex 1 (TORC1) and TOR complex 2 (TORC2) [41, 42]. The two complexes are formed of distinct and shared proteins and are responsible for regulating cellular processes in response to the environment. In mammalian cells, one *TOR* gene is present whereas in yeast Tor1 and Tor2 form TORC1 and TORC2 respectively [42, 43]. In mammalian cells, acute treatment with rapamycin inhibits mTORC1 only [27] through inhibition of its interaction with Raptor (regulatory associated protein of mTOR) [44]. In sustained treatment, both mTORC1 and mTORC2 are inhibited.

### 1.1.3 Yeast *TOR*

In yeast, TOR is a large protein present in two distinctive complexes. The TOR complex 1 (TORC1) includes Tor1 or Tor2, the scaffolding protein kontroller of growth 1 (Kog1) and the nutrient sensitive permease sorting factor Lst8 [45, 46]. On

the other hand, the TOR complex 2 (TORC2) includes Tor2, Avo1 (adheres voraciously), Avo2, Avo3 and Lst8 [47].

The TOR signalling pathway responds to nutrients such as carbon or nitrogen in order to promote cell growth [48]. TOR regulates gene expression depending on the availability of the nitrogen source. For example, in the presence of poor nitrogen sources such as urea or proline, a subset of genes involved in processing these sources are upregulated following inactivation of TOR [49]. Treatments with rapamycin or inhibition of TOR proteins results in reduced ribosome biogenesis, upregulation of autophagy, transcriptional modifications and increased mRNA turnover [45]. TORC2 regulates the cell-cycle-dependent polarization of the actin cytoskeleton, and this function of TORC2 is rapamycin-insensitive [50].

Both yeast Tor1 and Tor2 upregulate protein synthesis through activation of the translation initiation factor eIF4E as well as through transcriptional activation of ribosomal proteins. Inhibition of autophagy mediated by TOR phosphorylation of Apg1 is a mechanism to promote protein stability [51]. TOR also controls protein ubiquitylation by keeping nitrogen permease reactivator 1 (Npr1) in an inactive form [52]. The function of Npr1 is to stabilize plasma membrane amino acid transporters such as Bap2 [53], Mep2 [54], Tat2 [52] or Gap1 [55] against ubiquitylation-dependent degradation. Finally, TOR is involved in regulating transcription through the inhibition of starvation specific genes. For example, TOR phosphorylates the GATA-type transcription factor Gln3 in order to maintain it in the cytoplasm. When TOR is inhibited, during rapamycin treatment or starvation conditions, unphosphorylated Gln3 translocates to the nucleus and activates the transcription of

genes involved in the metabolism of secondary nitrogen sources [26, 56]. TOR also inhibits general stress transcription factors by keeping them in the cytoplasm, such as the multicopy suppressor of SNF1 mutation 2-4 (Msn2 and Msn4) [57] and the heterodimeric retrograde regulation 1-3 (Rtg1-Rtg3) [58].

TOR is important for the rapid regulation of a variety of protein phosphatase 2A (PP2A) complexes. The type 2A associated protein-42kDa (Tap42) binds to Sit4 phosphatase, a PP2A-related catalytic subunit in order to inhibit the dephosphorylation of both Npr1 and Gln3. The interaction between Tap42 and Sit4 is promoted by TOR phosphorylation of Tap42-interacting protein (Tip41). Treatment with rapamycin or inhibition of TOR leads to inactivation of Tip41, resulting in its binding to Tap42, which releases Sit4. Free Sit4 is then able to dephosphorylate transcription factors as well as Tip41, creating a fast response to stress conditions [59]. Our lab and others have shown that Rrd1 interacts with Sit4 phosphatase, and this interaction will be discussed in a later section [23, 60].

### 1.1.4 Mammalian TORC composition

In mammalian cells, both TOR complexes contain mTOR, mLST8/GβL and deptor [41, 42]. Deptor acts as an inhibitor of both mTORC1 and mTORC2 [61, 62], whereas mLST8/GβL binds to the mTOR kinase domain [63]. Raptor, a scaffold protein that links mTOR kinase to mTORC1 components [64], and PRAS40, an inhibitor or competitive substrate of mTORC1, are both specific to the mTORC1 complex. On the other hand, Rictor (rapamycin-insensitive companion of mTOR) and

mSin1, which are important for mTORC2 assembly and signaling and PRR5/protor, are part of the mTORC2 complex [65].

#### *1.1.4.1 mTORC1*

Anabolism is promoted through integration of both extra- and intracellular signals by the mTORC1. Nutrients and growth factors modulate mTORC1 activity in order to increase protein synthesis, cell growth, cell proliferation and cell metabolism [66]. Inactivation of mTORC1 leads to macroautophagy, or the degradation of cell proteins or organelles into amino acids or simple molecules [67].

Growth factors signal through phosphatidylinositol 3-kinase (PI3K) to activate phosphoinositide-dependent kinase 1 (PDK1), leading to phosphorylation of Akt and subsequent inhibitory phosphorylation of the Tuberous Sclerosis Complex (TSC) (including TSC1 and TSC2). TSC2 contains a GTPase-activating protein [31] and has been shown to stimulate the Ras homologue enriched in brain (Rheb). TSC1 stabilizes the TSC2 protein [68]. Inactivation of TSC complex allows the GTP-bound form of Rheb to interact and activate mTORC1 [69]. Amino acids, such as leucine, also activate mTORC1 through Rag proteins. These proteins bind to raptor and promote the interaction between Rheb and mTORC1 [70].

Finally, mTORC1 is important for ribosomal protein synthesis [71]. The ribosomal S6 kinase 1 (S6K1) can be phosphorylated on $Thr^{389}$ and the eukaryotic initiation factor 4E binding protein 1(4EBP1) can be phosphorylated on multiple sites ($Thr^{37/46}$, $Thr^{70}$ and $Ser^{65}$) by mTORC1 kinase activity to promote cell growth and

proliferation. mTOR activation is usually measured by assaying these downstream substrates [72].

#### *1.1.4.2 mTORC2*

The main substrate of mTORC2 is the serine/threonine protein kinase Akt, which promotes cell proliferation, survival and migration [73]. It is interesting that Akt regulation is both upstream (mTORC1) and downstream (mTORC2) of mTOR [41]. mTORC2 activates Akt through phosphorylation on serine 473 which allow the kinase to phosphorylate other substrate such as mTORC1 [74]. mTORC2 is also known for controlling actin cytoskeleton organization during cell growth through the activation of protein kinase C α (PKC-α) [75, 76].

### 1.1.5 Rrd1 and TOR

Rrd1 forms a complex with the TOR pathway members Tap42 and Sit4 [23, 60, 77]. Overexpression of Tap42 suppresses the rapamycin resistance seen in *rrd1Δ* mutant strains [77]. Moreover, both Sit4 and Rrd1 work in the same pathway to mediate resistance to oxidative stress induced by 4-nitroquinoline 1-oxide (4NQO) and UVA [23].

As mentioned previously, rapamycin also induces a reorganisation in the transcription profile of genes [56]. Our lab showed that *rrd1*Δ mutant cells fail to downregulate expression of genes encoding ribosomal proteins such as *RPS26A, RPL30* and *RPL9* when treated with rapamycin. Preliminary data also revealed that

RNA pol II was not degraded as efficiently as in a WT strain following rapamycin treatment [78]. So far, the exact mechanism by which Rrd1 deletion leads to rapamycin resistance remains unknown, and investigating this phenomenon is a main goal of this thesis.

## 1.2 RRD1/PTPA OVERVIEW

*RRD1* homologs exist in a variety of species such as *Xenopus laevis* [79], *Drosophila melanogaster,* and *Schizosaccharomyces pombe* [80]. It is highly conserved from yeast to human, showing 40% sequence identity with its human homolog PTPA (**p**hosphatase **t**wo A **p**hosphatase **a**ctivator). An alignment is shown in Figure 1.1.

*S. cerevisiae* also contains an *RRD1* homolog called *RRD2* showing 25% sequence identity. *rrd2Δ* mutant strains are also resistant to rapamycin and caffeine, but the phenotypes are weaker than *rrd1Δ* [24]. Deletion of both genes is lethal [24, 25], suggesting functional redundance and revealing their critical importance for the cell. Expression of the mammalian counterpart PTPA can rescue lethality in the *rrd1Δ rrd2Δ* double mutant [24, 81]. We chose to focus on Rrd1 because the *rrd2*Δ rapamycin phenotype is less severe, *rrd2*Δ cells are not sensitive to oxidative stress, and a mammalian homolog for *RRD2* has not yet been identified.

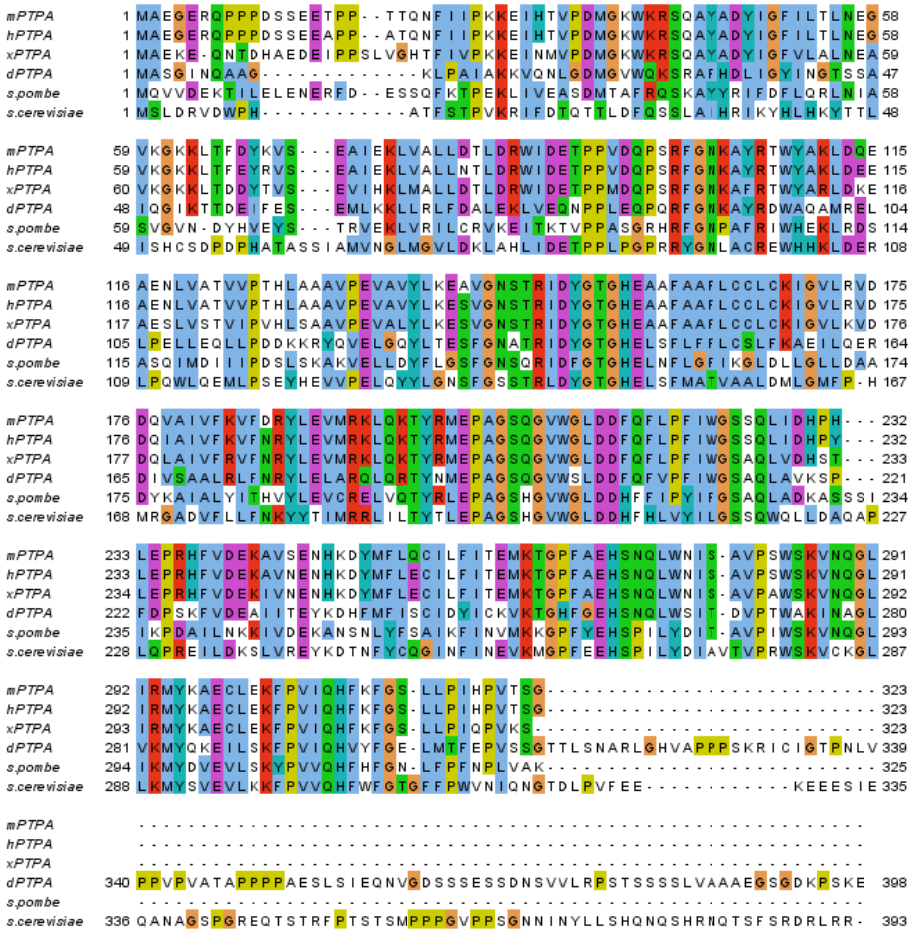


**Figure 1.1: Multiple sequence alignment between PTPA proteins**

Alignment was performed with CLUSTAL X2 [82] and represented with JALVIEW [83]. Proteins from *Mus musculus* (mPTPA), *Homo sapiens* (hPTPA), *Xenopus laevis* (xPTPA), *Drosophila melanogaster* (dPTPA), *Schizosaccharomyces pombe* (s.pombe) and *Saccharomyces cerevisiae* (s.cerevisiae) are presented.

### 1.2.1 PTPA

PTPA is encoded by a single gene on chromosome 9q34 in human (chromosome 2 in mouse) and consists of 10 exons and 9 introns [84]. The transcription factor yin yang 1 (YY1) is involved in the regulation of transcription of the PTPA gene [85]. PTPA also possesses multiple splicing sites resulting in seven distinctive products leading to the expression of 4 protein isoforms. However, only 2 of these proteins are detectable *in vivo* [86].

### 1.2.2 Rrd1/PTPA structure

Crystal structures of yeast Rrd1 and Rrd2 and human PTPA were solved using truncated peptides where non-structured regions were removed. Interestingly, the overall structures of all three proteins were very similar and were organized into an α-helical compact structure [87]. Comparison with known structures revealed no obvious similarity with any other previously analyzed proteins [88].

Mammalian truncated PTPA protein contains 17 α helices and 4 short β strands. The structure is organized in 3 main domains: the core, the lid and the linker. The core is linked to the lid, located at the C-terminus, by the linker forming a large cleft. The structure also revealed a deep pocket of conserved amino acid residues between the core domain and the linker possibly representing a protein interaction domain [88]. It was previously reported that the conserved region $_{200}$GVWGLD$_{205}$ is essential for the peptidyl prolyl isomerase activity (discussed in detail below) as well as activation of PP2A [80, 89].

### 1.2.3 PP2A

As indicated by its name, PTPA was first described as an activator of protein phophatase 2A (PP2A) in rabbit skeletal muscle and *Xenopus laevis* oocytes [79]. The weak phosphatase activity of PP2A could be stimulated by PTPA in an ATP and $Mg^{2+}$-dependent manner *in vitro* [90]. Mutational analysis has also shown that specific amino acids (V209D, E270A,V281D, G290D and M294D) are important for both the interaction between PP2A and PTPA and the ATPase activity of the complex [88]. The mechanism by which this occurs is still poorly understood [79, 90]. PP2A complexes are conserved serine/threonine phosphatases ubiquitously expressed in the cell and are important for the regulation of numerous signalling pathways [91]. Deregulation of PP2A is associated with cancer and Alzheimer's disease [92-95]. The PP2A heterodimeric complex (Figure 1.2) is formed of a core dimer ($PP2A_D$) containing a structural or scaffolding A subunit ($PP2A_A$) and a catalytic C subunit ($PP2A_C$), and each subunit can be found in 2 distinct isoforms ($\alpha$ or $\beta$). The core dimer can also associate with a regulatory B subunit ($PP2A_B$) to form the heterotrimeric holoenzyme. There are 4 structurally different families of $PP2A_B$ : B (PR55), B' (PR61), B'' (PR48, PR72 or PR130, G5PR) and B''' (PR93 or PR110) and each family consists of 2 to 5 different isoforms [96].

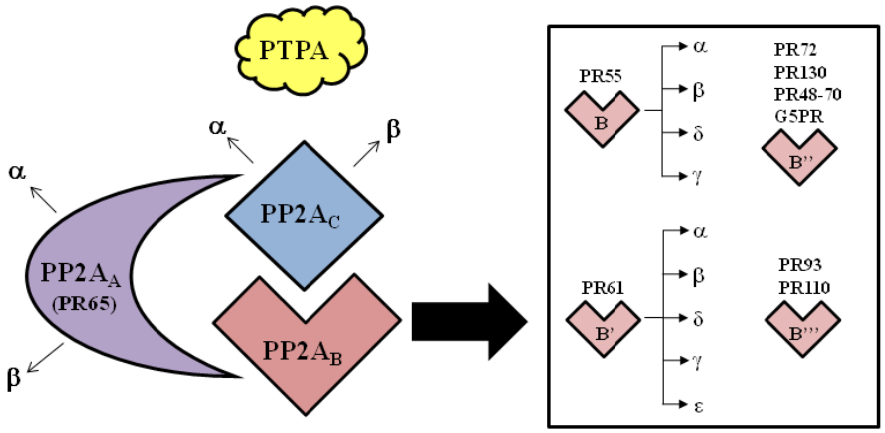


**Figure 1.2: PP2A holoenzyme composition**

The scaffolding subunit PP2A$_A$ is composed of 15 HEAT (huntington-elongation-A subunit-TOR) repeats, and the catalytic subunit PP2A$_C$ interacts with the conserved ridge of HEAT repeats 11-15 [97]. Mutations in this interaction site have been found in some tumors [98, 99]. The core dimer is the main target of the carcinogenic toxin okadaic acid [100] that inhibits PP2A by interacting with and blocking active site of the catalytic subunit [97].

The diversity of the regulatory subunit is important for substrate specificity and the localization of the holoenzyme in the cell [101, 102]. Each of the regulatory B subunits interacts differently with the core dimer, sometimes interacting with the scaffolding subunit, the catalytic subunit or both. The different combinations of these subunits give rise to over 70 different heterotrimeric holoenzymes displaying independent functions in the cell [91].

#### *1.2.3.1 Post-translational modifications*

Methylation of the PP2A holoenzyme is an essential mechanism of regulation [103-106]. Methylation of the carboxy-terminal Leu309 of the catalytic subunit ($PP2A_C$) is important for recognition by some of the regulatory subunits ($PP2A_B$) such as the regulatory subunit Bα [107, 108]. There is also evidence that inhibition of the methylation site in yeast leads to decreased formation of the holoenzyme [100]. Methylation is mediated by a conserved protein, a PP2A-specific leucine carboxyl methyltransferase (LCMT-1), and demethylation is catalysed by a PP2A-specific methylesterase (PME-1) [103, 109, 110]. Levels of this methyltransferase vary during the cell cycle, suggesting a role in cell-cycle regulation [91, 111]. The methylation status is critical for the differentiation of neuroblastoma cells and could possibly play an important role in Alzheimer disease [112, 113].

The catalytic subunit C of PP2A is also targeted for phosphorylation on Tyrosine 307 by tyrosine kinases such as $pp60^{v\text{-}src}$, $pp56^{lck}$, epidermal growth factor (EGF) and insulin receptors. Phosphorylation results in inactivation of the enzyme [114] and it has been associated with Alzheimer disease [115]. Phosphorylation of a regulatory B' subunit (B56α) by the serine/threonine kinase PKR [116] seems to be important for apoptosis. In short, phosphorylation of B56α on Serine 28 activates the phosphatase activity required for dephosphorylation of Bcl2 and inhibition of apoptosis [117, 118].

### 1.2.4 Rrd1/PTPA and PP2A

In yeast, Rrd1 was found to interact with the PP2A-like phosphatases Pph3 and Ppg1 [77]. It was also shown that Rrd1 and Pph3 act synergistically to induce rapamycin resistance in yeast [78]. The interaction with Pph3 was confirmed using affinity purification coupled with mass spectrometry analysis in mammalian cells [119].

Deletion of PTPA homologs in yeast results in accumulation of PP2A and PME-1 complex and decreased methylation of the catalytic subunit ($PP2A_C$) [120]. Also, inactive PP2A can be re-activated by PTPA in a Mg2+/ATP dependent manner *in vitro* [79], suggesting that PTPA inhibits the methyltransferase activity of PME-1 [110]. It remains unknown how PTPA performs this activation, but an attractive possibility is that it acts as a *cis/trans* peptidyl prolyl isomerase on Proline 190 close to the active site of the catalytic C subunit of PP2A and this function will be discussed later [87, 89].

Finally, a recent study suggested that depletion of PTPA with RNAi results in cell transformation caused by a defect in PP2A catalytic subunit C methylation. This defect altered the assembly of the catalytic subunit C with the scaffolding subunit A and suggests a novel role for PTPA as a tumor suppressor [121].

### 1.2.5 PTPA and apoptosis

To gain more insight into a possible function of PTPA, our laboratory previously monitored the biological response following transient overexpression of

PTPA labelled with the green fluorescent protein (GFP). The results showed cell death of the PTPA-overexpressing cells via p53-independent apoptosis in a time-dependant manner. This apoptosis was independent of the mitogen-activated protein kinase (MAPK). Surprisingly, inhibition of PP2A with okadaic acid did not prevent the PTPA overexpressing cells from dying through apoptosis. The exact mechanism leading to apoptosis remains unknown, but nonetheless these data reveal that a specific level of PTPA is required for normal homeostasis of the cells [122].

### 1.2.6 Rrd1/PTPA as a peptidyl prolyl isomerase (PPIase)

A key breakthrough was the discovery that PTPA and its yeast homolog, Rrd1, possesses peptidyl prolyl *cis*/*trans* isomerase (PPIase) activity. PPIases are enzymes that convert proline residues between their two distinct isoforms, *cis* or *trans* (Figure 1.3). PPIases are highly conserved from yeast to human [123]. These ubiquitous enzymes can be divided into four structurally different families: cyclophilins (Cyps), FK-506 binding proteins (FKBPs), parvulins and the Ser/Thr phosphatase 2A (PP2A) activator PTPA. The catalytic domain of PTPA is an α-helix fold whereas the other PPIases are characterized by a central β-sheet [87, 124, 125].

Typically, the peptide bond linking amino acid residues in a protein adopts the *trans* isoform since this is the less energetic conformation as compared to the *cis* conformation. Interestingly, proline residues are the only amino acids in which both conformations are relatively energetically equivalent [126]. The possibility of having two distinct structures (*cis* and *trans*) can act as a molecular switch, similar to other post-translational modifications, enabling proteins to perform different functions

within the cell (Figure 1.3). PPIases are known to be accelerating agents that regulate dynamic processes in the cell [127].

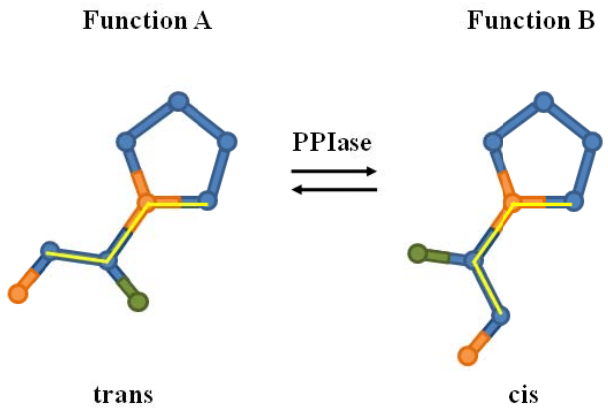


**Figure 1.3 : Peptide bond preceding a proline adopts the *cis* or *trans* conformation**

The Cyps and FKBPs have been widely studied since the immunosuppressant drug cyclosporine A uses the cyclophilins as a target [128] whereas the immunosuppressant and anti-cancer rapamycin targets FKBPs [129]. Interestingly, these drugs do not directly inhibit the isomerase activity of PPIases but instead prevent the formation of a ternary complex leading to dysfunction of a specific pathway. Moreover, these 2 families of PPIase are dispensable for viability in budding yeast since the disruption of each enzyme individually or all together is not lethal [130]. Analysis throughout the years has suggested that cyclophilins and FKBPs only perform non-essential and redundant roles as chaperones or in protein folding, and therefore interest in these molecules as therapeutic targets has decreased.

Another PPIase, Pin1, is a member of the parvulin family and has been found to play roles in a variety of cellular processes. Pin1 is the only enzyme that can isomerise a specific motif formed of phosphorylated Ser/Thr-Pro [131]. Pin1 *cis/trans* isomerisation is involved in the control of cell growth regulation, genotoxic stress, and the immune response. Deregulation of Pin1 has been linked to Alzheimer's disease, cancer and aging [132]. Interestingly, it has been shown that Pin1 binds to a pSer-Pro motif on the C-terminal domain (CTD) of the large subunit of RNA pol II and regulates the phosphorylation status of this domain through inhibition of the phosphatase FCP1 and stimulation of the kinase cdc2/cyclin B [133-135].

The PPIase activity of PTPA was discovered when PTPA was shown to isomerize synthetic PP2A catalytic subunit peptides *in vitro* [87, 89]. This led to a model in which isomerisation by PTPA could activate the phosphatase activity of PP2A. It is likely that PTPA and its yeast homolog Rrd1 isomerize other substrates, and identification of such substrates could explain its role in 4-NQO sensitivity and rapamycin resistance. Our lab previously showed that transcription of a subset of genes is deregulated in *rrd1*Δ cells [78]. This, along with the knowledge that Pin1 isomerizes the CTD of RNA pol II, led us to investigate whether the CTD is also an Rrd1 substrate. We found that isomerisation of the CTD by Rrd1 does indeed play a major role in transcription regulation, and these data will be presented in Chapter 2. To provide the necessary background, a review of the transcription mechanism in eukaryotic cells is presented in the next section.

## 1.3 TRANSCRIPTION

### 1.3.1 Overview

Transcription is the mechanism by which RNA is synthesized from the DNA template. During this process, RNA polymerase reads from one strand of the double-stranded DNA, called the template strand, whereas the other strand is termed the coding strand. The template strand is read in a 3' to 5' direction and the RNA is synthesized from 5' to 3', comparable to DNA replication. The resulting RNA is single-stranded and its sequence is identical to the coding strand except that uracil is substituted for thymine. Transcription can be divided into three major steps: initiation, elongation and termination. Since it leads to gene expression and is one of the most frequent events in a cell, it is highly regulated.

In eukaryotic cells, there are three different RNA polymerases, which each transcribe a specific type of RNA. Each RNA polymerase is composed of 4 to 14 polypeptides and requires the aid of distinct additional factors to perform its function. Ribosomal RNAs (rRNA) are synthesized by RNA pol I in the nucleus and are part of the 18S, 5.8S and 28S ribosomal subunits. These subunits are required for the assembly of the full ribosome, which is involved in the translation of messenger RNA (mRNA). Transcription of the rRNA represents around 60% of the transcription in a cell. Transfer RNA (tRNA) and the 5S subunit of rRNA are transcribed by RNA pol III and account for about 10% of the total transcription [136]. tRNAs are involved in transferring each amino acid to the polypeptide chain at the ribosome during translation, and 5S rRNA is another constituent of the ribosomal complex. Finally,

RNA pol II transcribes the mRNA in the nucleus. Most mRNAs code for genes and are translated into proteins. RNA pol II also transcribes non-coding small RNAs such as small nuclear RNA (snRNA), small nucleolar RNA (snoRNA) and micro RNA (miRNA) [137]. Small non-coding RNAs are involved directly in many different cellular pathways, although the specific function of the majority of them remains unknown [138]. My thesis will focus on the regulation of RNA pol II.

### 1.3.2 Transcription initiation

Initiation of transcription occurs at the core promoter, which is usually located upstream of the gene and contains specific sequences that recruit initiation factors. The TATA box, which is involved in about 30% of gene transcription, is found around 25 bases upstream of the transcription start site and possesses the consensus sequence TATAWAAR [139]. The TATA box is often associated with an initiator element (Inr) and both can act synergistically to activate transcription of abundantly expressed genes [140]. Another element called the downstream promoter element (DPE) activates transcription coupled to the Inr in TATA-less promoters [141]. All these markers are important to correctly direct the pre-initiation complex [142] to the site of transcription initiation. There are also *cis*-acting DNA sequence such as enhancers, silencers and insulators [143] and *trans*-acting elements such as the RNA pol II pre-initiation complex [142], transcription factors and chromatin remodeling proteins [144]. Transcription is highly regulated and these different elements can either activate or repress transcription.

Various transcription factors join RNA pol II at gene promoters in an organized order. General transcription factors (GTFs) that are necessary for initiation include TFIIA, TFIIB, TFIID, TFIIE, TFIIF and TFIIH. The formation of the PIC occurs by stepwise recruitment of the different GTFs to the promoter region and is initiated by TFIID binding to the core promoter. TFIID is a multi-subunit complex containing 14 different factors including TBP (TATA-binding protein) [145]. TBP is known to bind to this AT-rich region and unwind the DNA, forming a single-stranded "bubble" for the transcription machinery. TFIIB and TFIIA are then recruited and stabilize TFIID at the promoter region, followed by the recruitment of the RNA pol II complex already bound to TFIIF. At this point, transcription can only initiate when TFIIE and TFIIH join the complex. The ATP-dependant helicase activity of TFIIH melts the promoter, forming an "open" initiation complex and leading to the release of RNA pol II. This is called promoter clearance and represents the beginning of transcription elongation [146].

### 1.3.3 Transcription elongation

Elongation is a highly regulated and critical step that is mandatory for the correct organization and integrity of the genome. This step begins when promoter escape is complete, when the new RNA associates stably with the transcription complex. TFIIB is important to stabilize this association as well as to allow elongation initiation [147, 148]. In yeast, DRB sensitivity-inducing factor (DSIF), consisting of Spt4, Spt5, and negative elongation factor (NELF) facilitates RNA Pol II pausing in the promoter-proximal region, and TFIIS also associates with the paused

polymerase [149]. This pause is necessary for capping enzymes to bind to the C-terminal domain (CTD) (discussed in depth below) of Rpb1 phosphorylated on serine 5 and Spt5 and to allow nascent RNA capping [150, 151]. Positive transcription-elongation factor-b (P-TEFb) is involved in the phosphorylation of DSIF, NELF and serine 2 on the CTD leading to productive elongation [152]. TFIIF, eleven-nineteen lysine-rich in leukemia (ELL) and Elongin are the main factors that stimulate RNA pol II elongation and inhibit pausing [153]. TFIIF seems to be important for RNA pol II release from a stalled state [154]. Elongin is not active until the RNA transcript is 8-9 nucleotides long and until TFIIF leaves the complex [155]. Finally, the last component of transcription elongation is topoisomerase I, which allows unwinding of the DNA throughout the process [156].

Transcribing RNA pol II adopts three different states: a pretranslocation state where the nucleotide added to the RNA chain is still in the addition site, a posttranslocation state where the addition site becomes free and a backtracked state where RNA pol II performs a retrograde motion [157, 158]. RNA pol II backtracking of one residue is favorable whereas longer backtracking leads to a possible irreversible arrest [158] and this is detailed in a later section.

Nucleosomes protect the DNA by keeping it in a tightly closed form, and this process must be reversed to let RNA pol II access the gene to be transcribed. Several factors involved in remodeling the chromatin have been identified and will be discussed briefly here. The first category includes ATP-dependent chromatin remodelling complexes that use ATP hydrolysis to modify the chromatin structure [159, 160]. In yeast, the Swi/Snf (Switch/Sucrose nonfermentable) complex moves

nucleosomes around and can repress or activate transcription depending on the situation [161, 162]. Histones chaperones, such as FACT and Spt6, regulate intracellular histone dynamics, histone storage and replication-associated chromatin assembly [163, 164].

Acetylation of histones H3 and H4 is important during transcription elongation, and histone acetyltransferases (HATs) and deacetylases (HDACs) have been found in the coding region of multiple genes [165] although they are more commonly associated with the promoter and 5' region [166]. It has also been documented that histone methylation, mostly on H3, is important for progression of transcription [167]. One example is Set2, which interacts with active genes through its affinity for RNA pol II phosphorylated on both serine 2 and 5 of the CTD [168-170]. Finally, ubiquitylation of either histone H2B [171] or phosphorylation of histone H3 [172] are possible modification required for transcription elongation regulation.

### 1.3.4 Transcription termination

There are two proposed models for the termination of transcription: the torpedo model and the anti-terminator model. In the first model, cleavage of the polyadenylated site creates a new 5'-end, and exonuclease or helicase activity leads to the dissociation of RNA pol II [173]. The second model states that the appearance of the polyadenylation sequence on the RNA strand triggers a reorganisation in the binding factors, leading to a decrease in RNA pol II elongation [174]. Pausing of RNA pol II is also important for termination and occurs at the 3'-end of the gene, 10-

30 nucleotides downstream from the hexanucleotide AAUAAA. This specific sequence is recognized by the CPSF (cleavage and polyadenylation specific factor) group of proteins [175-178]. RNA pol II CTD regulation is also involved in 3'-end processing, but this will be discussed in a later section.

Recent research supports a combination of both models. As mentioned previously, the torpedo model involves the activity of the yeast 5'-3' exonuclease Rat1 (Xrn2 in mammals) in order to recruit the 3'-end processing factors, but this nuclease does not seem to be essential for cleavage at the poly(A) site [179, 180]. Xrn2 interacts with p54nrb and PSF (protein-associated splicing factor), which are involved in transcription, splicing and polyadenylation [181, 182]. Xrn2 action on the cleavage of the RNA product precedes the release of RNA pol II, as predicted by the second model [183].

### 1.3.5 RNA polymerase II structure

The RNA pol II holoenzyme is composed of 12 conserved subunits (termed Rpb1-12) resulting in a large complex of about 550 kDa [184]. All subunits are necessary for yeast cells to grow normally [185]. Three of the subunits are unique to RNA pol II: Rpb4, Rpb7 and Rpb9, whereas the rest are common to all RNA polymerases. The two major subunits Rpb1 and Rpb2 form the catalytic center and are homologous to subunits of the bacterial RNA polymerase [185]. Mutational analysis revealed that Rpb1 and Rpb2 are both required throughout the entire transcription process, from initiation to termination [186]. The largest subunit Rpb1 contains a unique C-terminal domain (CTD) containing a heptapeptide repeated 26-

27 times in yeast [187] and 52 times in mouse and human. This consensus sequence Tyr-Ser-Pro-Thr-Ser-Pro-Ser is highly conserved in eukaryotes and is essential in all organisms [188].

#### *1.3.5.1 RNA pol II C-terminal domain*

RNA pol II CTD regulation is critical for correct transcription of genes and occurs through direct or indirect interaction with different RNA processing factors. Through each step of transcription, the CTD is reversibly modified in a specific manner. These modifications include: 1) phosphorylation and dephosphorylation on tyrosine (Tyr1), threonine (Thr4) and all three serines (Ser2, 5 or 7) by kinases and phosphatases and 2) glycosylation of serines (Ser2, 5 or 7) and threonine (Thr4) by glycosyltransferase and deglycosylases. Research on Pin1 as well as mine has revealed a third category of CTD modification, isomerisation of the peptide bond of proline (Pro3 or 6) by peptidyl prolyl *cis/trans* isomerases (PPIases) activity. Therefore, the possibility to regulate the CTD through modifications of each amino acid leads to a vast possibility of combinations and regulates interacting partners [189]. This is summarized in the diagram below (Figure 1.4).

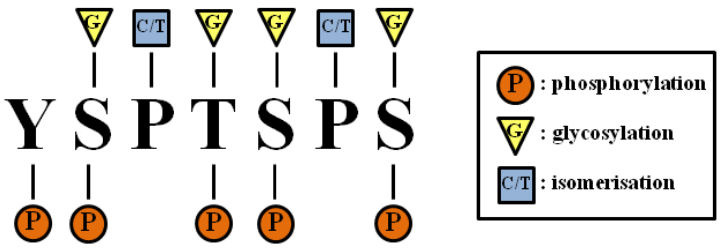


**Figure 1.4: Possible modifications of the CTD**

Rpb1 can be found in two main forms during transcription: IIa, the hypophosphorylated form and IIo, the hyperphosphorylated form. The IIa form is preferentially found at the pre-initiation site whereas the IIo form is found later in the elongation process where each repeat is phosphorylated at least once [190]. Chromatin immunoprecipitation (ChIP) analysis revealed that Ser5 phosphorylation by the cyclin-dependant kinase (Cdk)7 (Kin28 in yeast), was higher at the 5' region of a gene [191]. This phosphorylation is required to release the mediator complex from pol II, allowing recruitment of capping enzymes [192, 193].

Once the transcription machinery leaves the initiation site, Ser2 phosphorylation by Cdk9 (Ctk1 in yeast) is initiated and is important for binding of the 3'-RNA processing machinery. This phosphorylation is most abundant at the 3' end of the transcribed gene [194, 195]. Phosphatases such as SCP1 (Ssu72 in yeast) and Fcp1 dephosphorylate Ser5 and Ser2, respectively, and are required for the recycling of RNA pol II following transcription of a gene. Once RNA pol II reaches the polyadenylation signal, most of the Ser5 phosphorylation is gone and only Ser2 remains. In this state, pol II interacts with the polyadenylation cleavage factor (Pcf)1. Interaction of the CTD with the 3' processing factors is important for transcription termination. More recently, several studies have shown that Ser7 play a role in transcription of snRNA genes [196] as well as some protein coding genes, and that this phosphorylation site seems to be important for termination [197].

Throughout transcription, histone modifications are related to the phosphorylation status of the RNA pol II CTD. For example, the histone methyl-transferases Set1 and Set2 are recruited by phosphorylated Ser5 and Ser2/Ser5,

respectively [198]. Taken together, the phosphorylation status of the CTD of RNA pol II is a useful tool to monitor each step in the transcription of a gene and a better knowledge of this mechanism represents the challenge of future investigation. Determining the precise phosphorylation status of each heptapeptide will be an important future goal for the transcription field.

Each repeat of the RNA pol II CTD contains 2 possible prolines that could be isomerised. The mammalian Pin1 (Ess1 in yeast) PPIase isomerizes prolines preceded by phospho-serine or phosphor-threonine residues and inhibits Fcp1 phosphatase activity leading to inhibition of transcription [199]. It has been shown that Ess1 and Pin1 preferentially recognize both the Ser2 and Ser5 phosphorylated form of RNA pol II *in vitro* [200].

Finally, reversible addition of a monosaccharide *N*-acetylglucosamine (O-GlcNAc) has been found on serine and threonine residues, but it is still unknown how glycosylation regulates transcription [201]. Interestingly, both glycosylation and phosphorylation cannot be found simultaneously on the CTD [202].

### 1.3.6 RNA pol II arrest

Transcription elongation can be interrupted at any point and various consequences can result from this arrest. As mentioned above, programmed transcriptional pausing is important for factors to join the complex, but unscheduled pausing can become problematic and lead to transcriptional arrest. DNA compaction into chromatin, DNA-binding proteins and DNA lesions can all represent possible

obstacles to transcription elongation. The interaction between RNA pol II and the DNA is highly stable and a blockage in transcription leading to an arrest can be lethal. Consequently, cells have developed a plethora of mechanisms to counteract these events.

TFIIS is a general elongation factor (GEF) known to be important for releasing RNA pol II from transcriptional arrest. This arrest is recognized by the loss of contact between the 3'-end of the elongating RNA transcript and the RNA pol II active site following retrograde motion of RNA pol II. At this point, TFIIS re-established this link by endonucleolytically cleaving the RNA, known as cleavage-resynthesis [157]. Elongation factors are recruited and released dynamically throughout the elongation process and the association of RNA pol II with them is usually difficult to detect because of their transient nature. In the case of TFIIS, treatment with 6-azauracil (6AU), which decreases GTP and UTP intracellular levels and leads to an inhibition of elongation [203], can allow detection of TFIIS associated with RNA pol II by ChIP analysis [204].

#### *1.3.6.1 Transcription-coupled nucleotide excision repair (TC-NER)*

Transcription-coupled nucleotide excision repair (TC-NER) is an efficient mechanism to recognize transcription-blocking lesions on DNA and ultimately repair them. TC-NER is able to efficiently remove the two main photolesions induced by UV-C: cyclobutane pyrimidine dimers (CPD) and 6-4 photoproducts (6-4PP) [205]. The interaction between the elongating RNA pol II and the Cockayne's syndrome B (CSB) protein (Rad26 in yeast) becomes more stable when the complex is blocked at

a lesion [206]. CSB allows the interaction of CSA with DNA which in turn recruits XPA-binding protein 2 (XAB2), the high-mobility-group nucleosomal binding protein (HMGN1) and TFIIS [207]. Other factors, such as SWI/SNF histone acetyl transferase and p300/CBP, modify the chromatin around the lesion to allow access to the repair machinery [208]. Defects in TC-NER result in Cockayne syndrome characterised by growth failure, impaired development of the nervous system, photosensitivity and premature aging [209]. It was shown that when the TC-NER complex is unable to repair the damage, Def1 recruits the ubiquitylation machinery and RNA pol II is degraded to allow repair of the lesion by global genomic repair (GGR), a separate branch of NER that is independent of transcription [210].

#### *1.3.6.2 RNA pol II ubiquitylation*

When RNA pol II becomes irreversibly stalled during transcription, as occurs in Cockayne syndrome patients, its removal from chromatin is the only option. The main mechanism for displacement of RNA pol II is ubiquitylation, leading to proteasomal degradation of the protein. Ubiquitin is a highly conserved (96% identity between yeast and human) protein of 76 amino acids found in all eukaryotes. Ubiquitylation is a protein post-translational modification process where ubiquitin is covalently linked to a lysine residue of the targeted protein. Ubiquitin is first activated by an E1 ubiquitin-activating enzyme using ATP, and is then transferred to an E2 ubiquitin-conjugating enzyme. The E2 enzyme transiently carries the activated ubiquitin to an E3 ubiquitin ligase. The E3 transfers the ubiquitin from the E2 to the lysine of a specific substrate. The addition of multiple ubiquitins onto a substrate,

called polyubiquitylation, directs this substrate for degradation by the proteasome [142, 211].

Rpb1 can be ubiquitylated on only two lysines: K330 and K695 [212]. In yeast, the only known E1 is Uba1 and the E2 can be either Ubc5 or Ubc4 [213]. Rsp5 (NEDD4 in mammals) is the E3 required for monoubiquitylation of Rpb1, whereas Elc1 is required for polyubiquitylation in response to DNA damage [214, 215]. The Rpb1 CTD is required for ubiquitylation, but it is unknown how the protein is recognized [213]. Finally, it has been shown that ubiquitylation of RNA pol II is also present without DNA damage, indicating that Rpb1 degradation occurs during normal, unperturbed transcription. The 19S regulatory particle of the proteasome is required for active transcription, supporting this idea [216].

#### *1.3.6.3 RNA pol II sumoylation*

It has been shown that RNA pol II elongation arrest following DNA damage leads to ubiquitylation-dependant degradation of the protein in order for TC-NER to take place [217]. RNA pol II degradation is still effective in CS cells, however, in which the ubiquitylation pathway is altered, indicating that RNA pol II can be degraded by a separate ubiquitin-independent mechanism [218]. Recent findings have shown that sumoylation of Rpb1 following UV radiation is necessary for this degradation [219]. Thus, sumoylation may be involved in Rpb1 degradation in some circumstances. In yeast, the small ubiquitin-like modifier (SUMO) protein works in a three step enzymatic reaction similar to ubiquitin.

Based on the literature, we hypothesized that Rrd1 plays a role in regulating RNA pol II transcription through its peptidyl prolyl *cis*/*trans* isomerase activity and that this mechanism is conserved in mammals. The first objective is to determine whether Rrd1 localizes on the chromatin and interacts with RNA pol II. The second objective is to investigate the role of Rrd1 in the CTD isomerisation in response to rapamycin both *in vivo* and *in vitro*. Finally, the last objective is to understand the mechanism of RNA pol II degradation following rapamycin treatment. A better understanding of its role in transcription could explain the 4-NQO sensitivity as well as the rapamycin resistance of cells lacking Rrd1. The following two chapters (Chapter 2 and 3) of my thesis focus on papers published on a role for Rrd1 as a regulator of RNA pol II. The main finding of this work is that Rrd1 is associated with the chromatin and interacts with RNA polymerase II. *In vitro* and *in vivo* analysis with circular dichroism revealed that Rrd1 mediates structural changes of the C-terminal domain of the large subunit of RNA pol II, Rpb1, in response to rapamycin and 4-NQO. Consistently, we demonstrated that Rrd1 is required to alter RNA pol II occupancy on rapamycin responsive genes. We also showed that upon rapamycin exposure Rrd1 mediates the degradation of RNA polymerase II and that this mechanism is ubiquitin-independent.

We also hypothesised that Rrd1 function is conserved in mammalian cells. We first investigated PTPA function by performing its knockdown in mammalian cells using RNA interference (RNAi). The second objective of this work is to identify proteins interacting with PTPA. Finally, the last objective is to analyse the effects of PTPA knockout in mice. Chapter 4 presents results where PTPA knockdown did not

affect sensitivity to rapamycin, 4-NQO or $H_2O_2$. We also tried to find protein interaction partners for PTPA using tandem affinity purification, but no stable partners for PTPA were found. Finally, we attempted to study PTPA in a mouse model. We first determined that PTPA was expressed in a tissue-specific manner and was most abundant in the bone marrow, thymus and brain. We pursued creation of a knockout mouse and successfully generated chimeras, but the mutated allele was not transmitted to the germline.

# CHAPTER 2

## Article 1

## Rrd1 isomerizes RNA polymerase II in response to rapamycin

**Nathalie Jouvet*, Jeremie Poschmann*, Julie Douville, Lisa Bulet**

**and Dindial Ramotar**

* authors contributed equally

Article published in *BMC Molecular Biology* (2010); 11: 92

# 2 ARTICLE #1

## 2.1 AUTHORS' CONTRIBUTIONS

**NJ** carried out chromatin extraction, co-immunoprecipitation, Western blot analysis, protein purification and CD analysis, and drafted the manuscript. JP carried out Western blot analysis, genetic analysis, ChIP analysis, *in vitro* chromatin assay and drafted the manuscript. JD carried out chromatin extraction, co-immunoprecipitation, Western blot analysis, protein purification and CD analysis, and drafted the first version of the manuscript. LB carried out the GST-CTD and Rrd1-MYC pull-down. DR helped conceive the study, participated in its design and coordination, and assisted with writing the manuscript. All authors read and approved the final manuscript.

## 2.2 ABSTRACT

**Background**

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.

**Results**

We now provide evidence that Rrd1 is associated with the chromatin and it interacts with RNA polymerase II. Circular dichroism revealed that Rrd1 mediates structural changes onto the C-terminal domain (CTD) of the large subunit of RNA polymerase II (Rpb1) in response to rapamycin, although this appears to be independent of the overall phosphorylation status of the CTD. *In vitro* experiments, showed that recombinant Rrd1 directly isomerizes purified GST-CTD and that it releases RNA polymerase II from the chromatin. Consistent with this, we demonstrated that Rrd1 is required to alter RNA polymerase II occupancy on rapamycin responsive genes.

**Conclusion**

We propose as a mechanism, that upon rapamycin exposure Rrd1 isomerizes Rpb1 to promote its dissociation from the chromatin in order to modulate transcription.

## 2.3 BACKGROUND

Rapamycin is an immunosuppressant that was recently approved for treating kidney carcinomas [1]. It is known to inhibit the Tor1 (Target of Rapamycin) kinase signalling pathway leading to growth inhibition [2]. In *S. cerevisiae*, several factors have been identified through genome-wide screens that when deleted cause resistance to rapamycin [3]. 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 [4]. Mutants deficient in Rrd1 are also unable to undergo rapamycin-induced growth arrest and therefore exhibit marked resistance to the drug [5]. 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 [6, 7]. We and others reported that Rrd1 can physically interact with the Ser/Thr phosphatase Sit4, a PP2A like phosphatase [8-10]. 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 [11-13]. 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 [13-15]. 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 signalling pathway [12, 16-18].

Recent data indicate that Rrd1 exerts an effect at the transcriptional level [12, 16-18]. 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 *rrd1*Δ mutants [12, 16-18]. 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 [19]. PPIases are ubiquitous proteins that catalytically facilitate the *cis/trans* isomerization of peptide bonds N-terminal to proline residues within polypeptide chains [20, 21]. Both Rrd1 and PTPA can independently change the structure of short peptides including the synthetic substrate ($^{186}$LQEPHEGPMCDL$^{198}$) representing a conserved sequence amongst PP2A phosphatases [19]. As such, it has been suggested that Rrd1/PTPA could activate PP2As *via* this PPIase activity [19]. 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 [22, 23]. In yeast, the CTD consists of 26 repeats of the $YS_2PTS_5PS_7$ heptad sequence and Ess1 has been shown to stimulate the dephosphorylation of Ser-5 to efficiently terminate transcription of a subset of genes [24].

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.

## 2.4 METHODS

### 2.4.1 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*Δ*200 leu2*Δ*1 trp1 ura3-52 gal2 galΔ108*), 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 [25]. 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 [8]. pGST-CTD was constructed by amplifying the murine CTD from plasmid pGCTD [26] 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 [27].

### 2.4.2 Spot test analysis

The assay was done as previously described, except that plates contained rapamycin [28].

### 2.4.3 Extraction of chromatin-associated proteins

Extraction of proteins bound to chromatin was done as previously described, except for the high salt extraction [29].

### 2.4.4 Co-Immunoprecipitation experiments

Co-immunoprecipitation was done as previously described [8], except using 8WG16 antibody (Covance) covalently coupled to AminoLink matrix (Pierce) and total extracts [30] prepared from cells expressing either MYC- or GFP-tagged form of the indicated proteins or from the untagged parent or *rrd1*Δ mutant 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 blot using either anti-MYC, -GFP (Clontech), or -ubiquitin (Rockland). The remaining half of the matrix was analyzed separately by Western blot probed with 8WG16 antibody.

### 2.4.5 GST and GST-CTD purification

Strains bearing either pGST (this laboratory) or pGST-CTD plasmid were subcultured in 500 ml selective media to an $OD_{600}$ of ~1.0, then treated with the appropriate drug for the indicated time. Cells were centrifuged, washed once with sterile water, and resuspended in 1.5 ml of yeast extraction buffer and extracts were prepared as above. The extracts were centrifuged at 3000 rpm in an Eppendorf centrifuge at 4°C for 3 min. Lysates were diluted 3-4 folds in PBS and Triton X-100 was added to a final concentration of 0.2%. One and half ml of glutathione sepharose 4B matrix (Pharmacia) was equilibrated with 50 ml of PBS in 50 ml Falcon tube then the lysate (~80 mg) was added and allowed to bind for one hour at room temperature on a rotating platform. The matrix was washed 3 times with PBS then transferred to

10 ml disposable column (BioRad). Excess of PBS was allowed to flow through, then GST-CTD was eluted with 10 fractions each of 150 µl of 50 mM Tris-HCl pH 9.0, 20 mM reduced glutathione (Sigma). Peak fractions were pooled to a total volume of 750 µl and the buffer was exchanged to 500 µl phosphate buffer using centricon (Millipore). Purity of the samples was verified by SDS-PAGE followed by silver staining.

### 2.4.6 Purification of Rpb1-TAP

Proteins were extracted from untreated or rapamycin-treated (200 ng/ml for 1 h) cells as above and 2 mg were added to 40 µl of pre-equilibrated calmodulin affinity beads (Stratagene, USA). Purifications and washes were performed as described for the batch purification protocol provided by the manufacturer (Stratagene, USA). Eluates (50 µl) were collected, boiled and loaded onto SDS-PAGE for Western analysis. After probing with H5 or H14 antibody (Covance) membranes were stripped and re-probed with anti-PAP antibody (Sigma, USA).

### 2.4.7 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).

### 2.4.8 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 2 h) 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 using anti-MYC monoclonal antibody (SantaCruz).

### 2.4.9 Circular dichroism spectroscopy

Continuous far-UV circular dichroism spectra (197-250 nm) of the GST and the GST-CTD fusion protein (2.0 µg and 4.32 µg, respectively, in 100 µl 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 [31].

### 2.4.10 Limited chymotrypsin digestion assay

The purified GST-CTD (~100 ng) derived from parent cells untreated or treated with rapamycin (200 ng/ml for 2 h) was subjected to digestion with 5 ng chymotrypsin [32] 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.

### 2.4.11 *In vitro* isomerase assay

Purified HIS-Rrd1 (from *E. coli* using Talon affinity column according to the manufacturer (GE) protocol) 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 µl. The proteins were incubated for 1 h at 30°C the GST-CTD was recovered by GST-affinity purification and then subjected to CD analysis.

### 2.4.12 *In Vitro* Rpb1 release assay

Exponentially growing culture (200 ml) 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 µl 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 30°C. 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 µl of benzonase (Novagen) was added and tubes were incubated for 30 min at 37°C. Supernatant (SOL) and chromatin [11] fractions were loaded onto SDS-PAGE gels for Western blot analysis with 4H8 (Cell Signaling) and anti-MYC antibodies.

### 2.4.13 ChIP assay

The ChIP assay was done as previously described [33]. 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.

## 2.5 RESULTS

### 2.5.1 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 [18]. To corroborate our earlier findings that Rrd1 acts separately from the Sit4-Gln3 signaling pathway, we deleted the *RRD1* gene in the *gln3*Δ background (known also to be resistant to rapamycin) and examined the resulting *gln3Δ rrd1*Δ double mutant for the level of resistance to the drug [34]. This genetic analysis revealed that the *gln3Δ rrd1*Δ double mutant was significantly more resistant to rapamycin than either of the single mutants (Figure S2.1), 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 [18]. 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 Figure 1.1A, 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 [8]. 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) [35, 36].

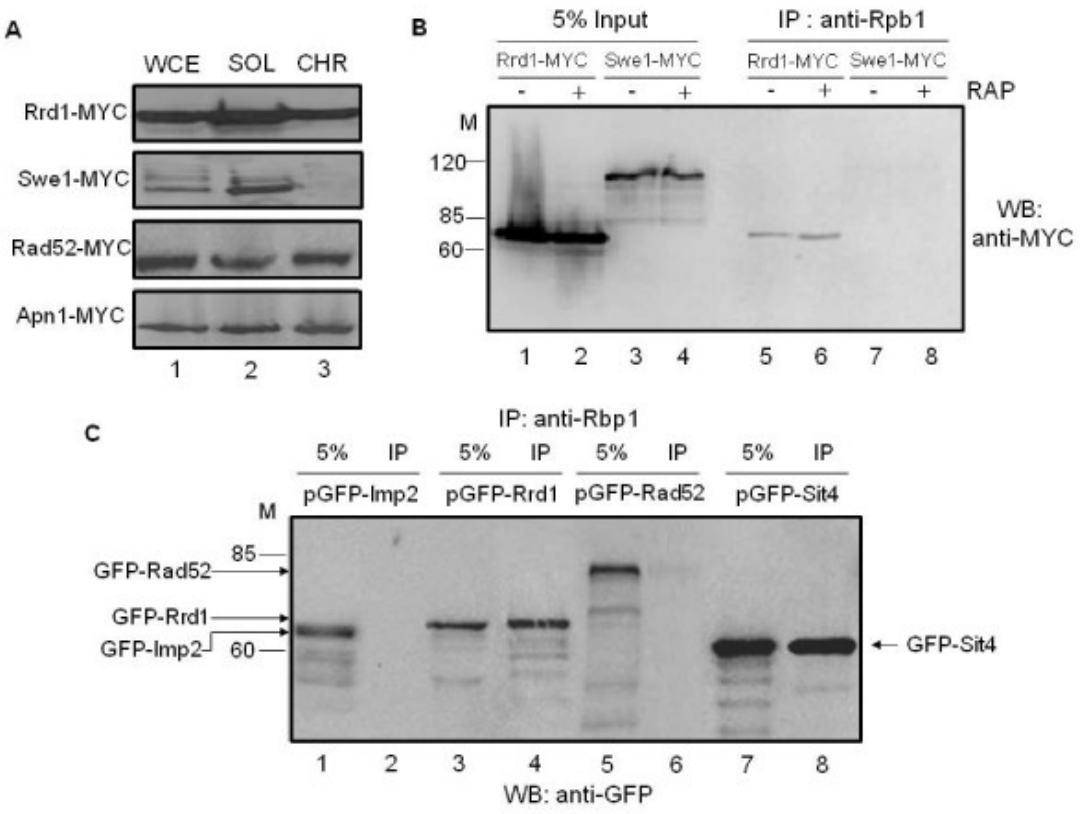


**Figure 2.1: Rrd1 is associated with the chromatin and interacts with Rpb1**.
**A)** Rrd1 is bound to chromatin. Whole cell extract (WCE), soluble (SOL) and chromatin [11] fractions were derived (see Methods) from the parent cells expressing either Rrd1-MYC, Swe1-MYC, Rad52-MYC or Apn1-MYC and the distribution 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 using GFP antibody.

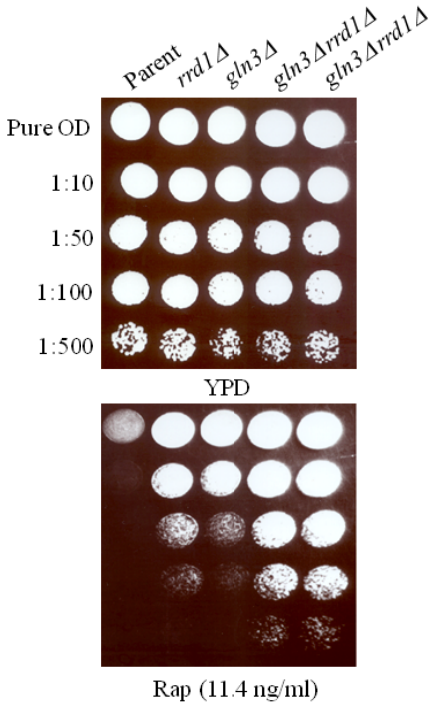


**Figure S2.1: *gln3*Δ *rrd1*Δ double mutant is more resistant to rapamycin than either single mutant.** The assay was done as previously described, except that plates contained rapamycin. Cells were serially diluted and spotted onto YPD plates without and with rapamycin at 11.4 ng/ml (Rap). Photos were taken after two days of growth at 30$^{o}$C.

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 (Figure 2.1B). 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) (Figure 2.1B).

Anti-Rpb1 also co-immunoprecipitated Rrd1 from parent cells carrying a plasmid expressing GFP-tagged Rrd1 (Figure 2.1C). In addition, the Sit4 phosphatase known to physically interact with Rrd1 [8] co-immunoprecipitated with Rpb1 from parent cells expressing this protein as GFP fusion (Figure 2.1C). 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 (Figure 2.1C, 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.

### 2.5.2 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 [22, 23, 37], 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 Methods) and has been shown to undergo post-translational modifications including Ser-5 and Ser-2 phosphorylation, isomerization and ubiquitylation [24, 26, 32, 38]. Introduction of this plasmid into the parent and *rrd1*Δ 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 (Figure 2.2A, see also Figure S2.2). 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 (Figure 2.2A), consistent with previous studies that the GST-CTD can be functionally modified *in vivo* [26, 32, 38, 39]. 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 *rrd1*Δ mutant or from cells that were pretreated with rapamycin (Figure 2.2A, Figure S2.2).

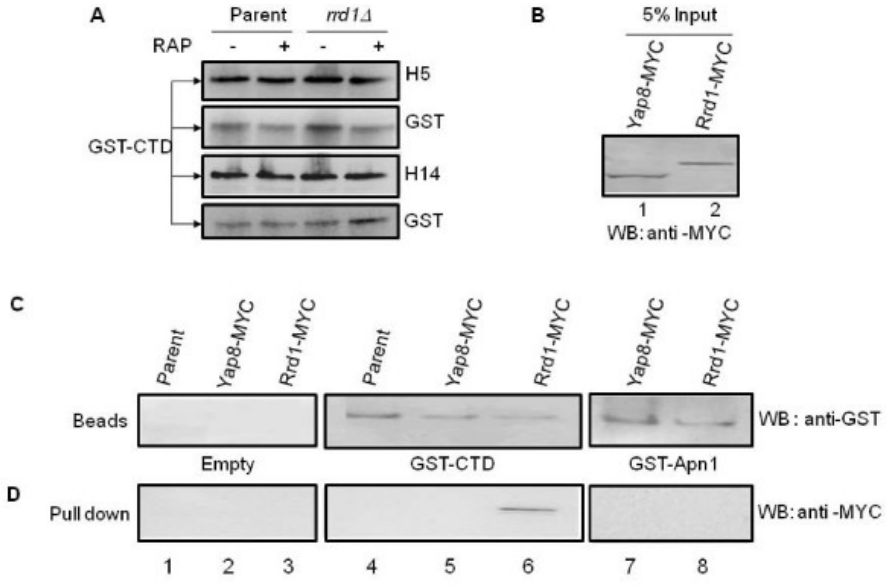


**Figure 2.2** : **Analysis of the GST-CTD and its interaction with Rrd1.**
**A)** Comparison of the expression and phosphorylation status of the GST-CTD between parent and *rrd1*Δ mutant cells following rapamycin exposure. The indicated

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 (5% of the total amount of protein extracts added to the beads) of parent cells expressing Yap8-MYC and Rrd1-MYC from the endogenous locus. Western blot analysis was done using anti-MYC antibody. **C)** and **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 (see Methods). The beads were then washed and an aliquot examined for retention of the MYC tagged proteins using anti-GST antibodies (C) or anti-GST antibodies (D). Results shown are representative of two independent experiments.

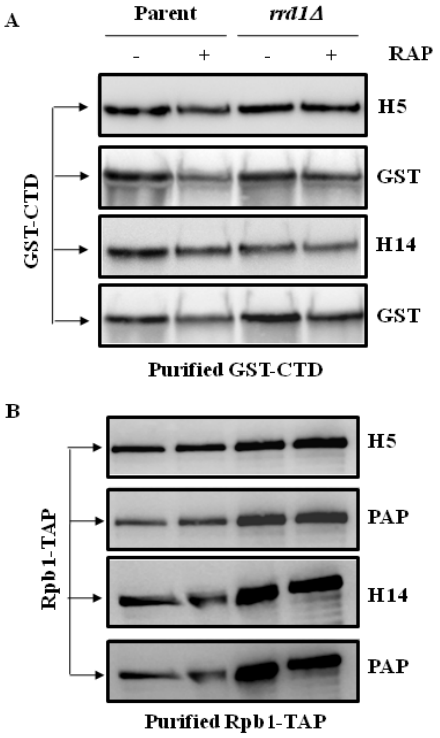


**Figure S2.2: Analysis of the phosphorylation status of purified GST-CTD and Rpb1-TAP.**
**A)** Comparison of the phosphorylation status of the purified GST-CTD derived from the parent and *rrd1*Δ mutant following rapamycin exposure (200 ng/ml for 30 min). The purified GST-CTD was subjected to Western 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.

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 (Figure 2.2B, 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 Figure 2.2C and 2.2D, 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 (Figure 2.2B, 2.2C and 2.2D). As expected, the empty beads did not pull down Rrd1-MYC from the total extract nor did the control beads carrying GST-Apn1 (Figure 2.2C and 2.2D). 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 [31]. We first purified the GST-CTD from the parent and the *rrd1*Δ 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 *rrd1*Δ mutant (Figure 2.3A, 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 also contained both phosphorylated forms, Ser-2 and Ser-5, but showed no alteration in response to rapamycin (Additional file 1 Figure S2A). 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 (Additional file 1 Figure S2B). However, this approach may not distinguish between subtle phosphorylation differences that may occur amongst the heptad repeats [40]. 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 *rrd1*Δ mutant were indistinguishable, and displayed a minimum at 202 nm (Figure 2.3B). 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 (Figure 2.3B), suggesting that the GST-CTD underwent a detectable change in its secondary structure. Remarkably, rapamycin treatment of the *rrd1*Δ mutant failed to induce this conformational change onto the GST-CTD (Figure 2.3B). Introduction of a single copy plasmid expressing functional Rrd1 in the *rrd1*Δ mutant restored the change in the spectral pattern of the GST-CTD (Figure 2.3C) [4]. 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 (Figure 2.3D). 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 [41]. As shown in Figure 3E, 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.

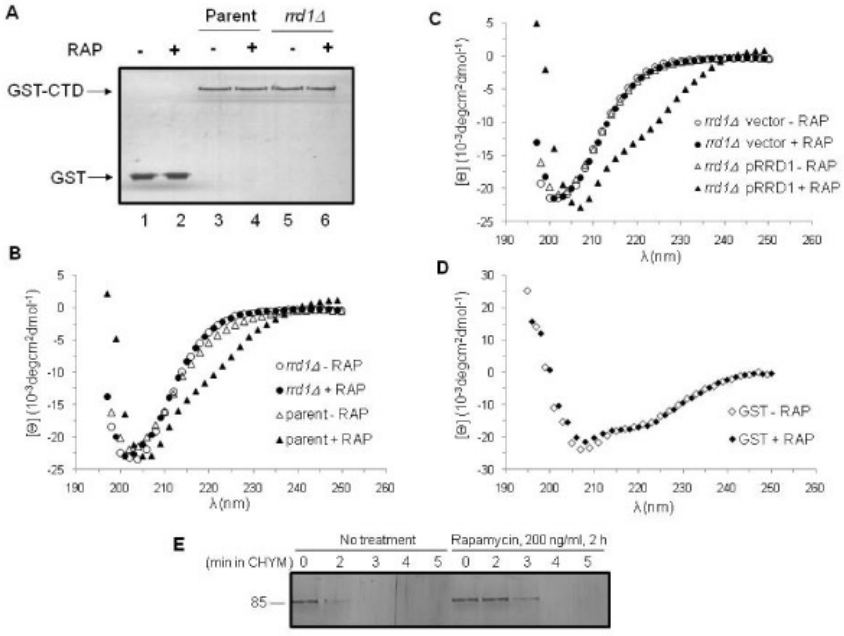


**Figure 2.3** : *rrd1*Δ **mutants are unable to induce conformational changes to the GST-CTD in response to rapamycin.**
**A)** Silver stained gel of purified GST and GST-CTD. The indicated strains carrying either the GST (lanes 1 and 2) or GST-CTD expressing plasmid (lanes 3-6) were untreated (-) or treated (+) with rapamycin (RAP) (200 ng/ml for 30 min). **B)** Far-UV circular dichroism (CD) spectral analysis of purified GST-CTD. The purified GST-CTD (0.45 μM) was derived from the parent strain (triangle) or *rrd1*Δ mutant (circle) that were untreated (opened symbol) or treated (closed symbol) with rapamycin. **C)** Far-UV CD spectral analysis of purified GST-CTD. The purified GST-CTD (0.45 μM) was derived from the *rrd1*Δ mutant carrying the empty vector (circle) or the

pRRD1 plasmid (triangle) that were untreated (opened symbol) or treated (closed symbol) with rapamycin. **D)** CD analysis of purified GST (0.76 μM) derived from untreated (opened symbol) and rapamycin treated (closed symbol) 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.

### 2.5.3 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 *rrd1*Δ mutant was previously shown to be sensitive to the DNA damaging agent 4-NQO [4], which induces oxidative stress as well as creating bulky lesions onto the DNA [42], 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 *rrd1*Δ mutant following 4-NQO treatment (Figure 2.4A). We also tested another DNA damaging agent, methyl methane sulfonate (MMS) (Figure 2.4B), to which the *rrd1*Δ mutant displays parental sensitivity [4]. 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 (Figure 2.4B). On the basis of these findings, it would appear that this phenomenon might occur for other stress conditions besides exposure to rapamycin.

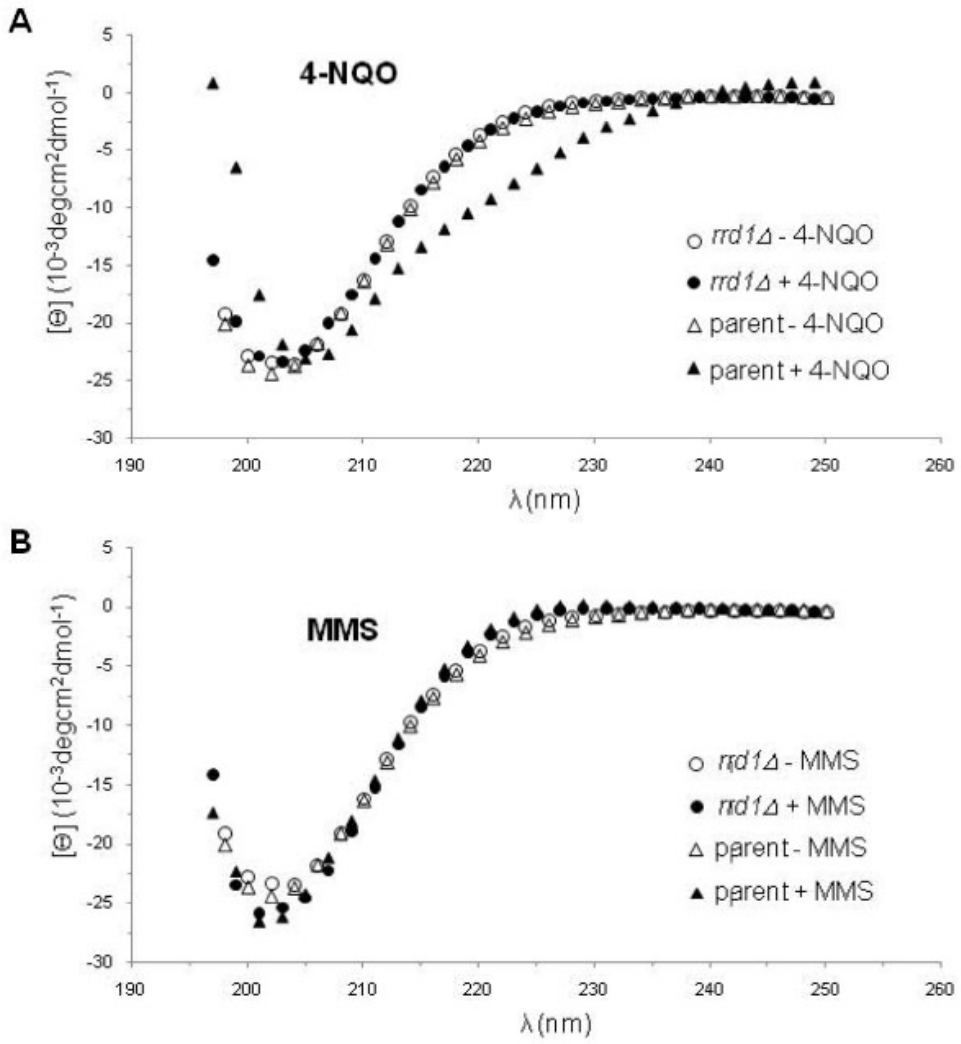


**Figure 2.4 : 4-NQO, but not MMS, induces structural changes onto the GST-CTD.**
**A and B)** CD analysis of the purified GST-CTD derived from exponentially growing parent (triangle) and *rrd1*Δ mutant (circle) that were untreated (opened symbol) or treated (closed symbol) with either 4-NQO (2 μg/ml 30 min) panel A or MMS (1% for 60 min) panel B.

### 2.5.4 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* (Figure 2.5A) with affinity purified GST-CTD derived from the *rrd1*Δ mutant at 30°C for 30 min, and then recovered the GST-CTD for CD analysis. As shown in Figure 2.5B, 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 $Mg^{2+}$ [19], 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 (Figure 2.5B). 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/$Mg^{2+}$ (Figure 2.5B). 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.

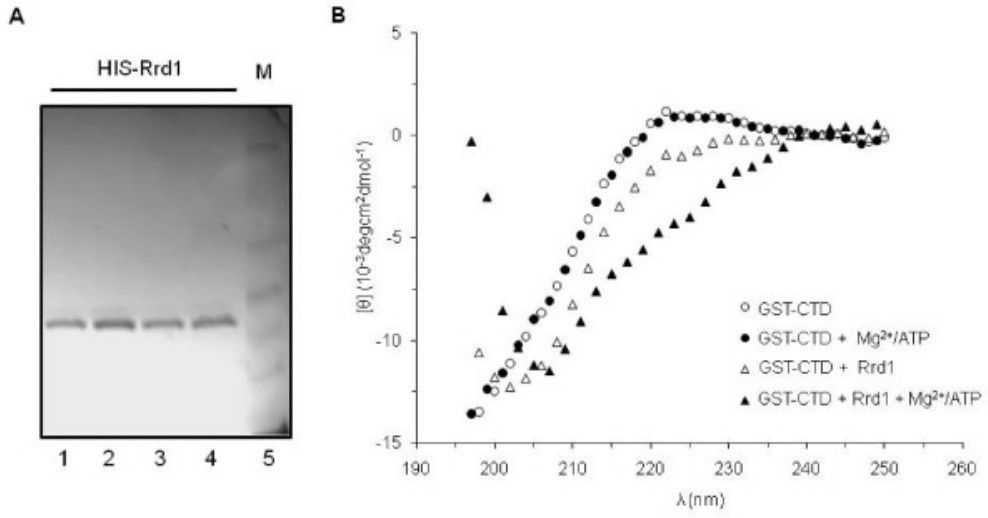


**Figure 2.5 : Purified recombinant Rrd1 alters the structure of purified GST-CTD *in vitro*.**

**A)** Silver stained gel of purified recombinant HIS-Rrd1 from *E. coli* expression system (see Methods). Lanes 1-2 and 3-4 are elution samples from two independent purifications obtained directly from Talon affinity column; lane 5, molecular weight standard. **B)** Equimolar amounts (4.5 μM) of purified GST-CTD derived from the *rrd1*Δ mutant and the purified recombinant HIS-Rrd1 (triangle) were incubated at 30°C in phosphate buffer in the absence (opened symbol) and presence (closed symbol) of $Mg^{2+}$/ATP. The resulting GST-CTD was re-purified free of the recombinant HIS-Rrd1 and subjected to CD analysis as in Figure 2.3. The result is the average of two independent experiments.

### 2.5.5 Comparison of RNA pol II occupancy at rapamycin-responsive genes

Since Rrd1 associates with and isomerizes the CTD, and that *rrd1*Δ 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* [33]. Since both genes are known to be rapidly downregulated and upregulated, respectively, within 30 min, we treated cells for this time period with rapamycin [11, 18]. In parent cells, the Rpb1-ChIP signal from the *RPS26A* gene was reduced by nearly 8-fold upon rapamycin treatment (Figure 2.6A). In contrast, Rpb1 remained associated with *RPS26A* in the *rrd1*Δ mutant (Figure 2.6A). 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 (Figure 2.6B). The occupancy of RNA pol II on these genes is consistent with the mRNA expression levels [11, 18]. These data raise the possibility that Rrd1 might displace Rpb1 in order to optimize rapid transcriptional changes caused by rapamycin.

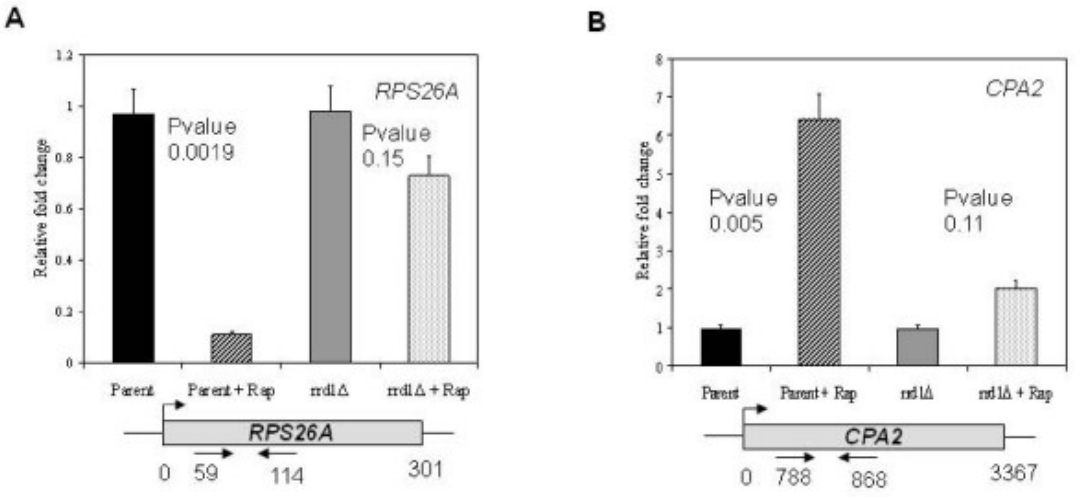


**Figure 2.6 : Comparison of RNA pol II occupancy at the indicated target genes in the parent and *rrd1*Δ mutant strain in response to rapamycin treatment.** Cells were untreated or treated with 200 ng/ml rapamycin for 30 min and Rpb1 localization was analyzed by ChIP assay (see Methods). Primer locations 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.

### 2.5.6 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 *rrd1*Δ 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 Figure 2.7 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.

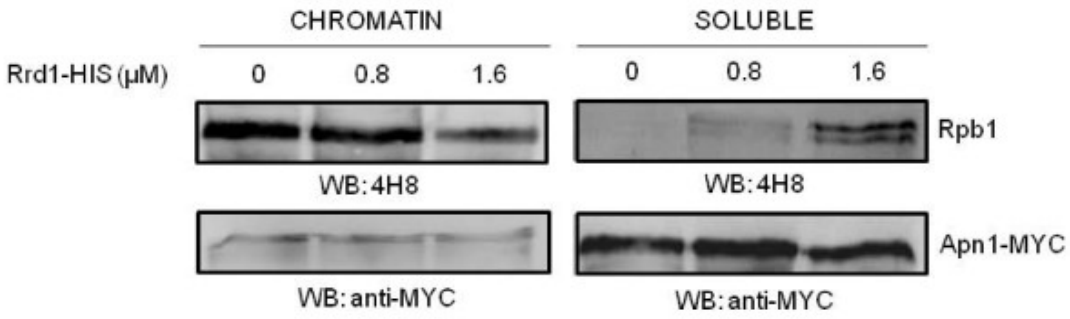


**Figure 2.7 : Purified recombinant Rrd1 dissociates Rpb1 from the chromatin *in vitro*.**
Increasing amounts of purified HIS-Rrd1 were added to the chromatin fraction isolated from *rrd1*Δ mutant strain expressing Apn1-MYC and incubated at 30°C for 1 h in phosphate buffer. Chromatin was recovered from 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.

## 2.6 DISCUSSION

In the present study, we show that Rrd1 is a chromatin bound protein, which associates with RNA pol II and 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. 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 [43]. 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 [43]. This shift also simultaneously caused an enrichment of RNA pol II onto mitochondrial genes [43]. 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 [23]. Under normal conditions, Pin1 interacts with the phosphorylated CTD of RNA pol II and this association is retained along the length of transcribed genes [23]. 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 [23, 44]. The dissociated RNA pol II accumulates in enlarged speckle-associated structures enriched for transcription and RNA processing factors [23, 45].

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 [24]. 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 [24]. 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 *rrd1*Δ mutant using the same set of antibodies (Figure 2.2A and Additional file 1 Figure S2). 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 [40]. 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 YSPTSPS. 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[46, 47]. Binding of these proteins to the CTD is modulated by serine phosphorylation and proline isomerization [40]. 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 [46-48]. 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 [48]. 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 throughout its purification (Lisa Miller, Brookhaven National Laboratories, personal communications) from untreated and rapamycin-treated cells (Figure 2.3). 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 [49]. Only peptidyl prolyl isomerases such as Pin1/Ess1 are known to trigger a switch between the *cis* and *trans* conformations of the CTD [50], and that in the absence of these enzymes the

conformational switch is slow [51]. 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* (Figure 2.5), 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 (Figure 2.4). 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 [4]. 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 [42]. 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 [51, 52]. 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.

## 2.7 CONCLUSION

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. These results provide the first direct evidence that Rrd1 acts *in vivo* as an isomerase and establish a physiological function for this activity.

## 2.8 ACKNOWLEDGMENTS

We thank Drs. Katherine Borden and Jim Daley for their helpful comments. We thank Drs. Ivan Topisirovic and Michael Osborne for their expertise with the circular dichroism analysis performed at the Institute for Research in Immunology and Cancer, and Xiaoming Yang for assistance with the pull down assay and purifying Rrd1. This work was supported by a grant (MOP-13152) from the Canadian Institutes of Health Research to D.R.

## 2.9 REFERENCES


1. Saylor, P.J. and M.D. Michaelson, *New treatments for renal cell carcinoma: targeted therapies.* J Natl Compr Canc Netw, 2009. **7**(6): p. 645-56.
2. Bjornsti, M.A. and P.J. Houghton, *The TOR pathway: a target for cancer therapy.* Nat Rev Cancer, 2004. **4**(5): p. 335-48.
3. Neklesa, T.K. and R.W. Davis, *Superoxide anions regulate TORC1 and its ability to bind Fpr1:rapamycin complex.* Proc Natl Acad Sci U S A, 2008. **105**(39): p. 15166-71.
4. Ramotar, D., et al., *A yeast homologue of the human phosphotyrosyl phosphatase activator PTPA is implicated in protection against oxidative DNA damage induced by the model carcinogen 4-nitroquinoline 1-oxide.* J Biol Chem, 1998. **273**(34): p. 21489-96.
5. Rempola, B., et al., *Functional analysis of RRD1 (YIL153w) and RRD2 (YPL152w), which encode two putative activators of the phosphotyrosyl phosphatase activity of PP2A in Saccharomyces cerevisiae.* Mol Gen Genet, 2000. **262**(6): p. 1081-92.
6. Janssens, V., et al., *PTPA regulating PP2A as a dual specificity phosphatase.* Methods Mol Biol, 1998. **93**: p. 103-15.
7. Cayla, X., et al., *Molecular cloning, expression, and characterization of PTPA, a protein that activates the tyrosyl phosphatase activity of protein phosphatase 2A.* J Biol Chem, 1994. **269**(22): p. 15668-75.
8. Douville, J., et al., *The yeast phosphotyrosyl phosphatase activator protein, yPtpa1/Rrd1, interacts with Sit4 phosphatase to mediate resistance to 4-nitroquinoline-1-oxide and UVA.* Curr Genet, 2004. **46**(2): p. 72-81.
9. Mitchell, D.A. and G.F. Sprague, Jr., *The phosphotyrosyl phosphatase activator, Ncs1p (Rrd1p), functions with Cla4p to regulate the G(2)/M transition in Saccharomyces cerevisiae.* Mol Cell Biol, 2001. **21**(2): p. 488-500.
10. Van Hoof, C., et al., *Specific interactions of PP2A and PP2A-like phosphatases with the yeast PTPA homologues, Ypa1 and Ypa2.* Biochem J, 2005. **386**(Pt 1): p. 93-102.
11. Hardwick, J.S., et al., *Rapamycin-modulated transcription defines the subset of nutrient-sensitive signaling pathways directly controlled by the Tor proteins.* Proc Natl Acad Sci U S A, 1999. **96**(26): p. 14866-70.
12. Heitman, J., N.R. Movva, and M.N. Hall, *Targets for cell cycle arrest by the immunosuppressant rapamycin in yeast.* Science, 1991. **253**(5022): p. 905-9.
13. Crespo, J.L. and M.N. Hall, *Elucidating TOR signaling and rapamycin action: lessons from Saccharomyces cerevisiae.* Microbiol Mol Biol Rev, 2002. **66**(4): p. 579-91, table of contents.
14. Schmelzle, T., et al., *Activation of the RAS/cyclic AMP pathway suppresses a TOR deficiency in yeast.* Mol Cell Biol, 2004. **24**(1): p. 338-51.
15. Beck, T. and M.N. Hall, *The TOR signalling pathway controls nuclear localization of nutrient-regulated transcription factors.* Nature, 1999. **402**(6762): p. 689-92.

16. Rohde, J., J. Heitman, and M.E. Cardenas, *The TOR kinases link nutrient sensing to cell growth.* J Biol Chem, 2001. **276**(13): p. 9583-6. Epub 2001 Feb 2.
17. Powers, T. and P. Walter, *Regulation of ribosome biogenesis by the rapamycin-sensitive TOR-signaling pathway in Saccharomyces cerevisiae.* Mol Biol Cell, 1999. **10**(4): p. 987-1000.
18. Douville, J., et al., *The Saccharomyces cerevisiae phosphatase activator RRD1 is required to modulate gene expression in response to rapamycin exposure.* Genetics, 2006. **172**(2): p. 1369-72.
19. Jordens, J., et al., *The Protein Phosphatase 2A Phosphatase Activator Is a Novel Peptidyl-Prolyl cis/trans-Isomerase.* J Biol Chem, 2006. **281**(10): p. 6349-6357.
20. Schmid, F.X., *Protein folding. Prolyl isomerases join the fold.* Curr Biol, 1995. **5**(9): p. 993-4.
21. Fischer, G., et al., *Conformational specificity of chymotrypsin toward proline-containing substrates.* Biochim Biophys Acta, 1984. **791**(1): p. 87-97.
22. Wilcox, C.B., A. Rossettini, and S.D. Hanes, *Genetic interactions with C-terminal domain (CTD) kinases and the CTD of RNA Pol II suggest a role for ESS1 in transcription initiation and elongation in Saccharomyces cerevisiae.* Genetics, 2004. **167**(1): p. 93-105.
23. Xu, Y.X. and J.L. Manley, *Pin1 modulates RNA polymerase II activity during the transcription cycle.* Genes Dev, 2007. **21**(22): p. 2950-62.
24. Singh, N., et al., *The Ess1 prolyl isomerase is required for transcription termination of small noncoding RNAs via the Nrd1 pathway.* Mol Cell, 2009. **36**(2): p. 255-66.
25. Wysocki, R., et al., *Transcriptional activation of metalloid tolerance genes in Saccharomyces cerevisiae requires the AP-1-like proteins Yap1p and Yap8p.* Mol Biol Cell, 2004. **15**(5): p. 2049-60.
26. Peterson, S.R., et al., *DNA binding provides a signal for phosphorylation of the RNA polymerase II heptapeptide repeats.* Genes Dev, 1992. **6**[113]: p. 426-38.
27. Wang, H. and D. Ramotar, *Cellular resistance to bleomycin in Saccharomyces cerevisiae is not affected by changes in bleomycin hydrolase levels.* Biochem Cell Biol, 2002. **80**(6): p. 789-96.
28. Aouida, M., et al., *A Genome-Wide Screen in Saccharomyces cerevisiae Reveals Altered Transport As a Mechanism of Resistance to the Anticancer Drug Bleomycin.* Cancer Res, 2004. **64**[113]: p. 1102-9.
29. Svejstrup, J.Q., T.G. Petrakis, and J. Fellows, *Purification of elongating RNA polymerase II and other factors from yeast chromatin.* Methods Enzymol, 2003. **371**: p. 491-8.
30. Vongsamphanh, R., P.K. Fortier, and D. Ramotar, *Pir1p mediates translocation of the yeast Apn1p endonuclease into the mitochondria to maintain genomic stability.* Mol Cell Biol, 2001. **21**(5): p. 1647-55.
31. Fasman, G.D., *Circular Dichroism and the Conformational Analysis of Biomolecules*. 1996, New York: Plenum Press.
32. Moisan, A., et al., *BRCA1 can modulate RNA polymerase II carboxy-terminal domain phosphorylation levels.* Mol Cell Biol, 2004. **24**(16): p. 6947-56.

33. Lee, T.I., et al., *Transcriptional regulatory networks in Saccharomyces cerevisiae.* Science, 2002. **298**(5594): p. 799-804.
34. Crespo, J.L., et al., *The TOR-controlled transcription activators GLN3, RTG1, and RTG3 are regulated in response to intracellular levels of glutamine.* Proc Natl Acad Sci U S A, 2002. **99**(10): p. 6784-9.
35. Bi, B., et al., *Human and yeast Rad52 proteins promote DNA strand exchange.* Proc Natl Acad Sci U S A, 2004. **101**(26): p. 9568-72.
36. Ramotar, D., et al., *Cellular role of yeast Apn1 apurinic endonuclease/3'-diesterase: repair of oxidative and alkylation DNA damage and control of spontaneous mutation.* Mol Cell Biol, 1991. **11**(9): p. 4537-44.
37. Morris, D.P., H.P. Phatnani, and A.L. Greenleaf, *Phospho-carboxyl-terminal domain binding and the role of a prolyl isomerase in pre-mRNA 3'-End formation.* J Biol Chem, 1999. **274**(44): p. 31583-7.
38. Bienkiewicz, E.A., A. Moon Woody, and R.W. Woody, *Conformation of the RNA polymerase II C-terminal domain: circular dichroism of long and short fragments.* J Mol Biol, 2000. **297**(1): p. 119-33.
39. Ursic, D., J.S. Finkel, and M.R. Culbertson, *Detecting phosphorylation-dependent interactions with the C-terminal domain of RNA polymerase II subunit Rpb1p using a yeast two-hybrid assay.* RNA Biol, 2008. **5**(1): p. 1-4.
40. Noble, C.G., et al., *Key features of the interaction between Pcf11 CID and RNA polymerase II CTD.* Nat Struct Mol Biol, 2005. **12**(2): p. 144-51.
41. Nelson, C.J., H. Santos-Rosa, and T. Kouzarides, *Proline isomerization of histone H3 regulates lysine methylation and gene expression.* Cell, 2006. **126**(5): p. 905-16.
42. Nunoshiba, T. and B. Demple, *Potent intracellular oxidative stress exerted by the carcinogen 4-nitroquinoline-N-oxide.* Cancer Res, 1993. **53**(14): p. 3250-2.
43. Pelechano, V., et al., *Regulon-specific control of transcription elongation across the yeast genome.* PLoS Genet, 2009. **5**(8): p. e1000614.
44. Xu, Y.X., et al., *Pin1 modulates the structure and function of human RNA polymerase II.* Genes Dev, 2003. **17**(22): p. 2765-76.
45. Lamond, A.I. and D.L. Spector, *Nuclear speckles: a model for nuclear organelles.* Nat Rev Mol Cell Biol, 2003. **4**(8): p. 605-12.
46. Hampsey, M. and D. Reinberg, *Tails of intrigue: phosphorylation of RNA polymerase II mediates histone methylation.* Cell, 2003. **113**(4): p. 429-32.
47. He, X., et al., *Functional interactions between the transcription and mRNA 3' end processing machineries mediated by Ssu72 and Sub1.* Genes Dev, 2003. **17**(8): p. 1030-42.
48. Buratowski, S., *The CTD code.* Nat Struct Biol, 2003. **10**(9): p. 679-80.
49. Jakob, R.P. and F.X. Schmid, *Molecular determinants of a native-state prolyl isomerization.* J Mol Biol, 2009. **387**(4): p. 1017-31.
50. Lu, K.P., et al., *Prolyl cis-trans isomerization as a molecular timer.* Nat Chem Biol, 2007. **3**(10): p. 619-29.
51. Gemmill, T.R., X. Wu, and S.D. Hanes, *Vanishingly low levels of Ess1 prolyl-isomerase activity are sufficient for growth in Saccharomyces cerevisiae.* J Biol Chem, 2005. **280**(16): p. 15510-7.

52. Gasch, A.P., et al., *Genomic expression programs in the response of yeast cells to environmental changes.* Mol Biol Cell, 2000. **11**(12): p. 4241-57.

# CHAPTER 3

## Article 2

## RNA polymerase II degradation in response to rapamycin is not mediated through ubiquitylation

**Nathalie Jouvet*, Jeremie Poschmann*, Julie Douville, Rim Marrakchi and Dindial Ramotar**

* authors contributed equally

Article published in *Biochemical and Biophysical Research Communication* (2011) 413 (2): 248-53.

# 3 ARTICLE #2

## 3.1 AUTHORS' CONTRIBUTION

**NJ** carried out all western blot and chromatin extraction experiments and drafted the manuscript. JP carried out the spot test analysis, the Rpb1-TAP experiment and drafted the manuscript. JD carried out the co-immunoprecipitation experiments. RM performed the *tor1-1* experiment. DR helped conceive the study, participated in its design and coordination, and assisted with writing the manuscript. All authors read and approved the final manuscript.

## 3.2 ABSTRACT

In *Saccharomyces cerevisiae*, the immunosuppressor rapamycin engenders the degradation of excessive RNA polymerase II leading to growth arrest but the regulation of this process is not known yet. Here, we show that this mechanism is dependent on the peptidyl prolyl *cis/trans* isomerase Rrd1. Strikingly this degradation is independent of RNA polymerase II polyubiquitylation and does not require the elongation factor Elc1. Our data reveal that there are at least two alternative pathways to degrade RNA polymerase II that depend on different type of stresses.

## 3.3 INTRODUCTION

Rapamycin is an immunosuppressant that was recently approved for treating kidney carcinomas [1]. It is known to inhibit the Tor1 (Target of rapamycin) kinase signaling pathway leading to growth inhibition [2]. In *Saccharomyces 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 [3-5]. We have reported that Rrd1 (rapamycin resistance deletion 1) is required for an efficient transcriptional response to rapamycin via the Tor1 signaling pathway [6, 7]. In fact, Tor1 mediated transcriptional changes are partially inhibited in *rrd1*Δ mutants [7]. Additional studies revealed that Rrd1 interacts with elongating RNA polymerase II (henceforth referred to as RNAPII) and that it regulates the rapamycin induced transcriptional response during the elongation of RNAPII [7]. Further, we demonstrated that Rrd1 isomerizes the C-terminal domain (CTD) of Rpb1 the major subunit of RNAPII and that it releases it from the chromatin as part of the regulatory process [8]. Interestingly, the response to rapamycin is kinetically associated with a diminished level of Rpb1, and which is blocked in the *rrd1*Δ mutants [6]. since RPB1 mRNA expression level was not significantly altered in response to rapamycin shown by three distinct genome wide expression analyses, and that RNAPII occupancy of the RPB1 gene was not changed as revealed by ChIP on chip analysis [3, 7, 9, 10], it thus appears that the decreased level of Rbp1 is associated with the degradation of the protein [6]. However, the exact function executed by Rrd1 that leads to RNAPII degradation has not been investigated yet. The degradation of RNAPII was initially observed in

response to DNA damage, such as UV radiation and by 4-nitroquinoline-1-oxide (4-NQO), an oxidant causing bulky adducts onto the DNA [11-15]. During this process RNAPII is polyubiquitylated on two lysine residues (K330 and K695) and this event is crucial for its degradation [16, 17]. This is mediated through an ubiquitin-ligase complex containing the elongation factor Elc1 [14, 15]. To date this is the only pathway known to degrade elongating RNAPII in response to stress [16, 18].

In this study, we asked the question whether Rrd1 is involved in the rapamycin induced degradation of RNAPII. Indeed, Rrd1 is required for this process and we show that this is neither strain specific nor an antibody artifact. More importantly, Rrd1 is required at the level of the chromatin, executing its function through its catalytic peptidyl-prolyl isomerase domain for the release of RNAPII from the chromatin. Surprisingly, we find that this mechanism is ubiquitylation independent as it does not require the ubiquitylation sites of RNAPII or the ubiquitin ligase complex as in the case of DNA damage. We propose a model where the isomerization of the CTD of RNAPII by Rrd1 leads to the dissociation of RNAPII from the chromatin resulting in transcriptional changes and the degradation of the surplus polymerase. This study provides insight into an alternative mechanism of RNAPII degradation in response to transcriptional stress.

## 3.4 MATERIALS AND METHODS

### 3.4.1 Strains, media, plasmids and antibodies

The strains used in this study were the BY4741 (Mat a, his3-1, leu2-0, met15-0, ura3-0), YDL401 (MATa his3Δ200leu2Δ1 trp1 ura3-52 gal2galΔ108), CY4029 (W303 background, SSD1-v1, MATa, ade2-1, can1-100, his3-11, 15, leu2-3, 112, trp1-1, ura3-1), and SEY6210 [19] and the isogenic mutants rrd1Δ. Strains were endogenously and independently tag at the following loci APN1 and RAD52 as previously described [19]. The tor1-1 allele was derived from the W303 parental background and provided by Dr. Joseph Heitman (Duke University Medical Center, Durham, NC, USA). Rpb4-MYC and Rpb1-TAP were provided by Tom Begley, Albany, USA. Strains bearing the native Rpb1 and the mutants K330R and K695R were provided by Svejstrup, UK [20]. The strains were grown in either rich (YPD) or selective (SD) media. Antibodies used for Rpb1 were 8WG16 (Covance), 4H8 (Cell signaling) and anti-PAP (Sigma). Anti-MYC antibodies were purchased from Santa Cruz and anti-ubiquitylation antibodies from assay designs.

### 3.4.2 Analysis of degradation of Rpb1 from whole cell extracts

Overnight cultures of the indicated strains were subcultured for 3 h and treated with rapamycin, 4-NQO or MMS (Sigma–Aldrich) for different times. The total protein extracts were then prepared as previously described using a mini-bead-beater [21] and separated by SDS–PAGE and analyzed by Western blot. Protein amounts from the Western blots were quantified using Multi Gauge V3.0 program [22].

### 3.4.3 Co-immunoprecipitation

Co-immunoprecipitation was done as previously described [23], except using 8WG16 antibody (Covance) covalently coupled to AminoLink matrix (Pierce) and total extracts [21] prepared from cells from the parent or rrd1Δ mutant 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 blot using anti-ubiquitin. The remaining half of the matrix was analyzed separately by Western blot probed with 8WG16 antibody.

### 3.4.4 Spot test analysis

Overnight cultures of the W303 strains bearing the native Rpb1 or the mutants K330R and K695R were diluted and spotted onto YPD solid agar plates without and with rapamycin.

### 3.4.5 Extraction of chromatin-associated proteins

Extraction of proteins bound to chromatin was done as previously described, except for the high salt extraction [24].

### 3.4.6 In vitro chromatin assay

Experiment was performed as described in Jouvet et al., besides that the plasmid expressing Rrd1 was mutated within the Rrd1 catalytic domain on residue D200G using the site directed mutagenesis kit (Stratagene) [8].

## 3.5 RESULTS AND DISCUSSION

### 3.5.1 Rrd1 is required for efficient RNAPII degradation in response to rapamycin

To begin investigating the underlying mechanism of how Rpb1 levels are decreased in response to rapamycin, we decided to look at its protein level using multiple genetic backgrounds [6]. Total cell extracts derived from the wild-type strain BY4741 and the isogenic *rrd1*Δ mutant were treated with rapamycin and examined for levels of Rpb1 using the antibody 8WG16 which recognizes the C-terminal domain (CTD) of the protein. In wild-type cells, rapamycin triggered a substantial decrease in the level of Rpb1 in a time-dependent manner, while the decrease was less prominent in the *rrd1*Δ mutant (Fig. 3.1A). At least 60% of Rpb1 disappeared in the wild-type within 60 min of rapamycin treatment, while the level diminished significantly less (no more than 30%) in the *rrd1*Δ mutant under the same conditions (see quantification data shown below panel A). Similar results were obtained in three other genetic backgrounds (W303, SEY6210 and FY56) (Fig. 3.1 and data not shown). As such, Rpb1 levels decrease in a Rrd1-dependent process, but independently of the genetic background.

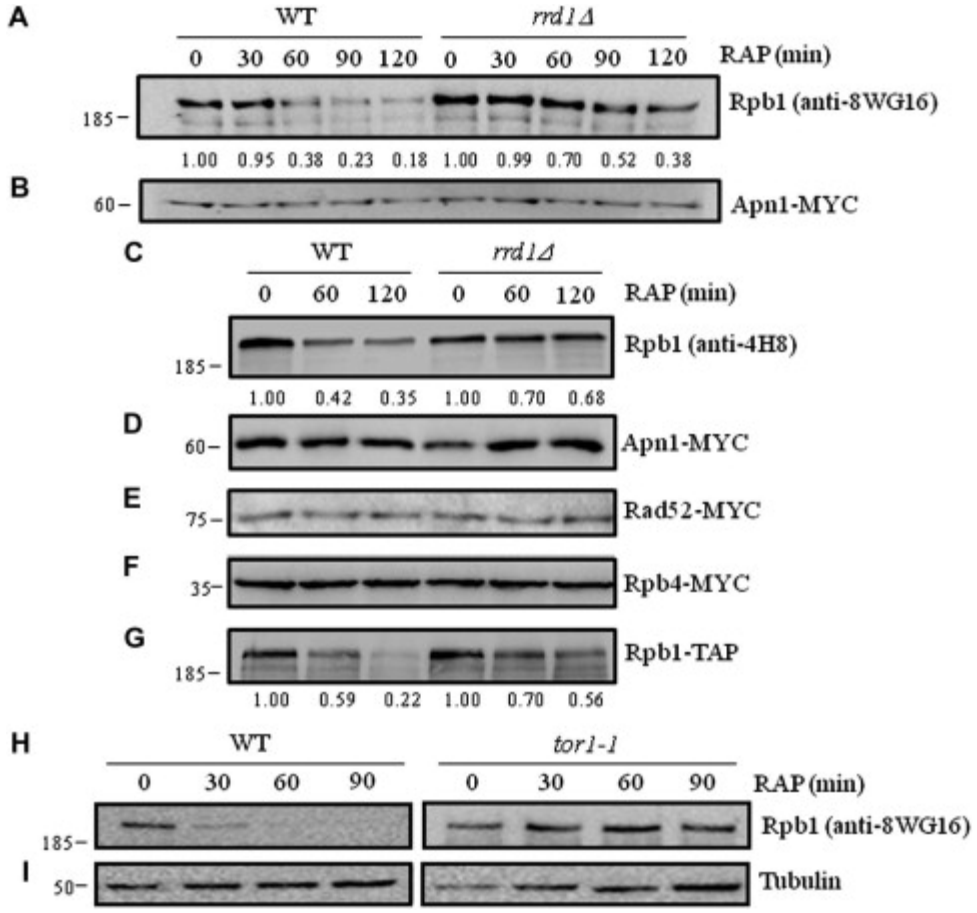


**Figure 3.1 : *rrd1*Δ or *tor1-1* mutants are unable to efficiently degrade RNAPII in response to rapamycin.**
**(A–I)** Exponentially growing wild-type yeast and the isogenic *rrd1*Δ mutant or the *tor1-1* mutant allele, expressing either Apn1-MYC, Rad52-MYC, Rpb4-MYC or Rpb1-TAP from the endogenous locus, were treated with rapamycin (200 ng/ml for 0–120 min). Total protein extracts were subjected to Western blot analysis using the indicated antibodies. RNAPII levels were calculated where the untreated is assigned an arbitrary unit of 1 and the treated calculated accordingly. The data is representative of at least three independent analyses.

To exclude the possibility that the disappearance of Rpb1 triggered by rapamycin is due to a general increase in protein degradation, we monitored the level of several other proteins including the DNA repair proteins Apn1 and Rad52, as well

as Rpb4, a component of RNAPII. The wild-type and *rrd1*Δ mutants expressing MYC-tagged proteins from the endogenous loci were treated with rapamycin as above and the total cell extracts probed with anti-MYC antibody. No significant changes were observed in the levels of these proteins (Fig. 3.1B, D, E and F), suggesting that rapamycin does not invoke a general protein degradation response, and that Rpb1 might be the only subunit of RNAPII complex that is degraded as reported for 4-NQO [25]. To ensure that the observed degradation of Rpb1 was not a reflection of the 8WG16 antibody preferentially recognizing the unphosphorylated form of the CTD [26-28], we conducted similar experiments in the same strain using a different antibody (4H8) which recognizes both the phosphorylated and unphosphorylated forms of the CTD of Rpb1 [29]. The 4H8 antibody also revealed that rapamycin triggered a decrease in Rpb1 level in the wild-type, but only modestly in the *rrd1*Δ mutant (Fig. 3.1C). In contrast, the control protein Apn1-MYC was not degraded (Fig. 3.1D). Additionally, we conducted similar experiments in a strain where Rpb1 was tagged with the tandem affinity purification tag (TAP) at the endogenous locus. We then monitored for Rpb1 level by using an antibody (anti-PAP) that recognizes the tag instead of the CTD of Rpb1. Anti-PAP also revealed that rapamycin triggered a decrease in Rpb1-TAP level in the wild-type, but only modestly in the *rrd1*Δ mutant (Fig. 3.1G). Thus, the various antibodies (8WG16, 4H8 and PAP) are indeed monitoring loss of Rpb1, instead of changes in post-translational modifications such as phosphorylation of the CTD.

The response to rapamycin is mediated through the TOR signaling pathway and thus we would expect less Rpb1 degradation if the TOR1 gene is mutated. As this

gene is essential, we used the conditional *tor1-1* mutant allele, which is known to be resistant to rapamycin [30]. We monitored whether Rpb1 is degraded over time in the *tor1-1* allele in response to rapamycin (Fig. 3.1H and I). Clearly, this *tor1-1* mutant allele was not able to degrade Rpb1 in response to rapamycin indicating that this is mediated through the TOR signaling pathway.

Since the loss of Rpb1 was monitored from total cell extracts, we next examined if this effect would be a reflection of the chromatin-bound Rpb1 in response to rapamycin. For this experiment, we used an established approach that separates soluble proteins from chromatin-bound [24]. In the untreated wild-type or *rrd1*Δ mutant, a significant amount of Rpb1 was found associated with the chromatin fraction (Fig. 3.2A, lane 3 and 9). Upon rapamycin treatment, nearly all of the Rpb1 in the soluble and chromatin fraction was lost in the wild-type cells (Fig. 3.2A lane 6 vs. 3); consistent with the notion that Rpb1 undergoes rapamycin-induced degradation. In contrast, the chromatin fraction derived from rapamycin-treated *rrd1*Δ mutant contained a substantial level of Rpb1 (Fig. 3.2A, lane 12 vs. 9), while the level in the soluble fraction was almost undetectable (Fig. 3.2A, lane 11). In these experiments, no major changes were observed for the control protein Apn1-MYC after rapamycin treatment (Fig. 3.2B).

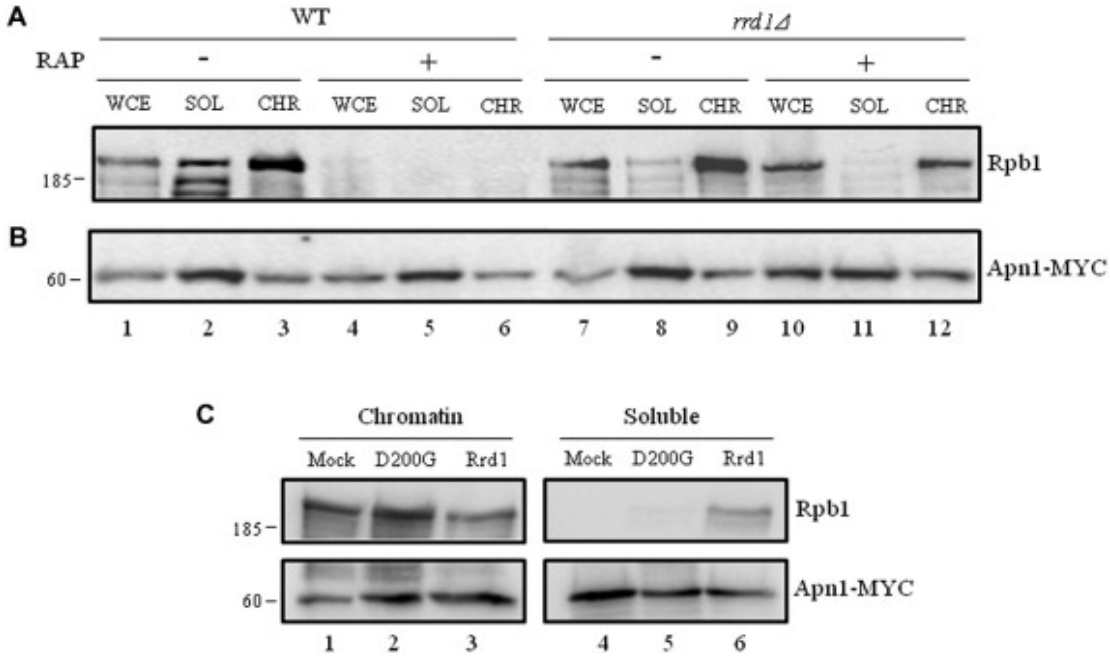


**Figure 3.2** : **Rrd1 is required for the release of RNAPII from chromatin.**
**(A and B)** Wild-type and *rrd1*Δ mutant strains expressing Apn1-MYC were challenged with (+) and without (−) rapamycin (200 ng/ml for 120 min) and whole cell extract (WCE), soluble (SOL) and chromatin [3] fractions (see Section 3.4) were analyzed for RNAPII and Apn1-MYC by Western blots. The data is representative of three independent analyses. **(C)** Purified HIS–Rrd1 (native and mutant G200D at a concentration of 1.6 μM) were added to the chromatin fraction isolated from *rrd1*Δ 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.

Next we tested whether the catalytic activity of Rrd1 is required for the process of releasing RNAPII from the chromatin. Using an *in vitro* assay, we recently documented that purified Rrd1 is capable of releasing RNAPII from the chromatin [8]. Basically, the chromatin is isolated from whole cells and the amount of attached RNAPII is monitored by Western blot assay. Addition of purified Rrd1 was shown to release RNAPII from the chromatin into the supernatant [8]. The

question remained whether Rrd1 acts through its catalytic peptidyl prolyl isomerase domain. To test this, we mutated a key residue in the catalytic center of the isomerase domain (D200G), known to abolish isomerase activity and tested whether this catalytic inactive mutant of Rrd1 is able to release RNAPII from the chromatin (Fig. 3.2C) [31-33]. The result showed that the wild-type form of Rrd1 was capable of releasing RNAPII from the chromatin (lane 3) into the soluble form (lane 6), but not the mutant D200G (lane 2 vs. 5), suggesting that indeed isomerization is an essential step for the release of RNAPII. Taken together, the data suggest that chromatin-bound RNAPII is degraded in a Rrd1-dependent manner in response to rapamycin. Our recent observation that Rrd1 acts as an elongation factor to regulate gene expression in response to rapamycin [7] raised the possibility that under starvation conditions, such as rapamycin treatment, the excessive RNAPII is removed from anabolic genes and moves to stress responsive and catabolic genes. Thus, when starvation persists the excessive RNAPII is degraded. Based on these findings, we believe that the transcriptional changes are associated and likely causal for the degradation of RNAPII [6, 7].

### 3.5.2 Rapamycin induces degradation of Rpb1 independent of ubiquitylation

Several stress conditions are known to induce degradation of Rpb1 through the ubiquitin/proteasome pathway [11, 12, 34]. For example, DNA damaging agents such as 254 nm-UV and 4-NQO cause elongating RNAPII to stall, leading to ubiquitylation-dependent RNAPII degradation [11-15]. Since this is the only known pathway of RNAPII degradation in response to stress, we reasoned that ubiquitylation

would mediate the loss of RNAPII following rapamycin treatment and this process could be defective in the *rrd1*Δ mutant, thereby leading to stable RNAPII levels. To examine the ubiquitylated form of RNAPII in total extracts, we immunoprecipitated RNAPII with anti-RNAPII antibody from rapamycin treated cell extracts and probed with a monoclonal anti-ubiquitin antibody [25]. To avoid bias introduced by rapamycin-induced RNAPII degradation, we only treated cells up to 60 min since further treatment caused substantial degradation of RNAPII (Fig. 3.1). In addition, we used an excess of total protein extract (2 mg) in comparison to a limiting amount of beads (20 μl) conjugated to anti-8WG16 to perform the immunoprecipitation. This resulted in the immunoprecipitation of similar amounts of RNAPII which were then probed with anti-ubiquitin (Fig. 3.3A). As shown in Fig. 3.3B, a basal level of ubiquitylated RNAPII existed in both the wild-type and the *rrd1*Δ mutant (lane 5 vs. 7), which is thought to represent RNAPII engaged in elongation [20]. Following rapamycin treatment for 60 min, the level of ubiquitylated RNAPII was unaltered in either strain (Fig. 3.3B, lane 6 vs. 8), suggesting that rapamycin does not appear to induce ubiquitylation of Rpb1. In control experiments, 254 nm-UV increased the level of ubiquitylated RNAPII to the same extent in both the wild-type and the *rrd1*Δ mutant, indicating that the ubiquitylation process of RNAPII is not impaired in the mutant (data not shown).

To further investigate if ubiquitylation is associated with RNAPII in response to rapamycin, we examined the RNAPII mutants K330R and K695R, which cannot be ubiquitylated, for degradation [17]. Total cell extracts derived from the wild-type carrying either the native RNAPII or its mutants showed a similar extent of degradation of the protein in response to rapamycin, again suggesting that rapamycin-

induced degradation of RNAPII is independent of ubiquitylation (Fig. 3.3C). In addition, we tested the RNAPII mutants for resistance to rapamycin (Fig. 3.3D). *Rrd1*Δ mutants are highly resistant to rapamycin and we would expect, if Rrd1 acts through ubiquitylation of RNAPII that the mutants K330R and K695R to be resistant. However, these mutants showed wild-type sensitivity to rapamycin and deleting the *RRD1* gene in this strain background increased the resistance to rapamycin indicating that the ubiquitylation pathway is not associated with Rrd1 function (Fig. 3.3D).

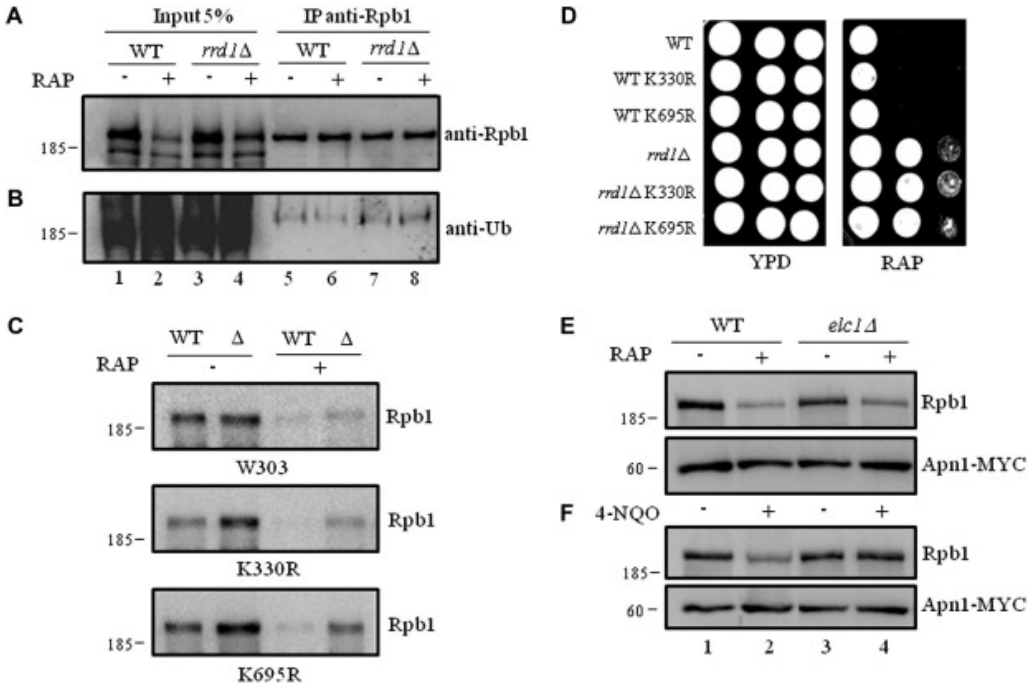


**Figure 3.3 : Ubiquitylation of RNAPII is not required for its response to rapamycin.**
**(A)** Total extracts (lanes 1–4) from either wild-type or *rrd1*Δ cells treated with (+) and without (−) rapamycin (200 ng/ml for 60 min) were immunoprecipitated with 8WG16 antibodies (lanes 5–8). **(B)** The total extracts and immunopreciptates from panel A were probed with anti-ubiquitin antibodies. The data is representative of three independent analyses. **(C)** Western blot analysis of RNAPII degradation in RNAPII mutants from wild-type (WT) and *rrd1*Δ mutant cells (Δ) without (−) and with (+) 200 ng/ml rapamycin for 2 h. **(D)** Spot test analysis of the wild-type and the RNAPII mutants on YPD or YPD media containing 5 ng/ml of rapamycin (RAP).

**(E)** Exponentially growing Apn1-MYC tagged wild-type (WT) or *elc1*Δ mutant strain were untreated (−) or treated (+) with rapamycin (200 ng/ml, 120 min) and total protein extracts were probed with anti-RNAPII (8WG16) to monitor the level of Rpb1. The membranes were probed with anti-MYC antibody to monitor for equal protein loading. **(F)** Same as in **(E)** except that cells were untreated (−) or treated (+) with 4-NQO (3 μg/ml, 60 min).

We finally decided to monitor for RNAPII degradation in an *elc1*Δ mutant strain since Elc1 is essential for the polyubiquitylation of RNAPII following DNA damage [14]. The different strains carrying Apn1-MYC endogenously tagged were treated with rapamycin (200 ng/ml, 120 min) (Fig. 3.3E) or 4-NQO (3 μg/ml, 60 min) (Fig. 3.3F) and whole cell protein extracts were monitored using 8WG16 antibody. Our results showed that RNAPII was degraded after rapamycin treatment in the *elc1*Δ mutant strain similar to the wild type strain (Fig. 3.3E). On the other hand, in the same *elc1*Δ mutant strain, RNAPII was not degraded in response to 4-NQO (Fig. 3.3F, lane 4) demonstrating the Elc1 requirement for RNAPII degradation for this specific DNA damaging agent. Apn1-MYC expression was monitored as a loading control. Taken together our results exclude a role of the Elc1-ubiquitylation pathway in the degradation of RNAPII following rapamycin treatment.

To summarize, (i) we confirmed that RNAPII is degraded in response to rapamycin in several strain backgrounds, (ii) that isomerization by Rrd1 is required for this process at the level of the chromatin and that (iii) this happens through an ubiquitylation independent pathway. The question still remains how RNAPII is degraded in response to rapamycin. One exciting possibility is that Rrd1 isomerizes

the CTD of Rpb1, already shown by our group, so that RNAPII becomes more susceptible for cleavage of its CTD resulting in its degradation [8].

It was previously demonstrated that in response to UVC radiation RNAPII is also degraded [25]. However, this degradation is dependent on ubiquitylation and not on the function of Rrd1 (data not shown), clearly indicating a different degradation pathway of RNAPII. UVC radiation induces specific DNA lesions and if these are not removed they can effectively block the movement of RNAPII during transcription. As a consequence, the stalled RNAPII is ubiquitylated and subsequently degraded [20, 25]. So far, there is no direct evidence that rapamycin can generate DNA lesions and, as such, we believe that this drug induces degradation of the excess RNAPII that arises as a result of the massive transcriptional reorganization. Despite the distinct modes of degradation of RNAPII triggered by the two different stresses, DNA damage and rapamycin, there seems to be some commonalities in the mechanisms. Both modes of degradation of RNAPII are mediated through elongation factors (Elc1 and Rrd1) and in both cases transcription of RNAPII is affected.

In conclusion, we propose that RNAPII is degraded by different mechanisms depending on the type of stress. In light of recent reports, we believe that rapamycin could cause a different marking of RNAPII such as acetylation and the resulting marked RNAPII is degraded via the autophagy pathway [35, 36].

## 3.6 ACKNOWLEDGMENT

This work was supported by a Grant (MOP-13152) from the Canadian Institutes of Health Research to D.R.

## 3.7 REFERENCES


1. Saylor, P.J. and M.D. Michaelson, *New treatments for renal cell carcinoma: targeted therapies.* J Natl Compr Canc Netw, 2009. **7**(6): p. 645-56.
2. Bjornsti, M.A. and P.J. Houghton, *The TOR pathway: a target for cancer therapy.* Nat Rev Cancer, 2004. **4**(5): p. 335-48.
3. Hardwick, J.S., et al., *Rapamycin-modulated transcription defines the subset of nutrient-sensitive signaling pathways directly controlled by the Tor proteins.* Proc Natl Acad Sci U S A, 1999. **96**(26): p. 14866-70.
4. Heitman, J., N.R. Movva, and M.N. Hall, *Targets for cell cycle arrest by the immunosuppressant rapamycin in yeast.* Science, 1991. **253**(5022): p. 905-9.
5. Crespo, J.L. and M.N. Hall, *Elucidating TOR signaling and rapamycin action: lessons from Saccharomyces cerevisiae.* Microbiol Mol Biol Rev, 2002. **66**(4): p. 579-91, table of contents.
6. Douville, J., et al., *The Saccharomyces cerevisiae phosphatase activator RRD1 is required to modulate gene expression in response to rapamycin exposure.* Genetics, 2006. **172**(2): p. 1369-72.
7. Poschmann, J., et al., *The Peptidyl Prolyl Isomerase Rrd1 Regulates the Elongation of RNA Polymerase II during Transcriptional Stresses.* PLoS One, 2011. **6**(8): p. e23159.
8. Jouvet, N., et al., *Rrd1 isomerizes RNA polymerase II in response to rapamycin.* BMC Mol Biol, 2010. **11**: p. 92.
9. Fournier, M.L., et al., *Delayed correlation of mRNA and protein expression in rapamycin-treated cells and a role for Ggc1 in cellular sensitivity to rapamycin.* Mol Cell Proteomics, 2010. **9**(2): p. 271-84.
10. Cardenas, M.E., et al., *The TOR signaling cascade regulates gene expression in response to nutrients.* Genes Dev, 1999. **13**(24): p. 3271-9.
11. Jung, Y. and S.J. Lippard, *RNA polymerase II blockage by cisplatin-damaged DNA. Stability and polyubiquitylation of stalled polymerase.* J Biol Chem, 2006. **281**[113]: p. 1361-70.
12. Inukai, N., et al., *A novel hydrogen peroxide-induced phosphorylation and ubiquitination pathway leading to RNA polymerase II proteolysis.* J Biol Chem, 2004. **279**(9): p. 8190-5.
13. Malik, S., et al., *Elongating RNA polymerase II is disassembled through specific degradation of its largest but not other subunits in response to DNA damage in vivo.* J Biol Chem, 2008.
14. Ribar, B., L. Prakash, and S. Prakash, *Requirement of ELC1 for RNA polymerase II polyubiquitylation and degradation in response to DNA damage in Saccharomyces cerevisiae.* Mol Cell Biol, 2006. **26**(11): p. 3999-4005.
15. Ribar, B., L. Prakash, and S. Prakash, *ELA1 and CUL3 are required along with ELC1 for RNA polymerase II polyubiquitylation and degradation in DNA-damaged yeast cells.* Mol Cell Biol, 2007. **27**(8): p. 3211-6.
16. Selth, L.A., S. Sigurdsson, and J.Q. Svejstrup, *Transcript Elongation by RNA Polymerase II.* Annu Rev Biochem, 2010. **79**: p. 271-93.

17. Somesh, B.P., et al., *Communication between distant sites in RNA polymerase II through ubiquitylation factors and the polymerase CTD.* Cell, 2007. **129**(1): p. 57-68.
18. Svejstrup, J.Q., *Contending with transcriptional arrest during RNAPII transcript elongation.* Trends Biochem Sci, 2007. **32**(4): p. 165-71.
19. Wysocki, R., et al., *Transcriptional activation of metalloid tolerance genes in Saccharomyces cerevisiae requires the AP-1-like proteins Yap1p and Yap8p.* Mol Biol Cell, 2004. **15**(5): p. 2049-60.
20. Somesh, B.P., et al., *Multiple mechanisms confining RNA polymerase II ubiquitylation to polymerases undergoing transcriptional arrest.* Cell, 2005. **121**(6): p. 913-23.
21. Vongsamphanh, R., P.K. Fortier, and D. Ramotar, *Pir1p mediates translocation of the yeast Apn1p endonuclease into the mitochondria to maintain genomic stability.* Mol Cell Biol, 2001. **21**(5): p. 1647-55.
22. Arima, Y., et al., *Transcriptional blockade induces p53-dependent apoptosis associated with translocation of p53 to mitochondria.* J Biol Chem, 2005. **280**(19): p. 19166-76.
23. Douville, J., et al., *The yeast phosphotyrosyl phosphatase activator protein, yPtpa1/Rrd1, interacts with Sit4 phosphatase to mediate resistance to 4-nitroquinoline-1-oxide and UVA.* Curr Genet, 2004. **46**(2): p. 72-81.
24. Svejstrup, J.Q., T.G. Petrakis, and J. Fellows, *Purification of elongating RNA polymerase II and other factors from yeast chromatin.* Methods Enzymol, 2003. **371**: p. 491-8.
25. Beaudenon, S.L., et al., *Rsp5 ubiquitin-protein ligase mediates DNA damage-induced degradation of the large subunit of RNA polymerase II in Saccharomyces cerevisiae.* Mol Cell Biol, 1999. **19**(10): p. 6972-9.
26. Jeong, S.J., et al., *Role of RNA polymerase II carboxy terminal domain phosphorylation in DNA damage response.* J Microbiol, 2005. **43**(6): p. 516-22.
27. Jones, J.C., et al., *C-terminal repeat domain kinase I phosphorylates Ser2 and Ser5 of RNA polymerase II C-terminal domain repeats.* J Biol Chem, 2004. **279**(24): p. 24957-64.
28. Phatnani, H.P. and A.L. Greenleaf, *Phosphorylation and functions of the RNA polymerase II CTD.* Genes Dev, 2006. **20**(21): p. 2922-36.
29. Kristjuhan, A., et al., *Transcriptional inhibition of genes with severe histone h3 hypoacetylation in the coding region.* Mol Cell, 2002. **10**(4): p. 925-33.
30. Lorenz, M.C. and J. Heitman, *TOR mutations confer rapamycin resistance by preventing interaction with FKBP12-rapamycin.* J Biol Chem, 1995. **270**(46): p. 27531-7.
31. Chao, Y., et al., *Structure and mechanism of the phosphotyrosyl phosphatase activator.* Mol Cell, 2006. **23**(4): p. 535-46.
32. Jordens, J., et al., *The Protein Phosphatase 2A Phosphatase Activator Is a Novel Peptidyl-Prolyl cis/trans-Isomerase.* J Biol Chem, 2006. **281**(10): p. 6349-6357.
33. Leulliot, N., et al., *Crystal structure of the PP2A phosphatase activator: implications for its PP2A-specific PPIase activity.* Mol Cell, 2006. **23**[113]: p. 413-24.

34. Ratner, J.N., et al., *Ultraviolet radiation-induced ubiquitination and proteasomal degradation of the large subunit of RNA polymerase II. Implications for transcription-coupled DNA repair.* J Biol Chem, 1998. **273**(9): p. 5184-9.
35. Dyavaiah, M., et al., *Autophagy-dependent regulation of the DNA damage response protein ribonucleotide reductase 1.* Mol Cancer Res, 2011. **9**(4): p. 462-75.
36. Robert, T., et al., *HDACs link the DNA damage response, processing of double-strand breaks and autophagy.* Nature, 2011. **471**(7336): p. 74-9.

# CHAPTER 4

## Article 3

## Investigating the biological role of PTPA in mammalian cells

Nathalie Jouvet and Dindial Ramotar

*Article in preparation*

# 4 ARTICLE #3

## 4.1 AUTHOR CONTRIBUTION

All of the experiments have been designed and performed by NJ under the supervision of DR. The manuscript was written by NJ.

## 4.2 ABSTRACT

PTPA, the mammalian homolog of Rrd1, has been shown to activate protein phosphatase 2A (PP2A). Our laboratory previously found that overexpression of PTPA leads to apoptosis independently of PP2A. The molecular function of Rrd1/PTPA is still largely unknown and our research in yeast reveals a novel role in transcription regulation and we believe it could be similar in mammals.

We showed that PTPA knockdown did not affect sensitivity to rapamycin, 4-NQO or $H_2O_2$. We also attempted to find protein interaction partners for PTPA using tandem affinity purification, but no stable partners for PTPA were found. In addition, mass spectrometry showed no evidence for phosphorylation or ubiquitylation. Finally, we attempted to study PTPA in a mouse model. We first determined that PTPA was expressed in a tissue-specific manner and was most abundant in the bone marrow, thymus and brain. We pursued creation of a knockout mouse and successfully generated chimeras, but the mutated cells were not transmitted to the germline.

My data and other data from our laboratory regarding the yeast work suggest a general role for Rrd1 in gene regulation. Whether PTPA has a similar function in mammalian cells remains unknown, and a different vision of what the protein does in mammalian cells will be required to adequately address this question in the future.

## 4.3 INTRODUCTION

After characterizing the role of Rrd1 in RNA Pol II regulation in yeast [1, 2], we next sought to investigate the human homolog of Rrd1, PTPA, using cell culture and mouse models. The *PTPA* gene is found at the 9q34 region in human and translocations resulting in leukemia have been reported at this locus [3]. The *PTPA* gene is highly expressed in several tissues such as the brain, kidney, liver, spleen and testis [4]. It has also been shown that *PTPA* transcription is higher in lymphoblastoid cells in comparison with monocytic cells [3]. In mammalian cells, *PTPA* was first characterized as a protein that stimulates the weak phosphotyrosyl phosphatase activity of the type 2A Ser/Thr phosphatase PP2A [5] dependant on $Mg^{2+}$ and ATP *in vitro*. PP2A family members are important Ser/Thr phosphatases that regulate a plethora of cellular pathways [6, 7] and their regulation still remains poorly understood.

The crystal structure of PTPA does not show similarity to any known family of proteins. PTPA has 17 α helices and 4 short β strands and adopts a compact α-helical structure [8] and this structure is conserved in yeast since comparison in the overall structures of Rrd1, Rrd2 and PTPA are essentially identical [11]. PTPA structure can be divided into three subdomains; lid, core and linker. The protein contains 323 amino acids and around 40 conserved residues among five species (*Homo sapiens*, *Xenopus laevis*, *Drosophila melanogaster*, *Caenorhabditis elegans* and *Saccharomyces cerevisiae)* are exposed on the surface of the PTPA protein between the core and the linker domains and could likely be an important binding site

for partners such as PP2A members. Analysis of 18 point mutation among the invariant amino acids described previously lead to differential interaction with PP2A suggesting a role for PTPA in the regulation of these phosphatases. For example, five mutations, V209D, E270A, V281D, G290D and M294D showed compromised binding to PP2A and indeed low or undetectable ATPase activity from the complex [8]. A recent study in which the PP2A interaction network was analysed identified multiple subunits of the chaperonin containing TCP-1 (CCT) as partners of PTPA, but the biological function of this interaction remains unknown [9]. Finally, studies previously performed by our lab showed that overexpression of PTPA in mammalian cells lead to apoptosis via a caspase 3-dependent pathway. This apoptosis was also independent of PP2A, MAP kinase, and p53 [10].

Like its yeast homolog Rrd1, PTPA is a peptidyl prolyl *cis-trans* isomerase (PPIase). PTPA has been shown to isomerize synthetic peptides derived from the catalytic subunit of PP2A [11]. The previous chapters of my thesis have shown that Rrd1 isomerizes the RNA pol II CTD in response to rapamycin, leading to Pol II degradation via a ubiquitylation independant pathway. Based on the findings in yeast and because PTPA is highly conserved, we hypothesized that PTPA performs a similar function in mammalian cells independently of its PP2A function. This chapter will describe our attempts to study PTPA in mammalian systems using a variety of approaches.

## 4.4 MATERIAL AND METHODS

### 4.4.1 Cells, culture conditions and siRNA transfection

HCT116 human colorectal carcinoma, HeLa cervical cancer cells, U2OS osteosarcoma cells, K562 human immortalised myelogenous leukemia cell line, and Phoenix virus-producing cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and penicillin-streptomycin. The small interfering RNA (siRNA) were purchased from Ambion (Austin, TX; catalog no.16704 and 4611) and transfection efficiency was measured using the Silencer FAM-Labeled Negative Control #1 siRNA (catalog no. 4620; Ambion). The small hairpin RNA (shRNA) were purchased from Sigma-Aldrich. Cells were transfected with either a nontargeting control or a PTPA RNA interference (RNAi) plasmid using Lipofectamine 2000 (Invitrogen).

### 4.4.2 Western blotting

Cells were transfected as above with either siRNA against PTPA or the nontargeting control siRNA and harvested by trypsinization at various times post-transfection followed by centrifugation at 1000 rpm for 1 min. Cell pellets were resuspended in 100 µl of lysis buffer containing 150 mM NaCl, 1 mM EDTA, 10 mM Tris HCl, 1% w/w sodium deoxycholate, 1% v/w Nonidet P-40, 2 µg/ml aprotinine, 1 µg/ml leupeptine, 1 mM sodium vanadate ($Na_3VO_4$) and 1 µM PMSF. Samples were incubated on ice for 1hr, centrifuged at 14000 rpm for 30 min, and total protein in the supernatant was quantified by the Bradford assay. Protein extracts were resolved on

10% denaturing polyacrylamide gels and transferred by electroblotting to a nitrocellulose membrane (Amersham Biosciences). After blocking with 5% nonfat dry milk in Tris buffered saline (1 X TBS containing 50 mM Tris and 150 mM NaCl pH 7.5), the membrane was incubated with the indicated primary antibodies overnight at 4°C. Primary monoclonal antibodies include anti-PTPA (Upstate) -p53 (DO-1, Santa Cruz), -p21 (Ab-1,Calbiochem), -PCNA (PC10, Santa Cruz), -COX VI (Cell Signaling Technology), -HA (Santa Cruz) and -Tubulin (Santa Cruz). Immunoreactive proteins were localized with horseradish peroxidase–conjugated secondary antibodies (Amersham Biosciences) for 1h at room temperature and visualized using enhanced chemiluminescence (Perkin Elmer Las, Boston, CA).

### 4.4.3 Colony formation assays.

HCT116 cells (~1 X $10^6$) were plated in 60 X 15 mm plates and incubated for 24h. Cells were treated with siRNA. Cells were trypsinized (Gibco) and treated with the appropriate drug or taken at various times post-transfection, centrifuged (1000 rpm for 1 min), diluted, and seeded in triplicate at a density of 500-1000 cells (Beckman cell counter) per 60 mm plate containing 5 ml of fresh media, and allowed to form colonies for 12 to 14 days. The colonies were stained with methylene blue (0.5% methylene blue in 50% methanol).

### 4.4.4 Chromatin assay

Cells (5 x $10^6$) were washed with PBS and resuspended in 150 µl of the lysis buffer (10 mM Hepes (pH 7.5), 10 mM KCl, 3 mM MgCl2, 0.35 M sucrose, 0.1%

NP40, 3 mM 2-mercaptoethanol, 0.4 mM PMSF, 1 μM pepstatin A, 1 μM leupeptin and 5 μg/ml aprotinin). The cells were incubated on ice for 5 min. Cytoplasmic proteins were removed from nuclei by centrifugation at 1300 g for 5 min. The nuclei pellet was resuspended in lysis buffer and nuclei were spun down by centrifugation. Isolated nuclei were resuspended in 200 μl of solution containing 3 mM EDTA, 0.2 mM EGTA, 1 mM dithiothreitol, 100 mM NaCl and 0.8% NP40. The nuclei were incubated on ice for 60 min, and soluble nuclear proteins (soluble fraction) were separated from chromatin by centrifugation at 1700 g for 5 min. The chromatin pellet was washed twice with the same solution as the above and spun down at high speed (10 000 g for 1 min), and the chromatin was resuspended in SDS sample buffer and sheared by sonication (chromatin fraction). Both fractions were subjected to SDS/PAGE and immunoblot analysis [12].

### 4.4.5 Isolation of RNA and RT-PCR

Total cellular RNA was isolated from HCT116 cells using TRIzol reagent (Invitrogen). A total of 5 μg of total RNA were reverse-transcribed in 20 μl of reaction mixture containing the RT buffer (Invitrogen), 10 mM dithiothreitol (Invitrogen), 1 mM each deoxynucleotide triphosphate (dNTP) (Amersham Biosciences), 40 units of RNaseOUT (Invitrogen), 0,6 μg of random primers (Invitrogen) and 20 units of Moloney murine leukemia virus reverse transcriptase (Invitrogen).

### 4.4.6 Mitochondrial fractionation

Cells transfected with the PTPA siRNA or nontargeting control siRNA were fractionated using the mitochondria isolation kit for cultured cells (PIERCE). The different fractions were subjected to western blotting analysis.

### 4.4.7 Cell cycle analysis

At various time points, PTPA siRNA or negative control siRNA transfected cells were washed with PBS containing 50 mM EDTA, trypsinized, resuspended in 1 ml of PBS/EDTA and fixed by addition of 3 ml of ice-cold 100% ethanol. Fixed cells were pelleted, washed with 4 ml of PBS/EDTA and stained with modified Krishan buffer [0.05 mg/ml propidium iodide (PI) (Sigma-Aldrich), 0.1% sodium citrate, 0.2 mg/ml RNase A and 0.3% v/v NP40] and analyzed using a FACScan flow cytometer equipped with CellQuest software (Beckton Dickinson).

### 4.4.8 Purification of PTPA-associated proteins and co-immunoprecipitation

HeLa cell line stably expressing Flag-HA-PTPA (WT) was generated following retroviral transduction and 3 rounds of selection using magnetic beads as previously described [13]. HeLa ($9 \times 10^9$) cells were used for the purification of PTPA-associated proteins, essentially as previously described [13] using the whole cell extract. Mass spectrometry analysis was provided by the Taplin Mass Spectrometry Facility (Harvard Medical School, Boston, MA). Standard coimmunoprecipitations using appropriate antibodies were conducted as previously described [14].

### 4.4.9 Plasmids

Retroviral constructs that express N-terminal Flag-hemagglutinin (HA)-tagged wild-type (WT) or mutant forms of human PTPA were generated by subcloning the cDNA into the POZ-N plasmid provided by E. Affar [13]. The catalytically inactive PTPA construct, POZ-PTPA (G205D), POZ-PTPA (V209D) and POZ-PTPA (G290D) were generated by site-directed mutagenesis using the QuikChange Lightning Kit (Stratagene).

### 4.4.10 Generation of knockout mice and genotyping

The mouse 129S2/SvPas embryonic stem cell (ESC) line D3H, carries a *Ptpa* allele disrupted by the insertion in the first intron of a gene trap vector (U3NeoSV1) kindly provided by the Sanger Institute Gene Trap Resource (SIGTR). The U3NeoSV1 promoter-trap provirus contains the ampicillin resistance (amp) gene and a plasmid origin of replication (Ori) flanked by the neomycin resistance (neo) gene in each long terminal repeat (LTR). Injection of these cells into C57Bl/6 blastocysts at the McIntyre Transgenic Core Facility Service from McGill University (Montreal, Canada) resulted in chimeric mice that were bred with CD1 mice to attempt creation of a germline transmission of the *Ptpa* mutant allele. Progeny were weaned at day 21. A PCR genotyping strategy was used to differentiate between the mutant and wild-type alleles from DNA extracted from tail tips. A common forward primer was used alongside reverse primers specific to each allele. Primer sequences were: forward (10511F), 5′- GAGGCTGCGTTCTTTATGAGACTC-3′; mutant reverse (neoIN10), 5′-CAGGTCGGTCTTGACAAAAAGAAC-3′; and wild type

reverse (10511R), 5′- AAAAAGGAAGTGTGGTGGAAGGTAC-3′. These primers amplified bands of 442 nt and 477 nt for the mutant and wild-type allele, respectively.

## 4.5 RESULTS

### 4.5.1 Reduction of PTPA by siRNA knockdown in HCT116 cells

To determine whether human PTPA functions similarly to yeast Rrd1, we first sought to transiently knock down PTPA in cultured human cells. We first assessed the effectiveness of siRNAs derived from three separate regions of PTPA in the colon cancer cell line HCT116. Briefly, cells were transfected, RNA was recovered and subjected to RT-PCR with PTPA-specific oligos. As shown in Figure 4.1A, the siRNA construct B used at a concentration of 30 nm was the most effective to deplete mRNA level of PTPA with an efficiency of about 80% (quantification in Figure 4.1B). We also monitored PTPA depletion at the protein level by Western blotting. PTPA was reduced by about 80% (quantification in Figure 4.1D) using the same siRNA construct B (Figure 4.1C). A FAM-labelled siRNA was used to monitor transfection efficiency and found that about 90% of the cells were transfected (Figure 4.1E).

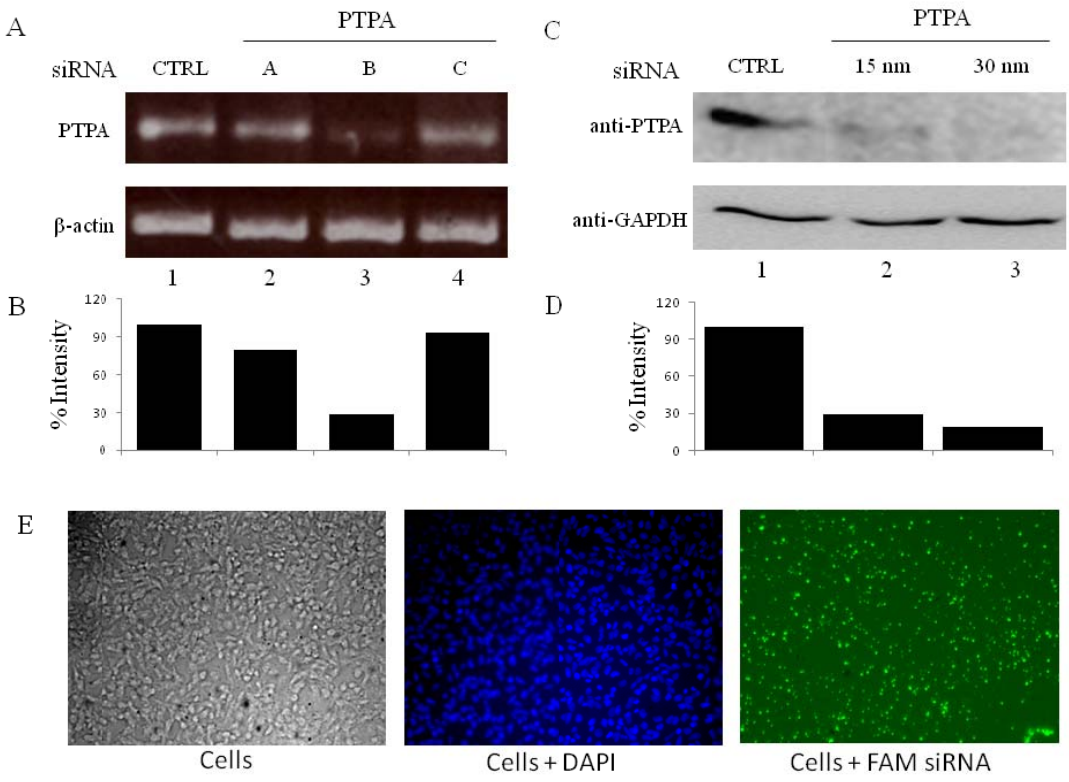


**Figure 4.1 : Reduction of PTPA by siRNA knockdown in HCT116 cells.**
Knockdown of PTPA mRNA in transiently transfected HCT116 cells. The different siRNA (A, B or C) or an siRNA negative control were transfected into 50-60% confluent HCT116. Total cell extracts were prepared and processed for RT-PCR using PTPA primers or β-actin primers (**A**). These extracts were also processed for Western blot analysis using anti-PTPA monoclonal antibody or GAPDH antibody (**C**). Each lane contained 100 µg of total protein extract. Quantification was done using alphaImager for RNA (**B**) or Multi Gauge V2.3 for protein (**D**). The results are representative of three independent analyses. **E)** HCT116 cells were transiently transfected using a FAM-labelled siRNA (green) and stained with DAPI (blue). Expression was observed using a fluorescence microscope.

### 4.5.2 PTPA siRNA diminishes colony formation

Following transfection with siRNA B, we noticed that more cells were dying in comparison with the negative control siRNA, which led us to investigate whether PTPA knockdown leads to a cell growth defect. We used clonogenic assays with

HCT116 cells to monitor colony formation after siRNA knockdown of PTPA for 36 hours. 60% of the cells survived after PTPA knockdown as compared to nearly 100% for the control siRNA. Thus, reduction of PTPA levels leads to a defect in cell proliferation.

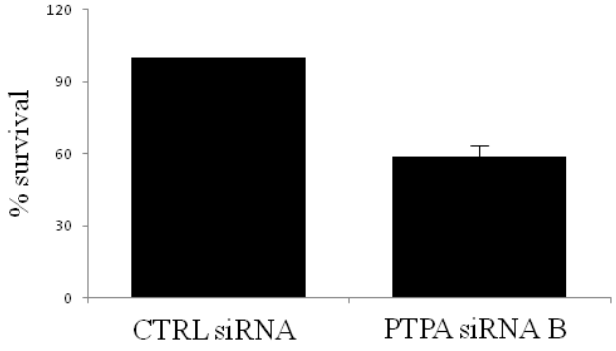


**Figure 4.2 : PTPA siRNA diminishes colony formation.**
HCT116 cells were transiently transfected with either siRNA B or siRNA negative control and viability was determined after 36 hours of transfection using the colony formation assay (see Methods). The means and standard deviations of at least three independent experiments are shown.

### 4.5.3 RNA pol II accumulates on chromatin after PTPA knockdown

In *rrd1Δ* yeast cells, RNA pol II accumulates on chromatin (see Chapter 3). We next attempted to determine if this accumulation also occurs in mammalian cells when the PTPA level is decreased. We used a technique in which the chromatin-bound proteins were isolated and separated from the rest of the cell extract. HCT116 cells were collected after treatment with siRNA against PTPA and chromatin

fractions were prepared and analyzed by Western blotting. In the Figure 4.3A, the total sample was loaded and analysed using the 8WG16 antibody against RNA pol II. RNA pol II accumulated more on chromatin in PTPA knockdown cells in comparison with untreated cells, similar to our results in yeast cells. The same membrane was probed with the H2A-phosphorylated antibody in the bottom panel as a control to distinguish between chromatin bound proteins and soluble proteins. The experiment was repeated using another loading control (APE1) and including cells untreated with siRNA, and similar results were obtained (Figure 4.3B). Taken together, our results show that RNA pol II accumulates on chromatin following PTPA depletion, which is compatible with our data in yeast.

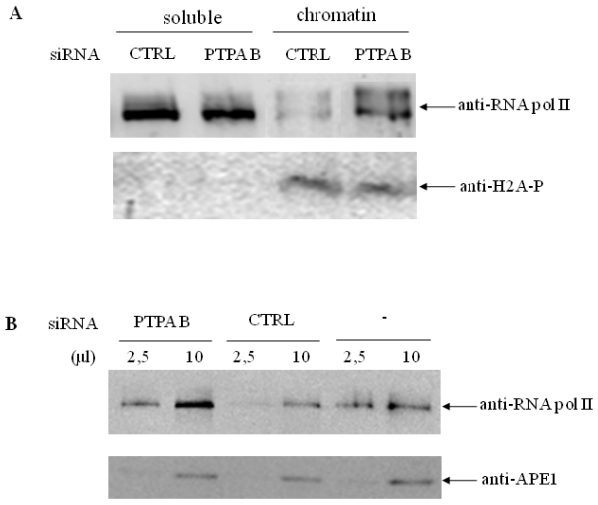


**Figure 4.3 : RNA pol II accumulation in PTPA knockdown HCT116 cells.**
**A)** HCT116 cells were transiently transfected with either the control (CTRL) or the PTPA siRNA B and proteins from the nucleus were extracted as described in material and methods. The proteins were analyzed by western blot with anti-8WG16 or H2A-P antibodies. **B)** The cells were processed as in panel A but only the chromatin fraction at different concentration was analyzed by western blot using the anti-8WG16 or anti-APE1. The result is representative of three independent analyses.

### 4.5.4 The growth defect in PTPA knockdown cells is p53-independent

In order to better understand the cause of this growth defect, we decided to look at the expression level of key proteins involved in cell proliferation and apoptosis. Since it is known that inhibition of RNA pol II on chromatin leads to accumulation of p53 [15], we assessed p53 levels by Western blotting following PTPA knockdown. p53 protein level was increased in the PTPA knockdown cells (Figure 4.4A). We also monitored the p21 (WAF1) level since its transcription is regulated by p53 [16]. p21 upregulation can lead to cell cycle arrest [17] which could explain the colony formation defect in the PTPA knockdown cells. p21 was unchanged following PTPA knockdown, however excluding this pathway to explain the growth defect (Figure 4.4B).

Since accumulation of p53 in mitochondria promotes apoptosis [18], we asked whether p53 accumulated in the mitochondria following PTPA knockdown. Cell fractionation was performed to obtain mitochondrial fractions, which were analyzed by Western blotting. As shown in Figure 4.4F, cells knocked down for PTPA had more p53 protein in the cytosol and mitochondria, consistent with Figure 4.4A, but there was no accumulation in the mitochondria.

Since PTPA knockdown led to an increase in p53 levels in both the cytosol and mitochondria, we next asked whether p53 depletion would rescue the PTPA growth defect. We therefore knocked PTPA down in p53 -/- HCT116 cells and monitored colony formation. As shown in Figure 4.4G, both wild-type and p53 -/-

cells showed the same growth defect after PTPA siRNA. Taken together, these results show this growth defect is p53-independent.

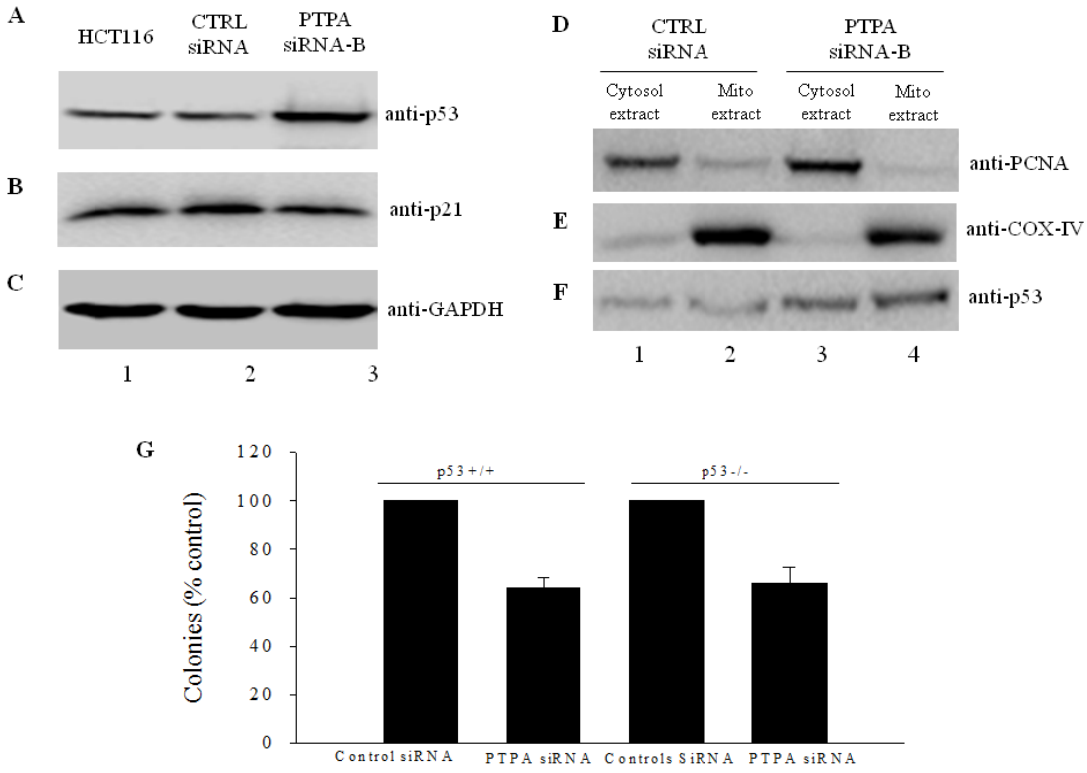


**Figure 4.4 : p53-independent cell death in PTPA knockdown cells.**
**A to C)** HCT116 cells were transiently transfected with the control siRNA (CTRL) or PTPA siRNA and whole cell protein extract were analyzed by westernblot with p53 **(A)**, p21 **(B)** and GAPDH (loading control) **(C)** antibodies. **D to F)** HCT116 cells were transfected as in A) and were fractionated using the mitochondria isolation kit for cultured cells (PIERCE). Each fraction was analyzed by westernblot using PCNA **(D)**, COX-IV **(E)** and p53 **(F)** antibodies. PCNA and COX-IV were used to validate the purification method. **G)** HCT116 cells were transiently transfected as above and viability was determined using trypan blue exclusion assay. The means and standard deviations of at least three independent experiments are shown for each panel.

### 4.5.5 PTPA knockdown cells accumulate in G1

In order to better define the growth defect in cells that have been depleted of PTPA, we chose to look at the cell cycle distribution. Cells were collected at different times following PTPA knockdown, dyed with propidium iodide (PI) and subjected to FACS analysis. Following PTPA knockdown, cells were delayed in G1 and did not progress through S phase as compared to control cells (Figure 4.5). Therefore, loss of PTPA causes a reduction in colony formation because the cells become arrested in G1.

These results support a model in which loss of PTPA leads to RNA pol II accumulation on chromatin and increased p53 levels. This leads to G1 arrest and decreased cell viability, although the colony formation defect is not p53-dependent. To further characterize the G1 arrest phenotype, we sought to follow the arrest to further time points and investigate activation of cell cycle checkpoints in PTPA-deficient cells. To accomplish this, we attempted to create cell lines in which PTPA was stably knocked down. A stable knockdown cell line would be useful for a variety of future studies and would avoid complications introduced by transient transfection of siRNA.

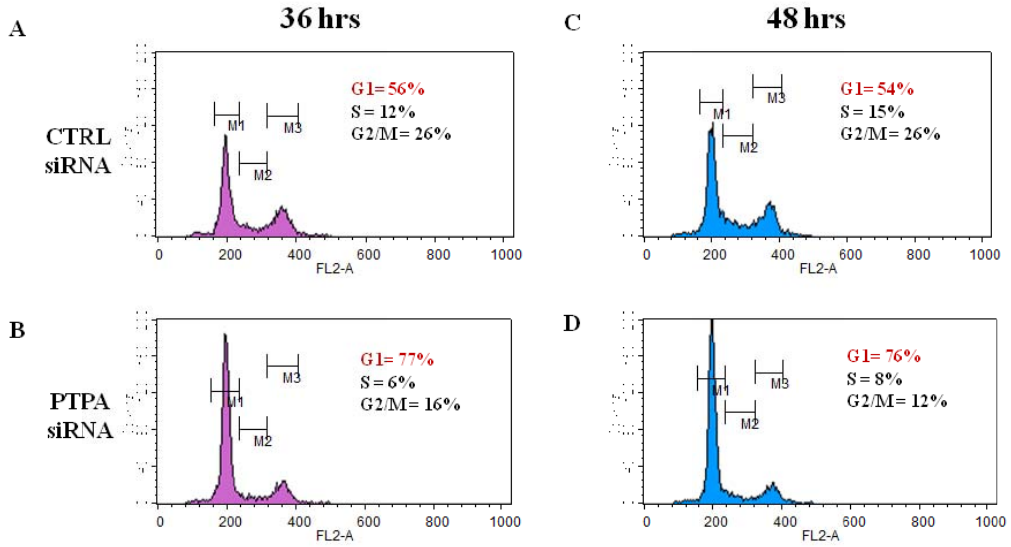


**Figure 4.5 : PTPA knockdown cells accumulate in G1 phase.**
HCT116 cells were transiently tranfected with the control siRNA (CTRL) (**A and C**) or PTPA siRNA (**B and D**) and were collected after 36 and 48 hours (hrs). Cells were fixed and the DNA was dyed using propidium iodide. The cells were analyzed using a FACScan flow cytometer equipped with CellQuest software. The data is representative of three independent experiments.

### 4.5.6 PTPA shRNA knockdown cells do not exhibit an *rrd1*Δ-like phenotype

We tested 4 different shRNA constructs against PTPA in HCT116 cells. Transient transfection of several of these efficiently reduced expression of PTPA to an extent similar to siRNA knockdown (Figure 4.6A), but surprisingly, we did not observe a growth defect with any of them (data not shown). This unexpected observation revealed that the growth defect seen with the siRNA could be due to off-target effects of the PTPA siRNA [19-21]. In order to circumvent this issue, we choose to continue our analysis of PTPA knockdown with shRNA because this

method is generally thought to produce fewer off-targets effects [22] and allows the possibility to create a stable knockdown cell line.

Since knockdown of PTPA with shRNA did not lead to a growth defect, we next assayed for other PTPA phenotypes using the yeast Rrd1 work as a guide. Since *rrd1Δ* yeast are sensitive to 4-NQO and $H_2O_2$ and resistant to rapamycin [23-26], we asked if the PTPA knockdown cells showed a similar response. Unfortunately, as shown in Figure 4.6B-E, cells knocked down for PTPA using shRNA did not show sensitivity or resistance to any of the drugs used (4-NQO, rapamycin, MMS or $H_2O_2$).

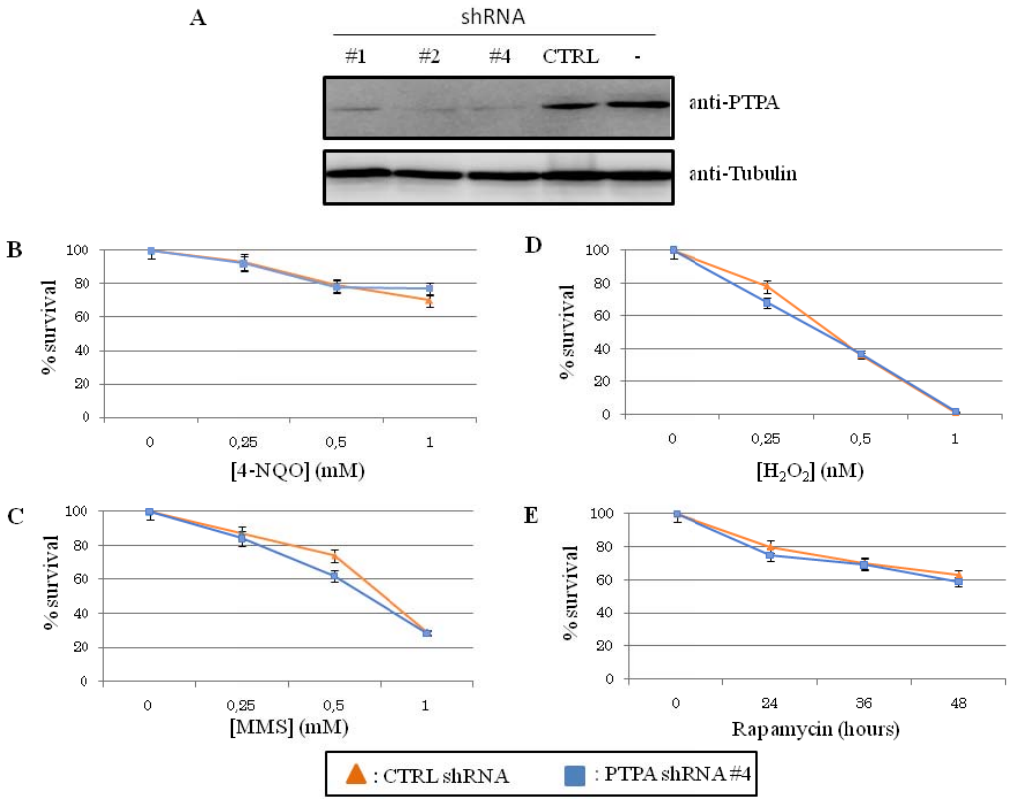


**Figure 4.6 : PTPA knockdown cells by shRNA does not lead to sensitivity to 4-NQO, MMS or $H_2O_2$ or resistance to rapamycin.**
(**A**) HCT116 cells were transiently transfected with control (CTRL) shRNA or with a different PTPA shRNA construct. PTPA or Tubulin (loading control) antibodies were used. HCT116 cells were transiently transfected with the control (CTRL)

shRNA or the PTPA shRNA #4 for 36 hours. The cells were treated with either 4-NQO (**B**), MMS (**C**) or $H_2O_2$ (**D**) for 1 hour at various concentrations and viability was determined using trypan blue exclusion assay. The cells were plated in triplicate following transfection and treated with rapamycin (**E**). The number of cells was determined every 12 hours. The means and standard deviations of at least three independent experiments are shown for each panel.

Since we did not see any phenotype with HCT116 cells, we decided to test other cell lines, since the function of PTPA might vary in different cell types. We knocked down PTPA in HeLa (cervical cancer) (Figure 4.7A), HDLF (normal human primary lung fibroblasts) (Figure 4.7B) and K562 (human immortalised myelogenous leukaemia) cells (Figure 4.7C). For each cell line we transfected two different PTPA shRNA constructs and checked for PTPA knockdown using western blot analysis. We did not follow up with further experiments on any of these cell lines because we were unable to get consistent knockdown.

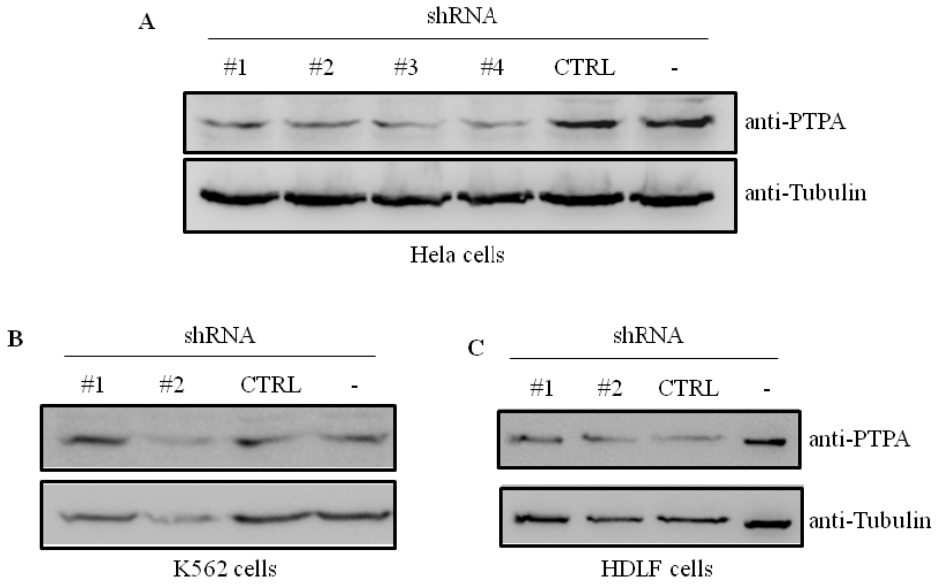


**Figure 4.7 : PTPA knockdown in various cell lines.**
Total cell extracts from HeLa cells (**A**), K562 cells (**B**) or HDLF cells (**C**) transiently transfected with different PTPA shRNA or the shRNA negative control (CTRL) were

prepared and processed for Western blot analysis using anti-PTPA monoclonal antibody or Tubulin antibody as a loading control. These blots are representative of at least 3 different experiments.

### 4.5.7 PTPA does not stably interact with other proteins and shows no evidence of post-translational modifications

To gain further insight into the function of PTPA, we decided to hunt for PTPA-interacting partners using affinity capture. The PTPA crystal structure revealed a conserved pocket which could be a possible protein-protein interaction domain [8]. Dr. El Bachir Affar's laboratory collaborated with us on this experiment.

We first tagged PTPA with FLAG-HA in an overexpression vector. Because overexpression of PTPA in mammalian cells leads to apoptosis [10], we decided to mutate key amino acids that would suppress the pro-apoptotic activity of PTPA, but would be expected to retain interactions with potential partners. We used 3 different mutations (G205D, V209D and G290D) that have been previously shown to be catalytically inactive [8]. HeLa cells were transiently transfected with each of the 3 mutants and wild-type PTPA and cell extracts were monitored for PTPA expression by Western blotting. As shown in Figure 4.8A, the cells transfected with the mutants expressed high levels of PTPA and grew normally (data not shown). On the other hand, overexpression of the wild-type PTPA killed most of the cells (data not shown) and made the collection of cell extracts difficult. Nonetheless, we were able to collect enough extract to perform a Western, which showed expression of tubulin (included

as a loading control) but not PTPA (Figure 4.8A). This is likely because the cells expressing PTPA died, and those that remained were untransfected.

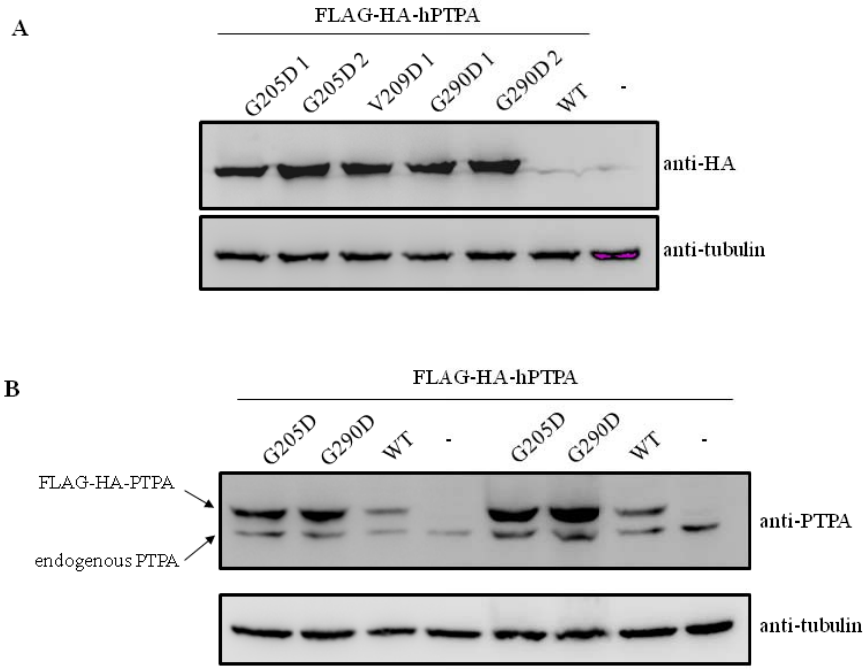


**Figure 4.8 : FLAG-HA-hPTPA overexpression in HeLa cells.**
**A)** HeLa cells were transiently transfected with plasmids expressing wild-type or mutant PTPA as indicated. Whole cell extracts were processed and analyzed by western blot using anti-PTPA and anti-Tubulin (loading control) antibodies. **B)** Whole cell protein extract from HeLa cells stably expressing either the native FLAG-HA-hPTPA construct or the site-directed mutated construct were analyzed by western blot with anti-PTPA and anti-tubulin (loading control) antibodies. Both the endogenous and the overexpressed version of PTPA are visible.

We then generated HeLa cells stably expressing each plasmid in order to proceed with the purification of protein complexes. Each plasmid was co-transfected with retroviral proteins in 293GPG cells, leading to stable retroviral expression of PTPA. We then used magnetic beads coupled to the anti-IL2 antibody to select the

cells that had incorporated the plasmid. After 3 rounds of selection using the magnetic beads, stable clones were assayed for PTPA expression using immunofluorescence and western blotting. As shown in Figure 4.8B, the PTPA antibody was able to recognize both the FLAG-HA labelled protein and the endogenous PTPA level. Surprisingly, we were able to create stable clones overexpressing wild-type FLAG-HA-hPTPA protein, although these cells expressed low levels of PTPA, which likely explains how they were able to survive. We chose to continue the purification using only the FLAG-HA-hPTPA wild-type construct, since using a wild-type protein is more likely to identify true interacting partners.

To identify potential complexes, we conducted a large-scale double immunopurification of the protein using anti-Flag and anti-HA columns. Unfortunately, silver staining of the eluted material revealed that no polypeptides copurified with PTPA when compared to the mock purification (Figure 4.9). Although there is a distinct band under PTPA, Western blotting revealed that this was a PTPA degradation product (data not shown).

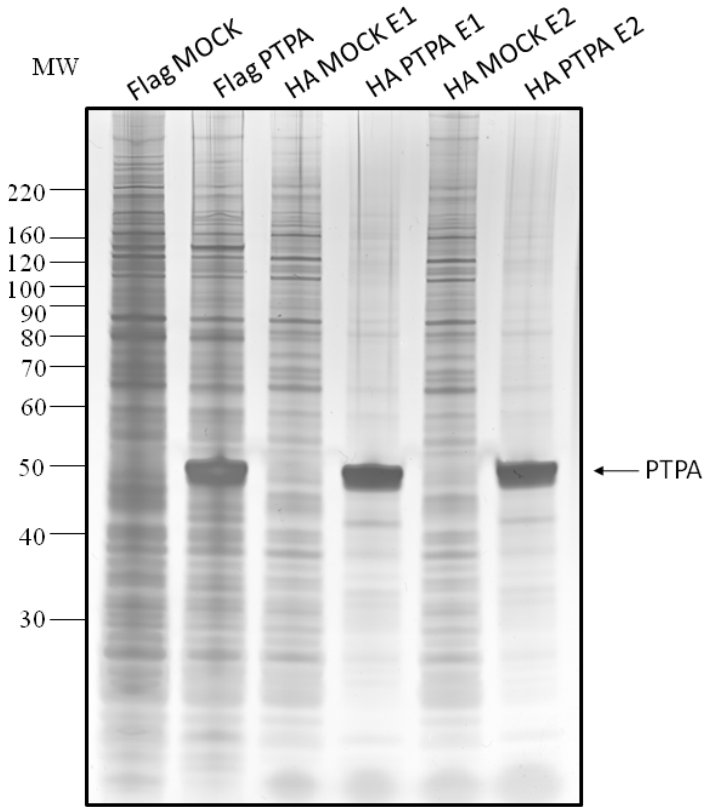


**Figure 4.9 : PTPA protein does not stably interact with any protein.**
The HeLa cell line stably expressing Flag-HA-hPTPA was used along with the control cells for double immunopurification of PTPA complexes. Silver staining was conducted on fractions from elution with a Flag peptide and two elutions (E1 and E2) with an HA peptide.

As mentioned previously, a recent study where the PP2A interaction network was analysed using affinity-purification coupled to mass spectrometry identified multiple subunits of the chaperonin containing TCP-1 (CCT) as partners of PTPA [9]. Since we were unable to identify new PTPA-interacting partners, we decided to use our stable PTPA-expressing HeLa cells to confirm this interaction using coimmunoprecipitation. We attempted to co-IP PTPA with one of the CCT subunit (β). As shown in Figure 4.10, we were not able to demonstrate this interaction. The

size of the CCT protein is the same as the heavy chain of the antibody, preventing us from assessing the interaction. Although there are methods to avoid binding of the heavy chain of the antibody to the beads, we choose not to invest more time or money on this particular experiment. The reciprocal coimmunoprecipitation did not show pulldown of PTPA by the CCT protein (Figure 4.10B). Taken together, the data did not give us any insight in a possible function for PTPA since we were unable to find a possible partner.

Finally, since we were able to express PTPA in a large amount and since we were able to obtain pure protein on a gel, we decided to send our protein sample for detection of post-translation modification by mass spectrometry. Unfortunately, the analysis revealed no evidence that PTPA is ubiquitylated. On the other hand, the protein might be phosphorylated, but at a level below the detection limit (data not shown).

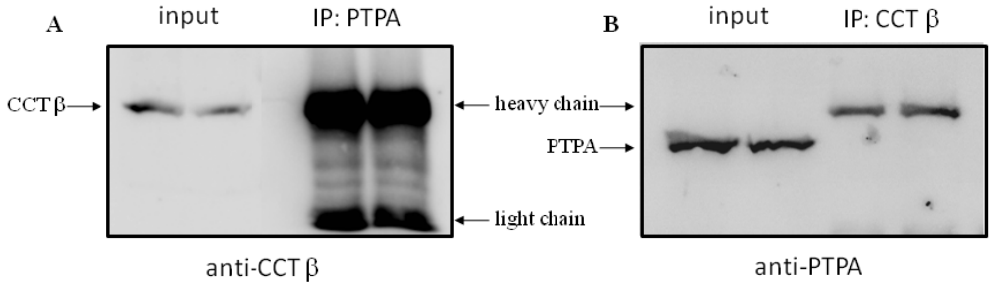


**Figure 4.10 : PTPA do not interact with the β subunit of the CCT protein.**
**A)** Protein extract from HeLa cells stably expressing FLAG-HA-PTPA were incubated with agarose beads containing anti-PTPA antibody in duplicate. The protein extract (input) and the washed beads (IP : PTPA) were analyzed by westernblot using the anti-CCT β antibody. **B)** Same as in A except that the beads contained the anti-CCT β antibody and that the westernblot was revealed using PTPA antibody.

### 4.5.8 Distribution of PTPA in the mouse organs

To gain further insight into the biological role of PTPA in mammals, we attempted to create a PTPA knockout mouse in parallel with the cell culture work described above. First, we analyzed the distribution of PTPA in various organs of the mouse by western blotting (Figure 4.11). PTPA was highly expressed in the brain, bone marrow, spleen and thymus (Figure 4.11). Thus, PTPA may function in neurons and the immune system.

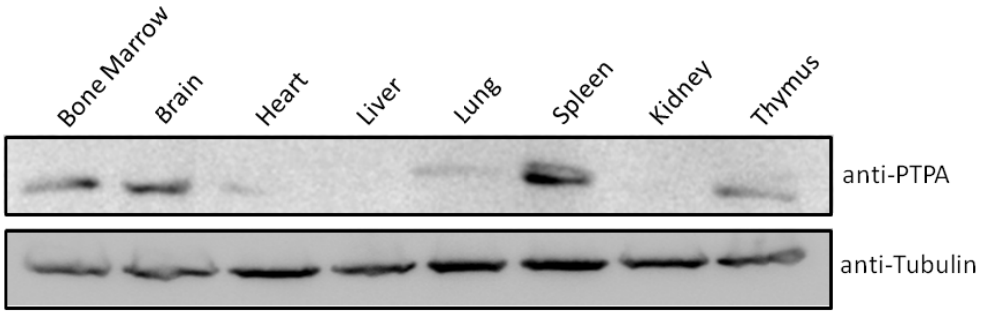


**Figure 4.11 : PTPA protein distribution in mouse organs.**
Different organs from a mouse were isolated and protein extract were generated and analyzed by western blot using anti-PTPA and anti-tubulin (loading control) antibodies. This result is representative of 3 independent mice.

### 4.5.9 Attempt to generate a PTPA knockout mouse

The publicly available gene trap resources, coordinated by the International Gene Trap Consortium (IGTC), generate gene trapped embryonic stem (ES) cell lines that can be used by researchers to determine the functions of genes of interest [27]. We used this tool to create a PTPA knockout mouse. The ES cells containing the

vector U3NeoSV1 integrated in the first intron of the PTPA allele were introduced into C57Bl/6 blastocysts. Chimeric mice were then mated with CD1 females and germline transmission was analyzed by PCR amplification (Figure 4.12A) with tail DNA from progeny.

From a total of 21 chimeras (both males and females), all the males were mated at least 4 times with CD1 females. All progeny were tested, but germline transmission of the PTPA knockout allele was not detected. An example of the PCR result from the F1 progeny is shown in Figure 4.12B. We also tried mating three female chimeras with CD1 males but there was still no germline transmission to the progeny. One possible explanation for this result could be that PTPA knockout is dominant lethal in mouse. To test whether loss of PTPA leads to embryonic lethality, we monitored plugs of females every 24 hours in order to establish an approximate mating starting point. At day 12, we isolated and genotyped the embryos from each female. The embryos tested had wild type PTPA allele and all were alive *in utero*. We believe that there was a germline transmission problem and after breeding for about 8 months, this line of research was discontinued due to lack of success and funding constraints.

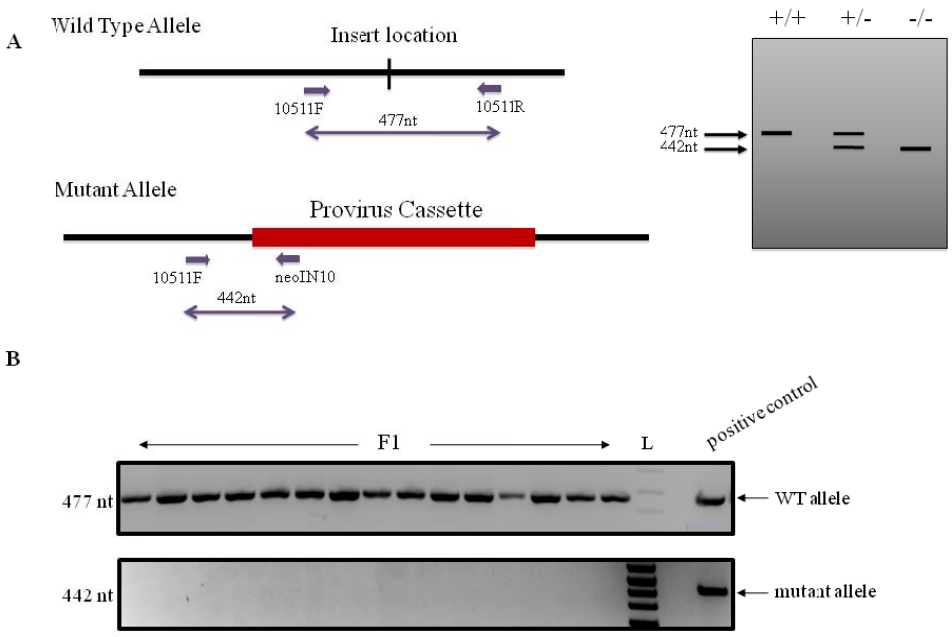


**Figure 4.12 : No germline transmission after mouse genotyping.**
**A)** Genotyping strategy to differentiate between the mutated allele with the insertion of the U3NeoSV1 vector and the wild type allele. Primer sequences are detailed in the material and methods section and the expected result in nucleotide (nt) is shown at the right side of the panel. **B)** Example of the F1 progeny PCR product loaded on a 2% agarose gel. The DNA for the positive control was obtained from a dead female chimera. L represents the DNA ladder.

## 4.6 DISCUSSION

We first attempted to study the role of PTPA in mammalian cells by using transient siRNA knockdown, and found that loss of PTPA led to a growth defect caused by G1 arrest. This result was not reproduced when PTPA was knocked down by shRNA, however. In addition, we were unable to reproduce an *rrd1Δ* mutant-like phenotype with these cells. Since shRNA uses the endogenous processing machinery, their effect is more sustainable and use low copy number resulting in less off-target effect than with siRNA [19-21]. We believe that the growth defect could be an off-target effect of siRNA. In the future, one could address this issue by using three different siRNA constructs to verify the phenotype and eliminate off-target effects. Another good option would have been to use a pool of siRNA instead of individual duplexes to analyze the effect of PTPA knockdown [28].

We believe there are multiple possible explanations for the differences observed between the yeast and mammalian results. The first potential problem with this work is the type of cells used for the knockdown. Since we do not know the function of PTPA in mammalian cells, it is challenging to choose the right cell line. We also noticed that PTPA is still expressed in the cells after the knockdown (Figure 4.1 and 4.7) and we cannot exclude the possibility that a lower expression level is sufficient for PTPA to perform its function. Finally, we think that there could be another yet unidentified protein compensating for PTPA loss of function.

As mentioned previously, using a pool of siRNA could have given us a better knockdown of PTPA and maybe a different phenotype. Ideally, the best option would

have been to create a knockout cell line in order to really avoid partial knockdown of PTPA. Nonetheless, we would still be facing the problem regarding the choice of cell line to study. So far, we believe that mouse embryonic fibroblast (MEF) would have been the best starting point. Finally, since deletion of both Rrd1 and Rrd2 is lethal in yeast [25] we do not know if complete deletion of PTPA in mammalian cells could also be lethal.

A possible key to understanding PTPA biological role is to identify proteins it isomerizes and looking for binding partners is a way to do it. The affinity purification experiment did not identify any protein partners of PTPA. Surprisingly, we were unable to detect known interacting proteins such as PP2A subunits. In the future, this experiment could be repeated with less stringent parameters in order to confirm that known partners of PTPA are pulled down. We also think that PTPA could be interacting only transiently with other proteins and unfortunately the sensitivity of this method is not sufficient. Using nuclear or cytoplasmic extracts rather than whole cell extract could help to eliminate non-specific proteins. Finally, the last problem with this experiment is the low expression of FLAG-HA-PTPA protein in the cell (Figure 4.8B) reducing the chance of finding interacting partners. The catalytic mutants could be a good alternative to repeat the experiment, despite the caveat that the mutations could affect protein-protein interactions. A positive aspect of this analysis is that it shows that regulation of PTPA level in the cell is important for its survival which is consistent with previous work by our lab [10]. In other words, only the cells that were overexpressing the FLAG-HA-PTPA to a level comparable to the endogenous PTPA level were able to survive.

We then decided to study PTPA in mouse and choose to monitor the expression level of PTPA in various organs of the mouse (Figure 4.11). We found that PTPA was highly expressed primarily in the bone marrow, the brain, the spleen and the thymus. We considered using T lymphocytes, but as shown in Figure 4.7, the DO11.1 cells were not a good model to perform siRNA studies. In this case, our best option was to create a knockout mouse and to derive cell lines from it.

As described in the result section, we were unable to successfully create a knockout mouse for PTPA. It seems that the mutated allele was unable to go germline and create heterozygous progeny. Many factors seemed problematic in our methodology. First, it seems that the ES cell line that we were using is not ideal and an expert from the Sanger Institute recommended using the E14 ES cells instead of D3H. Another possibility is that the ES cells could have differentiated before their implantation in the foster female, preventing transmission of the mutation to the progeny. Another explanation could be that deletion of one allele of PTPA is dominant lethal. Our observation that none of the live embryos carried the PTPA deletion supports this notion. Finally, it has been shown that Mycoplasma or viral contamination of ES cells can interfere with germline transmission [29, 30]. In the end, creation of the knockout mouse should be reattempted to address these issues. This could be done using a Cre/Lox construct allowing for conditional knockout in order to target specific organs or to avoid lethality during embryonic stages of development. Since PTPA is highly expressed in organs such as the thymus, the

spleen or the bone marrow (Figure 4.11), it would be relevant to start our investigation with them.

Taken together, our results fell short in determining the biological function of PTPA in mammalian cells. Our studies in yeast revealed an important function for Rrd1 in regulating transcription in response to rapamycin or 4-NQO, and it is likely that PTPA functions in a similar capacity in mammalian cells. Future work addressing some of the technical limitations we encountered could lend more insight into this important biological question.

## 4.7 REFERENCES


1. Jouvet, N., et al., *Rrd1 isomerizes RNA polymerase II in response to rapamycin.* BMC Mol Biol, 2010. **11**: p. 92.
2. Jouvet, N., et al., *RNA polymerase II degradation in response to rapamycin is not mediated through ubiquitylation.* Biochem Biophys Res Commun, 2011. **413**(2): p. 248-53.
3. Van Hoof, C., et al., *Structure and chromosomal localization of the human gene of the phosphotyrosyl phosphatase activator (PTPA) of protein phosphatase 2A.* Genomics, 1995. **28**(2): p. 261-72.
4. Cayla, X., et al., *Molecular cloning, expression, and characterization of PTPA, a protein that activates the tyrosyl phosphatase activity of protein phosphatase 2A.* J Biol Chem, 1994. **269**(22): p. 15668-75.
5. Cayla, X., et al., *Isolation and characterization of a tyrosyl phosphatase activator from rabbit skeletal muscle and Xenopus laevis oocytes.* Biochemistry, 1990. **29**[113]: p. 658-67.
6. Janssens, V. and J. Goris, *Protein phosphatase 2A: a highly regulated family of serine/threonine phosphatases implicated in cell growth and signalling.* Biochem J, 2001. **353**(Pt 3): p. 417-39.
7. Lechward, K., et al., *Protein phosphatase 2A: variety of forms and diversity of functions.* Acta Biochim Pol, 2001. **48**(4): p. 921-33.
8. Chao, Y., et al., *Structure and mechanism of the phosphotyrosyl phosphatase activator.* Mol Cell, 2006. **23**(4): p. 535-46.
9. Glatter, T., et al., *An integrated workflow for charting the human interaction proteome: insights into the PP2A system.* Mol Syst Biol, 2009. **5**: p. 237.
10. Azam, S., E. Drobetsky, and D. Ramotar, *Overexpression of the cis/trans isomerase PTPA triggers caspase 3-dependent apoptosis.* Apoptosis, 2007. **12**(7): p. 1243-55.
11. Leulliot, N., et al., *Crystal structure of the PP2A phosphatase activator: implications for its PP2A-specific PPIase activity.* Mol Cell, 2006. **23**[113]: p. 413-24.
12. Lee, S.Y., et al., *A proteomics approach for the identification of nucleophosmin and heterogeneous nuclear ribonucleoprotein C1/C2 as chromatin-binding proteins in response to DNA double-strand breaks.* Biochem J, 2005. **388**(Pt 1): p. 7-15.
13. Nakatani, Y. and V. Ogryzko, *Immunoaffinity purification of mammalian protein complexes.* Methods Enzymol, 2003. **370**: p. 430-44.
14. Sui, G., et al., *Yin Yang 1 is a negative regulator of p53.* Cell, 2004. **117**(7): p. 859-72.
15. Ljungman, M., et al., *Inhibition of RNA polymerase II as a trigger for the p53 response.* Oncogene, 1999. **18**[113]: p. 583-92.
16. el-Deiry, W.S., et al., *WAF1, a potential mediator of p53 tumor suppression.* Cell, 1993. **75**(4): p. 817-25.
17. Roninson, I.B., *Oncogenic functions of tumour suppressor p21(Waf1/Cip1/Sdi1): association with cell senescence and tumour-promoting activities of stromal fibroblasts.* Cancer Lett, 2002. **179**(1): p. 1-14.

18. Arima, Y., et al., *Transcriptional blockade induces p53-dependent apoptosis associated with translocation of p53 to mitochondria.* J Biol Chem, 2005. **280**(19): p. 19166-76.
19. Snove, O., Jr. and T. Holen, *Many commonly used siRNAs risk off-target activity.* Biochem Biophys Res Commun, 2004. **319**(1): p. 256-63.
20. Jackson, A.L., et al., *Expression profiling reveals off-target gene regulation by RNAi.* Nat Biotechnol, 2003. **21**(6): p. 635-7.
21. Fedorov, Y., et al., *Off-target effects by siRNA can induce toxic phenotype.* RNA, 2006. **12**(7): p. 1188-96.
22. Rao, D.D., et al., *siRNA vs. shRNA: similarities and differences.* Adv Drug Deliv Rev, 2009. **61**(9): p. 746-59.
23. Ramotar, D., et al., *A yeast homologue of the human phosphotyrosyl phosphatase activator PTPA is implicated in protection against oxidative DNA damage induced by the model carcinogen 4-nitroquinoline 1-oxide.* J Biol Chem, 1998. **273**(34): p. 21489-96.
24. Douville, J., et al., *The yeast phosphotyrosyl phosphatase activator protein, yPtpa1/Rrd1, interacts with Sit4 phosphatase to mediate resistance to 4-nitroquinoline-1-oxide and UVA.* Curr Genet, 2004. **46**(2): p. 72-81.
25. Rempola, B., et al., *Functional analysis of RRD1 (YIL153w) and RRD2 (YPL152w), which encode two putative activators of the phosphotyrosyl phosphatase activity of PP2A in Saccharomyces cerevisiae.* Mol Gen Genet, 2000. **262**(6): p. 1081-92.
26. Poschmann, J., et al., *The Peptidyl Prolyl Isomerase Rrd1 Regulates the Elongation of RNA Polymerase II during Transcriptional Stresses.* PLoS One, 2011. **6**(8): p. e23159.
27. Nord, A.S., et al., *The International Gene Trap Consortium Website: a portal to all publicly available gene trap cell lines in mouse.* Nucleic Acids Res, 2006. **34**(Database issue): p. D642-8.
28. Parsons, B.D., et al., *A direct phenotypic comparison of siRNA pools and multiple individual duplexes in a functional assay.* PLoS One, 2009. **4**(12): p. e8471.
29. Markoullis, K., et al., *Mycoplasma contamination of murine embryonic stem cells affects cell parameters, germline transmission and chimeric progeny.* Transgenic Res, 2009. **18**(1): p. 71-87.
30. Mahabir, E., et al., *Impairment of germline transmission after blastocyst injection with murine embryonic stem cells cultured with mouse hepatitis virus and mouse minute virus.* Transgenic Res, 2009. **18**(1): p. 45-57.

# CHAPTER 5

# DISCUSSION

# 5 DISCUSSION

PTPA is a highly conserved protein from yeast to humans and its expression is ubiquitous [87, 88, 90]. Deletion of both yeast homologs, *RRD1* and *RRD2*, is lethal, revealing an important function for this gene in the cell [24]. PTPA was originally identified as an activator of $PP2A_i$ but we have identified multiple additional roles in the cell [77, 110]. My thesis has focused on unravelling Rrd1/PTPA function using yeast and mammalian models.

## 5.1 YEAST RRD1

### 5.1.1 Rrd1 and RNA pol II

The second chapter of my thesis focused on the role of Rrd1 in transcription in response to the immunosuppressant drug rapamycin. We showed that Rrd1 was bound to the chromatin in whole cell extract and co-immunoprecipitation assays revealed a weak interaction between RNA pol II and Rrd1 (Figure 2.1A and B). We also demonstrated that this interaction was present with or without rapamycin treatment (Figure 2.1B). Previous cellular localization studies showed that GFP-Rrd1 was found in the cytoplasm and in the nucleus [23]. Moreover, our lab found that Rrd1 colocalizes on actively transcribed genes with RNA pol II in a recent genome-wide location analysis study of RNA pol II on chromatin following rapamycin treatment [220]. These data support the idea that Rrd1 isomerizes RNA pol II while localized to the chromatin. The exact interacting domains between the two proteins remain to be found. Site-directed mutagenesis experiments and analysis of the

localisation of Rrd1 as well as its interaction with RNA pol II could provide insight into this. The CTD on RNA Pol II is the likely place where Rrd1 binds since the CTD is isomerized. Co-crystallisation of Rrd1 with RNA pol II peptides would be highly informative after the interacting domains are identified. Furthermore, it is still unclear how and under which conditions Rrd1 is recruited to the chromatin. A key question is why Rrd1 is recruited to some rapamycin-responsive genes and not others. Maybe identifying Rrd1 interacting proteins would help identify the mechanism.

Rrd1 was found to be a peptidyl prolyl *cis*/*trans* isomerase (PPIase), and prior to my work, its only known substrate was proline 190 of the PP2A catalytic subunit [88]. In this thesis, we showed that association of Rrd1 with RNA pol II allows isomerisation of the CTD of the large subunit Rpb1 following rapamycin or 4-NQO treatment (Figure 2.3B and 2.4). Circular dichroism experiments demonstrate a novel role for Rrd1 to induce this structural change both *in vitro* and *in vivo* (Figure 2.5B). The RNA pol II CTD is highly regulated by modifications such as phosphorylation, glycosylation and isomerisation and a plethora of factors interact throughout the transcription cycle. Ess1 in yeast (Pin1 in mammals) was already identified as an RNA pol II isomerase essential for the phosphatase Ssu72 to dephosphorylate the Serine 5 of the CTD [221]. Studies of Pin1 suggested that it recognized the phosphorylated CTD, and Pin1 overexpression led to transcription inhibition caused by dissociation of RNA pol II from the chromatin [135]. Our results suggest that a similar function is performed by Rrd1. However, the exact mechanism leading to the induction of Rrd1 isomerase activity is still poorly understood. Rrd1 is constitutively bound to RNA pol II, but our CD analysis showed that the CTD is only isomerized

after rapamycin treatment (Figure 2.3B and Figure 2.4). Therefore, Rrd1 must somehow be activated following stress conditions such as rapamycin or 4-NQO treatment. Unpublished yeast two-hybrid experiment from our lab revealed that Rrd1 interacts with a molecular chaperone belonging to the DnaJ family, Apj1, but the exact connection between the two proteins is still unknown. Surprisingly, we also co-immunoprecipitated this GFP-labelled chaperone with Rpb1 (Figure 2.1C). Further characterisation of a possible modification of Rrd1 by this chaperone would be interesting to investigate.

Interestingly, the loss of Rrd1 does not change the global RNA pol II CTD phosphorylation pattern. The analysis presented in the second chapter using specific antibodies that recognize RNA pol II phosphorylation status revealed that the level of Serine 2 and 5 phosphorylation was the same in wild type and *rrd1Δ* mutant cells (Figure 2.2A and S2.2). However, recent findings from our lab demonstrate that Rrd1 binds to the elongating RNA pol II and that the phosphorylation pattern changes in an *rrd1Δ* mutant at specific genes. ChIP-chip analysis for serine 5 and serine 2 phosphorylation using wild type and *rrd1Δ* mutant treated or not with rapamycin was used [220]. It seems that we were unable to detect these modifications using a general western blot experiment (Figure 2.2A) since the overall phosphorylation level in the cell does not change, but phosphorylation status varies locally at certain genes [220].

We showed that Rrd1 performs isomerisation of RNA pol II CTD following 4-NQO treatment as well as rapamycin (Figure 2.3B and 2.4). But how do these two distinct drugs result in the same response? Rapamycin treatment mimics starvation conditions whereas 4-NQO induces bulky DNA lesions as well as oxidative stress. In

the first case, rapamycin induces a reorganisation of transcription in order to respond to stress conditions. Ribosomal genes are inactivated to reduce translation and stress responsive genes are activated to counteract the effect of poor nitrogen sources [26, 56]. Our chromatin immunoprecipitation (ChIP) results suggest that in an *rrd1Δ* mutant strain, the cells do not arrest because of a defect in transcriptional reorganisation caused by the lack of RNA pol II isomerisation (Figure 2.6). We believe that a similar pattern occur following 4-NQO treatment where stress responsive genes are not activated in an *rrd1Δ* mutant strain leading to accumulation of genotoxic stress [222]. Oxidative stress level measurement with a superoxide anion-sensitive probe, dihydroethidium, revealed that *rrd1Δ* mutant cells exhibit elevated levels of superoxide anions which could explain sensitivity of these mutants to 4-NQO [223]. Sensitivity of *rrd1Δ* mutant cells to other oxidizing agents such as $H_2O_2$ and sodium arsenite (NaAs) was also shown in later experiments. Moreover, analysis of mRNA using GeXP multiplex PCR system from wild type and *rrd1Δ* mutant strains treated or not with rapamycin, $H_2O_2$ or NaAs revealed the importance of Rrd1 in transcriptional stress responses [220]. On the other hand, methyl methane sulfonate (MMS) treatment, which causes apurinic/apyrimidinic sites on DNA, did not induce RNA pol II isomerisation and *rrd1Δ* mutant cells showed parental sensitivity to this agent [22]. Taken together, these results indicate that Rrd1 responsiveness is highly specific to certain conditions and suggest additional levels of regulation via upstream factors.

### 5.1.2 RNA pol II loss

In chapter 3, we showed that total RNA pol II protein level decreased following extended rapamycin treatment in a wild type strain and that this loss was defective in an *rrd1Δ* mutant strain (Figure 3.1), confirming preliminary results [78]. The extensive analysis using different RNA pol II tag and yeast background showed accumulation of RNA pol II in an *rrd1Δ* mutant strain. Further characterisation of RNA pol II localisation revealed that the protein was still found in the chromatin extract in an *rrd1Δ* mutant strain whereas it was not visible in a wild type (Figure 3.2A). Importantly, loss of RNA pol II protein in our extract was seen after 2 hours of treatment with rapamycin, but previous analysis in the literature studied RNA pol II distribution only after 30 minutes [56]. Even though global RNA pol II degradation does not occur until later, ChIP on chip experiments performed by our lab at 30 minutes demonstrated a RNA pol II redistribution defect in the *rrd1Δ* mutant on rapamycin responsive genes [220]. Therefore, Rrd1 plays both early and late roles in the transcriptional response to rapamycin.

We demonstrate that loss of RNA pol II is independent of ubiquitylation, revealing the existence of a novel degradation mechanism. We did not see differences between the wild type and the rrd1 mutant strain following analysis of the overall ubiquitylation status of co-immunuprecipitated RNA pol II (Figure 3.3A and B). Indeed, site-directed mutagenesis of the 2 lysine residues on Rpb1 that could be ubiquitylation targets did not prevent RNA pol II loss after rapamycin treatment (Figure 3.3C and D). Moreover, RNA pol II was still degraded even in a strain

lacking Elc1 (Figure 3.3E), a component of the ubiquitin ligase complex and important for polyubiquitylation of RNA pol II following DNA damage [224]. It would be interesting to look for other modifications such as sumoylation or acetylation of RNA pol II to get more insight into a possible mechanism.

### 5.1.3 Rrd1 and TOR

We showed in chapter 2 that *rrd1Δ gln3Δ* double mutants were highly resistant to rapamycin as compared to the single mutants, suggesting an independent role for Rrd1 in the rapamycin response (Figure S2.1). Dephosphorylation by Sit4 of the nutrient-responsive transcriptional activator Gln3 allows translocation to the nucleus to activate *GLN1* and *MEP2* expression [57, 225]. Rrd1 is part of the Tap42 complex and interacts with Sit4 phosphatase and it is still unclear how this interaction affects the rapamycin response [23, 60]. Genetic studies revealed that both Rrd1 and Sit4 function in the same pathway to mediate protection against oxidative stress induced by 4-NQO, but rapamycin was not studied [23]. It is noteworthy to mention that GFP-Sit4 also interacts with Rpb1 (Figure 2.1C) which could mean that the interaction with this phosphatase plays a role in Rrd1 regulation and activity. We did not study this aspect in our analysis, but it would be interesting to investigate further the link between both proteins.

### 5.1.4 Model

Based on our results collected from these two papers, we propose the following model. When yeast cells are challenged with rapamycin or 4-NQO, Rrd1 bound to

transcribing RNA pol II becomes activated and induces the isomerisation of its CTD. Whether this occurs via the same upstream mechanism is unclear. In the case of 4-NQO treatment it could be induced by accumulation of oxidative stress and activation of the transcriptional stress response, whereas with rapamycin it would involves starvation conditions resulting in a transcriptional reorganization. This CTD structural change allows RNA pol II to interrupt transcription of specific genes and leave the chromatin in order to be recruited to stress responsive genes in both cases. This dynamic process occurs after the first 30 minutes of rapamycin treatment, and extended time in the presence of the drug eventually leads to degradation of excessive RNA pol II independent of ubiquitylation. A schematic representation of our model is shown in Figure 5.1.

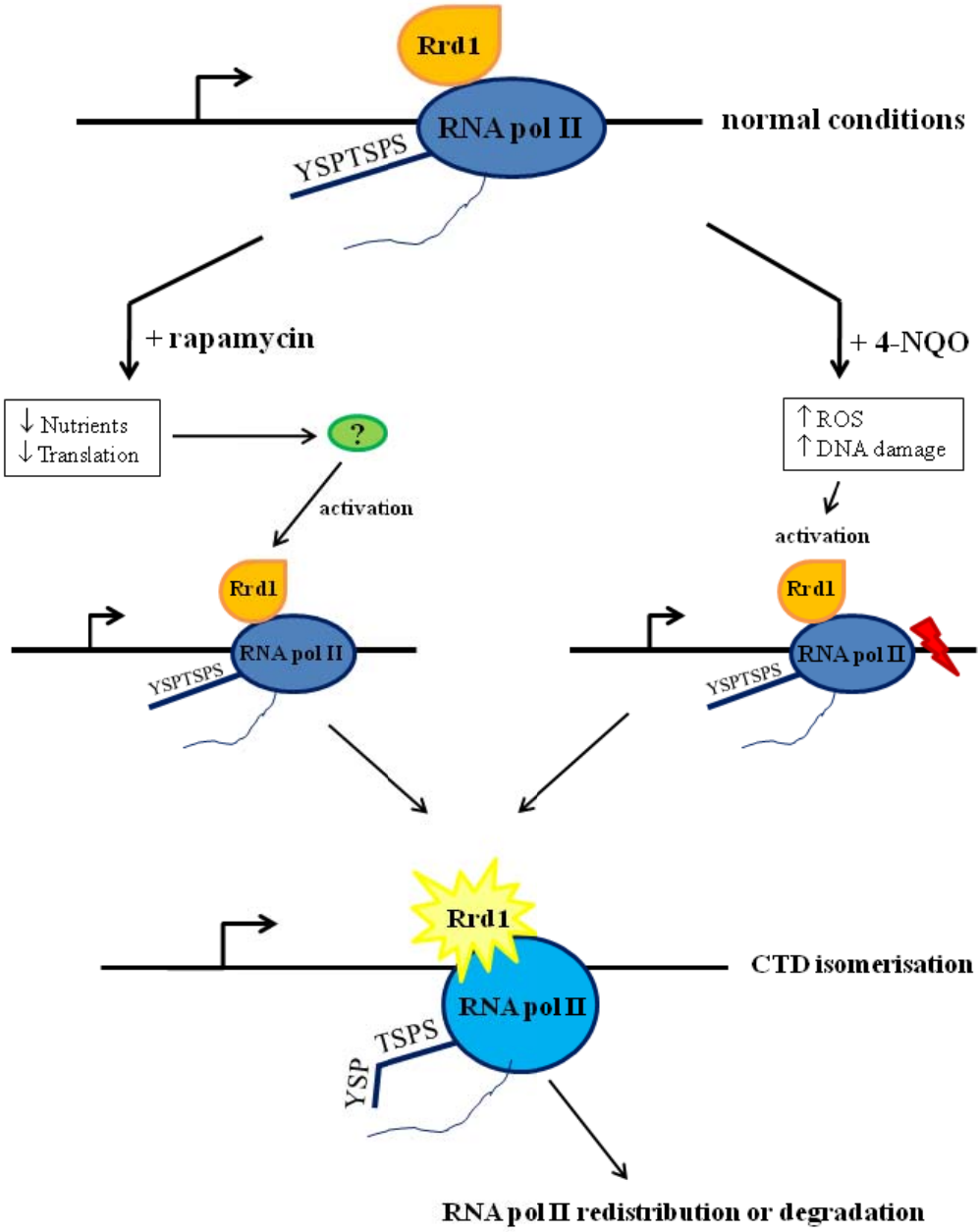


**Figure 5.1**: **Proposed model of Rrd1 activity on RNA pol II**

## 5.2 PTPA IN MAMMALIAN

### 5.2.1 PTPA knockdown

The results found in yeast and the high conservation of the *RRD1* gene amongst species led us to hypothesize that a mammalian PTPA would perform similar functions. The last chapter on my thesis focused on understanding the role of PTPA in mammals using RNAi and mouse genetics.

We first noticed that transient siRNA knockdown of PTPA led to a growth defect caused by G1 arrest. However, this result was not reproduced when PTPA was knocked down by shRNA and we believe that the growth defect could be an off-target effect of siRNA. Indeed, our results using shRNA revealed that PTPA knockdown did not affect the general survival of several cell lines. Additionally, PTPA knockdown HCT116 cells did not show sensitivity to 4-NQO, $H_2O_2$ or resistance to rapamycin (Figure 4.6). One possible explanation for this difference could be that the knockdown was not 100% efficient. Residual activity of the PTPA protein or compensation by an unidentified protein could be sufficient to prevent detection of a phenotype. Our analysis also revealed that PTPA overexpression leads to cell death (Figure 4.8) and that only cells expressing low PTPA survived [122]. This suggests that only a small amount of the protein is required for cell homeostasis.

A possible alternative approach for studying PTPA knockdown and establish a parallel with our yeast work, could be to choose a different cell line. Studies using cells from the knockout mouse, assuming it is not lethal, would be another option. At

last, the chicken B cell line DT40 represents another alternative since it allows efficient gene disruption [226].

PTPA knockdown analysis in HEK TER cells showed that PTPA is involved in the regulation of PP2A methylation leading to cell transformation [121]. This study revealed a new role for PTPA as a possible tumor suppressor gene and it would be interesting to check if this mechanism is also present in other cell lines. Since we found that PTPA was highly expressed in some mice organs such as the brain, the thymus or the bone marrow (Figure 4.11), it would be interesting to start with these cell lines.

### 5.2.2 PTPA substrates

We also attempted to isolate PTPA binding proteins by affinity purification in order to identify new possible targets for its PPIase activity. This first and only analysis where we used whole cell extract did not identify any stable partners for PTPA (Figure 4.9). Moreover, our assay did not isolate known interacting partners such as Pph3 or CCT complex proteins (Figure 4.10) found by another group [119]. This indicates that our pulldown conditions may have been too stringent. Using nuclear or cytoplasmic extracts rather than whole cell extract could help to eliminate non-specific proteins. We also believe that reducing the concentration of salt in the washes could increase our chance of finding partners that are not tightly bound. Finally, it is possible that PTPA only interacts transiently with its substrates making it difficult to detect these interactions by affinity capture. Several other techniques can be used to detect transient interactions, such as nuclear magnetic resonance (NMR)

[227] or fluorescence resonance energy transfer (FRET) [228], but these methods are not suitable for exploratory or screening-type applications [229]. The yeast two-hybrid system could also be a possible alternative. Our lab previously used Rrd1 as bait in Y2H and found that Apj1 could be a possible partner (unpublished data) and we believe that performing the same experiment using PTPA as a bait could identify new partners or substrates.

### 5.2.3 PTPA in mice

To better study the importance of PTPA in mice, we successfully generated chimeras from ES cells lines obtained from the International Gene Trap Consortium [230]. Unfortunately, we were unable to get germline transmission of the mutated allele to the progeny after breeding the chimeras for several rounds (Figure 4.12). We therefore asked whether PTPA heterozygosity could cause lethality. Indeed, the embryos at day 12 were all healthy and none of them carried the mutated allele. We believe that PTPA performs an important function in mammals and we cannot exclude the possibility that deletion of one allele causes lethality, especially given that deletion of both Rrd1 and Rrd2 in yeast is lethal.

The other possible complication could reside in the ES cells. It was shown that viral or Mycoplasma contaminations of ES cells can interfere with germline transmission and no such analysis were performed. Moreover, an expert from the Sanger Institute recommended using E14 ES cells instead of D3H to increase our success rate. The lack of funding resources led us to prematurely abort the project without further investigation.

In the future, a better alternative would be to create a conditional Cre-Lox knockout mouse in which a tissue-specific promoter induces expression of the Cre recombinase, deleting PTPA in that tissue only [231]. For example, it would be interesting to use tissue-specific promoter for the knockout in thymus, bone marrow or brain since PTPA expression is higher in those organs.

## 5.3 CONCLUSION

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. Indeed, Rrd1 interacts and isomerises RNA pol II and promote its dissociation from the chromatin. Furthermore, we showed that sustained treatment with rapamycin induces RNA pol II degradation in an ubiquitylation-independent pathway.

Our investigation in mammals did not allow us to define a model for PTPA function. Unfortunately, we were unable to reproduce an *rrd1Δ* mutant-like phenotype in mammalian cells. Moreover, the affinity purification experiment did not identify any protein partners of PTPA. Finally, we were unable to successfully create a knockout mouse for PTPA. But as mentioned previously, the high homology between the yeast and mammalian sequences and the conservation between species suggest an important role for PTPA in mammals. Indeed, our results in yeast could indicate that PTPA performs a possible similar function in mammalian cells in regulating transcription in response to specific stress. We still believe that we are very

close of discovering a related function of PTPA in mammals and finding a good model system will highly increase the chances of identifying its function in the cell.

## REFERENCES


1. Bos, J.L., *ras oncogenes in human cancer: a review.* Cancer Res, 1989. **49**(17): p. 4682-9.
2. Downward, J., *Targeting RAS signalling pathways in cancer therapy.* Nat Rev Cancer, 2003. **3**(1): p. 11-22.
3. Blum, R. and Y. Kloog, *Tailoring Ras-pathway--inhibitor combinations for cancer therapy.* Drug Resist Updat, 2005. **8**(6): p. 369-80.
4. Morin, M.J., *From oncogene to drug: development of small molecule tyrosine kinase inhibitors as anti-tumor and anti-angiogenic agents.* Oncogene, 2000. **19**(56): p. 6574-83.
5. Sherr, C.J., *Principles of tumor suppression.* Cell, 2004. **116**(2): p. 235-46.
6. Hollstein, M., et al., *p53 mutations in human cancers.* Science, 1991. **253**(5015): p. 49-53.
7. De Bont, R. and N. van Larebeke, *Endogenous DNA damage in humans: a review of quantitative data.* Mutagenesis, 2004. **19**(3): p. 169-85.
8. Friedberg, E.C., L.D. McDaniel, and R.A. Schultz, *The role of endogenous and exogenous DNA damage and mutagenesis.* Curr Opin Genet Dev, 2004. **14**(1): p. 5-10.
9. Caldecott, K.W., *Single-strand break repair and genetic disease.* Nat Rev Genet, 2008. **9**(8): p. 619-31.
10. Fortini, P., et al., *The base excision repair: mechanisms and its relevance for cancer susceptibility.* Biochimie, 2003. **85**(11): p. 1053-71.
11. de Laat, W.L., N.G. Jaspers, and J.H. Hoeijmakers, *Molecular mechanism of nucleotide excision repair.* Genes Dev, 1999. **13**(7): p. 768-85.
12. Kunkel, T.A. and D.A. Erie, *DNA mismatch repair.* Annu Rev Biochem, 2005. **74**: p. 681-710.
13. Sonoda, E., et al., *Differential usage of non-homologous end-joining and homologous recombination in double strand break repair.* DNA Repair (Amst), 2006. **5**(9-10): p. 1021-9.
14. Daley, J.M., et al., *Nonhomologous end joining in yeast.* Annu Rev Genet, 2005. **39**: p. 431-51.
15. Pastwa, E. and J. Blasiak, *Non-homologous DNA end joining.* Acta Biochim Pol, 2003. **50**(4): p. 891-908.
16. Botstein, D., S.A. Chervitz, and J.M. Cherry, *Yeast as a model organism.* Science, 1997. **277**(5330): p. 1259-60.
17. Galiegue-Zouitina, S., et al., *N2-guanyl and N6-adenyl arylation of chicken erythrocyte DNA by the ultimate carcinogen of 4-nitroquinoline 1-oxide.* Cancer Res, 1986. **46**(4 Pt 1): p. 1858-63.
18. Sugimura, T., K. Okabe, and M. Nagao, *The metabolism of 4-nitroquinoline-1-oxide, a carcinogen. 3. An enzyme catalyzing the conversion of 4-nitroquinoline-1-oxide to 4-hydroxyaminoquinoline-1-oxide in rat liver and hepatomas.* Cancer Res, 1966. **26**(8): p. 1717-21.
19. Prakash, S., P. Sung, and L. Prakash, *DNA repair genes and proteins of Saccharomyces cerevisiae.* Annu Rev Genet, 1993. **27**: p. 33-70.

20. Hall, A. and J. Ballantyne, *Characterization of UVC-induced DNA damage in bloodstains: forensic implications.* Anal Bioanal Chem, 2004. **380**(1): p. 72-83.
21. Balajee, A.S. and V.A. Bohr, *Genomic heterogeneity of nucleotide excision repair.* Gene, 2000. **250**(1-2): p. 15-30.
22. Ramotar, D., et al., *A yeast homologue of the human phosphotyrosyl phosphatase activator PTPA is implicated in protection against oxidative DNA damage induced by the model carcinogen 4-nitroquinoline 1-oxide.* J Biol Chem, 1998. **273**(34): p. 21489-96.
23. Douville, J., et al., *The yeast phosphotyrosyl phosphatase activator protein, yPtpa1/Rrd1, interacts with Sit4 phosphatase to mediate resistance to 4-nitroquinoline-1-oxide and UVA.* Curr Genet, 2004. **46**(2): p. 72-81.
24. Rempola, B., et al., *Functional analysis of RRD1 (YIL153w) and RRD2 (YPL152w), which encode two putative activators of the phosphotyrosyl phosphatase activity of PP2A in Saccharomyces cerevisiae.* Mol Gen Genet, 2000. **262**(6): p. 1081-92.
25. Van Hoof, C., et al., *The Saccharomyces cerevisiae homologue YPA1 of the mammalian phosphotyrosyl phosphatase activator of protein phosphatase 2A controls progression through the G1 phase of the yeast cell cycle.* J Mol Biol, 2000. **302**(1): p. 103-20.
26. Cardenas, M.E., et al., *The TOR signaling cascade regulates gene expression in response to nutrients.* Genes Dev, 1999. **13**(24): p. 3271-9.
27. Guertin, D.A. and D.M. Sabatini, *The pharmacology of mTOR inhibition.* Sci Signal, 2009. **2**(67): p. pe24.
28. Cao, W., et al., *Toll-like receptor-mediated induction of type I interferon in plasmacytoid dendritic cells requires the rapamycin-sensitive PI(3)K-mTOR-p70S6K pathway.* Nat Immunol, 2008. **9**(10): p. 1157-64.
29. Sinclair, L.V., et al., *Phosphatidylinositol-3-OH kinase and nutrient-sensing mTOR pathways control T lymphocyte trafficking.* Nat Immunol, 2008. **9**(5): p. 513-21.
30. Sauer, S., et al., *T cell receptor signaling controls Foxp3 expression via PI3K, Akt, and mTOR.* Proc Natl Acad Sci U S A, 2008. **105**(22): p. 7797-802.
31. Araki, K., et al., *mTOR regulates memory CD8 T-cell differentiation.* Nature, 2009. **460**(7251): p. 108-12.
32. Rao, R.R., Q. Li, and P.A. Shrikant, *Fine-tuning CD8(+) T cell functional responses: mTOR acts as a rheostat for regulating CD8(+) T cell proliferation, survival and differentiation?* Cell Cycle, 2010. **9**(15): p. 2996-3001.
33. Pearce, E.L., et al., *Enhancing CD8 T-cell memory by modulating fatty acid metabolism.* Nature, 2009. **460**(7251): p. 103-7.
34. Harrison, D.E., et al., *Rapamycin fed late in life extends lifespan in genetically heterogeneous mice.* Nature, 2009. **460**(7253): p. 392-5.
35. Hosoi, H., et al., *Rapamycin causes poorly reversible inhibition of mTOR and induces p53-independent apoptosis in human rhabdomyosarcoma cells.* Cancer Res, 1999. **59**(4): p. 886-94.
36. Shi, Y., et al., *Rapamycin enhances apoptosis and increases sensitivity to cisplatin in vitro.* Cancer Res, 1995. **55**(9): p. 1982-8.

37. Ishizuka, T., et al., *Rapamycin potentiates dexamethasone-induced apoptosis and inhibits JNK activity in lymphoblastoid cells.* Biochem Biophys Res Commun, 1997. **230**(2): p. 386-91.
38. Huang, S. and P.J. Houghton, *Targeting mTOR signaling for cancer therapy.* Curr Opin Pharmacol, 2003. **3**(4): p. 371-7.
39. Motzer, R.J., et al., *Efficacy of everolimus in advanced renal cell carcinoma: a double-blind, randomised, placebo-controlled phase III trial.* Lancet, 2008. **372**(9637): p. 449-56.
40. Hess, G., et al., *Phase III study to evaluate temsirolimus compared with investigator's choice therapy for the treatment of relapsed or refractory mantle cell lymphoma.* J Clin Oncol, 2009. **27**(23): p. 3822-9.
41. Bhaskar, P.T. and N. Hay, *The two TORCs and Akt.* Dev Cell, 2007. **12**(4): p. 487-502.
42. Jacinto, E. and A. Lorberg, *TOR regulation of AGC kinases in yeast and mammals.* Biochem J, 2008. **410**(1): p. 19-37.
43. Soulard, A., A. Cohen, and M.N. Hall, *TOR signaling in invertebrates.* Curr Opin Cell Biol, 2009. **21**(6): p. 825-36.
44. Oshiro, N., et al., *Dissociation of raptor from mTOR is a mechanism of rapamycin-induced inhibition of mTOR function.* Genes Cells, 2004. **9**(4): p. 359-66.
45. Loewith, R., et al., *Two TOR complexes, only one of which is rapamycin sensitive, have distinct roles in cell growth control.* Mol Cell, 2002. **10**(3): p. 457-68.
46. Roberg, K.J., et al., *Control of amino acid permease sorting in the late secretory pathway of Saccharomyces cerevisiae by SEC13, LST4, LST7 and LST8.* Genetics, 1997. **147**(4): p. 1569-84.
47. Cybulski, N. and M.N. Hall, *TOR complex 2: a signaling pathway of its own.* Trends Biochem Sci, 2009. **34**(12): p. 620-7.
48. Barbet, N.C., et al., *TOR controls translation initiation and early G1 progression in yeast.* Mol Biol Cell, 1996. **7**(1): p. 25-42.
49. Magasanik, B. and C.A. Kaiser, *Nitrogen regulation in Saccharomyces cerevisiae.* Gene, 2002. **290**(1-2): p. 1-18.
50. Schmidt, A., J. Kunz, and M.N. Hall, *TOR2 is required for organization of the actin cytoskeleton in yeast.* Proc Natl Acad Sci U S A, 1996. **93**(24): p. 13780-5.
51. Kamada, Y., et al., *Tor-mediated induction of autophagy via an Apg1 protein kinase complex.* J Cell Biol, 2000. **150**(6): p. 1507-13.
52. Schmidt, A., et al., *The TOR nutrient signalling pathway phosphorylates NPR1 and inhibits turnover of the tryptophan permease.* EMBO J, 1998. **17**(23): p. 6924-31.
53. Omura, F. and Y. Kodama, *The N-terminal domain of yeast Bap2 permease is phosphorylated dependently on the Npr1 kinase in response to starvation.* FEMS Microbiol Lett, 2004. **230**(2): p. 227-34.
54. Boeckstaens, M., B. Andre, and A.M. Marini, *The yeast ammonium transport protein Mep2 and its positive regulator, the Npr1 kinase, play an important role in normal and pseudohyphal growth on various nitrogen media through retrieval of excreted ammonium.* Mol Microbiol, 2007. **64**(2): p. 534-46.

55. De Craene, J.O., O. Soetens, and B. Andre, *The Npr1 kinase controls biosynthetic and endocytic sorting of the yeast Gap1 permease.* J Biol Chem, 2001. **276**(47): p. 43939-48.
56. Hardwick, J.S., et al., *Rapamycin-modulated transcription defines the subset of nutrient-sensitive signaling pathways directly controlled by the Tor proteins.* Proc Natl Acad Sci U S A, 1999. **96**(26): p. 14866-70.
57. Beck, T. and M.N. Hall, *The TOR signalling pathway controls nuclear localization of nutrient-regulated transcription factors.* Nature, 1999. **402**(6762): p. 689-92.
58. Komeili, A., et al., *Mechanism of metabolic control. Target of rapamycin signaling links nitrogen quality to the activity of the Rtg1 and Rtg3 transcription factors.* J Cell Biol, 2000. **151**(4): p. 863-78.
59. Jacinto, E., et al., *TIP41 interacts with TAP42 and negatively regulates the TOR signaling pathway.* Mol Cell, 2001. **8**(5): p. 1017-26.
60. Zheng, Y. and Y. Jiang, *The yeast phosphotyrosyl phosphatase activator is part of the Tap42-phosphatase complexes.* Mol Biol Cell, 2005. **16**(4): p. 2119-27.
61. Laplante, M. and D.M. Sabatini, *mTOR signaling at a glance.* J Cell Sci, 2009. **122**(Pt 20): p. 3589-94.
62. Peterson, T.R., et al., *DEPTOR is an mTOR inhibitor frequently overexpressed in multiple myeloma cells and required for their survival.* Cell, 2009. **137**(5): p. 873-86.
63. Guertin, D.A., et al., *Ablation in mice of the mTORC components raptor, rictor, or mLST8 reveals that mTORC2 is required for signaling to Akt-FOXO and PKCalpha, but not S6K1.* Dev Cell, 2006. **11**(6): p. 859-71.
64. Dunlop, E.A. and A.R. Tee, *Mammalian target of rapamycin complex 1: signalling inputs, substrates and feedback mechanisms.* Cell Signal, 2009. **21**(6): p. 827-35.
65. Alessi, D.R., L.R. Pearce, and J.M. Garcia-Martinez, *New insights into mTOR signaling: mTORC2 and beyond.* Sci Signal, 2009. **2**(67): p. pe27.
66. Ma, X.M. and J. Blenis, *Molecular mechanisms of mTOR-mediated translational control.* Nat Rev Mol Cell Biol, 2009. **10**(5): p. 307-18.
67. Chang, Y.Y., et al., *Nutrient-dependent regulation of autophagy through the target of rapamycin pathway.* Biochem Soc Trans, 2009. **37**(Pt 1): p. 232-6.
68. Benvenuto, G., et al., *The tuberous sclerosis-1 (TSC1) gene product hamartin suppresses cell growth and augments the expression of the TSC2 product tuberin by inhibiting its ubiquitination.* Oncogene, 2000. **19**(54): p. 6306-16.
69. Manning, B.D. and L.C. Cantley, *Rheb fills a GAP between TSC and TOR.* Trends Biochem Sci, 2003. **28**(11): p. 573-6.
70. Sancak, Y., et al., *The Rag GTPases bind raptor and mediate amino acid signaling to mTORC1.* Science, 2008. **320**(5882): p. 1496-501.
71. Inoki, K., et al., *Signaling by target of rapamycin proteins in cell growth control.* Microbiol Mol Biol Rev, 2005. **69**(1): p. 79-100.
72. Beugnet, A., X. Wang, and C.G. Proud, *Target of rapamycin (TOR)-signaling and RAIP motifs play distinct roles in the mammalian TOR-dependent phosphorylation of initiation factor 4E-binding protein 1.* J Biol Chem, 2003. **278**(42): p. 40717-22.

73. Manning, B.D. and L.C. Cantley, *AKT/PKB signaling: navigating downstream.* Cell, 2007. **129**(7): p. 1261-74.
74. Du, K. and P.N. Tsichlis, *Regulation of the Akt kinase by interacting proteins.* Oncogene, 2005. **24**(50): p. 7401-9.
75. Ikenoue, T., et al., *Essential function of TORC2 in PKC and Akt turn motif phosphorylation, maturation and signalling.* EMBO J, 2008. **27**(14): p. 1919-31.
76. Sarbassov, D.D., et al., *Rictor, a novel binding partner of mTOR, defines a rapamycin-insensitive and raptor-independent pathway that regulates the cytoskeleton.* Curr Biol, 2004. **14**(14): p. 1296-302.
77. Van Hoof, C., et al., *Specific interactions of PP2A and PP2A-like phosphatases with the yeast PTPA homologues, Ypa1 and Ypa2.* Biochem J, 2005. **386**(Pt 1): p. 93-102.
78. Douville, J., et al., *The Saccharomyces cerevisiae phosphatase activator RRD1 is required to modulate gene expression in response to rapamycin exposure.* Genetics, 2006. **172**(2): p. 1369-72.
79. Cayla, X., et al., *Isolation and characterization of a tyrosyl phosphatase activator from rabbit skeletal muscle and Xenopus laevis oocytes.* Biochemistry, 1990. **29**(3): p. 658-67.
80. Van Hoof, C., et al., *Functional analysis of conserved domains in the phosphotyrosyl phosphatase activator. Molecular cloning of the homologues from Drosophila melanogaster and Saccharomyces cerevisiae.* Biochemistry, 1998. **37**(37): p. 12899-908.
81. Mitchell, D.A. and G.F. Sprague, Jr., *The phosphotyrosyl phosphatase activator, Ncs1p (Rrd1p), functions with Cla4p to regulate the G(2)/M transition in Saccharomyces cerevisiae.* Mol Cell Biol, 2001. **21**(2): p. 488-500.
82. Larkin, M.A., et al., *Clustal W and Clustal X version 2.0.* Bioinformatics, 2007. **23**(21): p. 2947-8.
83. Waterhouse, A.M., et al., *Jalview Version 2--a multiple sequence alignment editor and analysis workbench.* Bioinformatics, 2009. **25**(9): p. 1189-91.
84. Van Hoof, C., et al., *Structure and chromosomal localization of the human gene of the phosphotyrosyl phosphatase activator (PTPA) of protein phosphatase 2A.* Genomics, 1995. **28**(2): p. 261-72.
85. Janssens, V., et al., *Functional analysis of the promoter region of the human phosphotyrosine phosphatase activator gene: Yin Yang 1 is essential for core promoter activity.* Biochem J, 1999. **344 Pt 3**: p. 755-63.
86. Janssens, V., et al., *Identification and characterization of alternative splice products encoded by the human phosphotyrosyl phosphatase activator gene.* Eur J Biochem, 2000. **267**(14): p. 4406-13.
87. Leulliot, N., et al., *Crystal structure of the PP2A phosphatase activator: implications for its PP2A-specific PPIase activity.* Mol Cell, 2006. **23**(3): p. 413-24.
88. Chao, Y., et al., *Structure and mechanism of the phosphotyrosyl phosphatase activator.* Mol Cell, 2006. **23**(4): p. 535-46.

89. Jordens, J., et al., *The Protein Phosphatase 2A Phosphatase Activator Is a Novel Peptidyl-Prolyl cis/trans-Isomerase.* J Biol Chem, 2006. **281**(10): p. 6349-6357.
90. Cayla, X., et al., *Molecular cloning, expression, and characterization of PTPA, a protein that activates the tyrosyl phosphatase activity of protein phosphatase 2A.* J Biol Chem, 1994. **269**(22): p. 15668-75.
91. Janssens, V. and J. Goris, *Protein phosphatase 2A: a highly regulated family of serine/threonine phosphatases implicated in cell growth and signalling.* Biochem J, 2001. **353**(Pt 3): p. 417-39.
92. Landrieu, I., et al., *Molecular implication of PP2A and Pin1 in the Alzheimer's disease specific hyperphosphorylation of Tau.* PLoS One, 2011. **6**(6): p. e21521.
93. Rudrabhatla, P. and H.C. Pant, *Role of Protein Phosphatase 2A in Alzheimer's Disease.* Curr Alzheimer Res, 2011.
94. Kalev, P. and A.A. Sablina, *Protein phosphatase 2A as a potential target for anticancer therapy.* Anticancer Agents Med Chem, 2011. **11**(1): p. 38-46.
95. Eichhorn, P.J., M.P. Creyghton, and R. Bernards, *Protein phosphatase 2A regulatory subunits and cancer.* Biochim Biophys Acta, 2009. **1795**(1): p. 1-15.
96. Shi, Y., *Serine/threonine phosphatases: mechanism through structure.* Cell, 2009. **139**(3): p. 468-84.
97. Xing, Y., et al., *Structure of protein phosphatase 2A core enzyme bound to tumor-inducing toxins.* Cell, 2006. **127**(2): p. 341-53.
98. Ruediger, R., H.T. Pham, and G. Walter, *Alterations in protein phosphatase 2A subunit interaction in human carcinomas of the lung and colon with mutations in the A beta subunit gene.* Oncogene, 2001. **20**(15): p. 1892-9.
99. Ruediger, R., H.T. Pham, and G. Walter, *Disruption of protein phosphatase 2A subunit interaction in human cancers with mutations in the A alpha subunit gene.* Oncogene, 2001. **20**(1): p. 10-5.
100. Wu, J., et al., *Carboxyl methylation of the phosphoprotein phosphatase 2A catalytic subunit promotes its functional association with regulatory subunits in vivo.* EMBO J, 2000. **19**(21): p. 5672-81.
101. Janssens, V., S. Longin, and J. Goris, *PP2A holoenzyme assembly: in cauda venenum (the sting is in the tail).* Trends Biochem Sci, 2008. **33**(3): p. 113-21.
102. Mayer-Jaekel, R.E. and B.A. Hemmings, *Protein phosphatase 2A--a 'menage a trois'.* Trends Cell Biol, 1994. **4**(8): p. 287-91.
103. Lee, J., et al., *A specific protein carboxyl methylesterase that demethylates phosphoprotein phosphatase 2A in bovine brain.* Proc Natl Acad Sci U S A, 1996. **93**(12): p. 6043-7.
104. Lee, J. and J. Stock, *Protein phosphatase 2A catalytic subunit is methyl-esterified at its carboxyl terminus by a novel methyltransferase.* J Biol Chem, 1993. **268**(26): p. 19192-5.
105. Xie, H. and S. Clarke, *Methyl esterification of C-terminal leucine residues in cytosolic 36-kDa polypeptides of bovine brain. A novel eucaryotic protein carboxyl methylation reaction.* J Biol Chem, 1993. **268**(18): p. 13364-71.

106. Xie, H. and S. Clarke, *An enzymatic activity in bovine brain that catalyzes the reversal of the C-terminal methyl esterification of protein phosphatase 2A.* Biochem Biophys Res Commun, 1994. **203**(3): p. 1710-5.
107. Bryant, J.C., R.S. Westphal, and B.E. Wadzinski, *Methylated C-terminal leucine residue of PP2A catalytic subunit is important for binding of regulatory Balpha subunit.* Biochem J, 1999. **339 ( Pt 2)**: p. 241-6.
108. Yu, X.X., et al., *Methylation of the protein phosphatase 2A catalytic subunit is essential for association of Balpha regulatory subunit but not SG2NA, striatin, or polyomavirus middle tumor antigen.* Mol Biol Cell, 2001. **12**(1): p. 185-99.
109. De Baere, I., et al., *Purification of porcine brain protein phosphatase 2A leucine carboxyl methyltransferase and cloning of the human homologue.* Biochemistry, 1999. **38**(50): p. 16539-47.
110. Longin, S., et al., *An inactive protein phosphatase 2A population is associated with methylesterase and can be re-activated by the phosphotyrosyl phosphatase activator.* Biochem J, 2004. **380**(Pt 1): p. 111-9.
111. Lee, J.A. and D.C. Pallas, *Leucine carboxyl methyltransferase-1 is necessary for normal progression through mitosis in mammalian cells.* J Biol Chem, 2007. **282**(42): p. 30974-84.
112. Sontag, J.M., et al., *Regulation of protein phosphatase 2A methylation by LCMT1 and PME-1 plays a critical role in differentiation of neuroblastoma cells.* J Neurochem, 2010. **115**(6): p. 1455-65.
113. Sontag, E., et al., *Downregulation of protein phosphatase 2A carboxyl methylation and methyltransferase may contribute to Alzheimer disease pathogenesis.* J Neuropathol Exp Neurol, 2004. **63**(10): p. 1080-91.
114. Chen, J., B.L. Martin, and D.L. Brautigan, *Regulation of protein serine-threonine phosphatase type-2A by tyrosine phosphorylation.* Science, 1992. **257**(5074): p. 1261-4.
115. Liu, R., et al., *Phosphorylated PP2A (tyrosine 307) is associated with Alzheimer neurofibrillary pathology.* J Cell Mol Med, 2008. **12**(1): p. 241-57.
116. Xu, Z. and B.R. Williams, *The B56alpha regulatory subunit of protein phosphatase 2A is a target for regulation by double-stranded RNA-dependent protein kinase PKR.* Mol Cell Biol, 2000. **20**(14): p. 5285-99.
117. Ruvolo, V.R., et al., *PKR regulates B56(alpha)-mediated BCL2 phosphatase activity in acute lymphoblastic leukemia-derived REH cells.* J Biol Chem, 2008. **283**(51): p. 35474-85.
118. Simizu, S., Y. Tamura, and H. Osada, *Dephosphorylation of Bcl-2 by protein phosphatase 2A results in apoptosis resistance.* Cancer Sci, 2004. **95**(3): p. 266-70.
119. Glatter, T., et al., *An integrated workflow for charting the human interaction proteome: insights into the PP2A system.* Mol Syst Biol, 2009. **5**: p. 237.
120. Hombauer, H., et al., *Generation of active protein phosphatase 2A is coupled to holoenzyme assembly.* PLoS Biol, 2007. **5**(6): p. e155.
121. Sablina, A.A., et al., *Identification of PP2A complexes and pathways involved in cell transformation.* Cancer Res, 2010. **70**(24): p. 10474-84.

122. Azam, S., E. Drobetsky, and D. Ramotar, *Overexpression of the cis/trans isomerase PTPA triggers caspase 3-dependent apoptosis.* Apoptosis, 2007. **12**(7): p. 1243-55.
123. Lu, K.P., et al., *Prolyl cis-trans isomerization as a molecular timer.* Nat Chem Biol, 2007. **3**(10): p. 619-29.
124. Kallen, J., et al., *Structure of human cyclophilin and its binding site for cyclosporin A determined by X-ray crystallography and NMR spectroscopy.* Nature, 1991. **353**(6341): p. 276-9.
125. Van Duyne, G.D., et al., *Atomic structure of FKBP-FK506, an immunophilin-immunosuppressant complex.* Science, 1991. **252**(5007): p. 839-42.
126. Pal, D. and P. Chakrabarti, *Cis peptide bonds in proteins: residues involved, their conformations, interactions and locations.* J Mol Biol, 1999. **294**(1): p. 271-88.
127. Fanghanel, J. and G. Fischer, *Insights into the catalytic mechanism of peptidyl prolyl cis/trans isomerases.* Front Biosci, 2004. **9**: p. 3453-78.
128. Takahashi, N., T. Hayano, and M. Suzuki, *Peptidyl-prolyl cis-trans isomerase is the cyclosporin A-binding protein cyclophilin.* Nature, 1989. **337**(6206): p. 473-5.
129. Heitman, J., N.R. Movva, and M.N. Hall, *Targets for cell cycle arrest by the immunosuppressant rapamycin in yeast.* Science, 1991. **253**(5022): p. 905-9.
130. Dolinski, K., et al., *All cyclophilins and FK506 binding proteins are, individually and collectively, dispensable for viability in Saccharomyces cerevisiae.* Proc Natl Acad Sci U S A, 1997. **94**(24): p. 13093-8.
131. Ranganathan, R., et al., *Structural and functional analysis of the mitotic rotamase Pin1 suggests substrate recognition is phosphorylation dependent.* Cell, 1997. **89**(6): p. 875-86.
132. Lu, K.P., *Pinning down cell signaling, cancer and Alzheimer's disease.* Trends Biochem Sci, 2004. **29**(4): p. 200-9.
133. Wu, X., et al., *The Ess1 prolyl isomerase is linked to chromatin remodeling complexes and the general transcription machinery.* EMBO J, 2000. **19**(14): p. 3727-38.
134. Kops, O., X.Z. Zhou, and K.P. Lu, *Pin1 modulates the dephosphorylation of the RNA polymerase II C-terminal domain by yeast Fcp1.* FEBS Lett, 2002. **513**(2-3): p. 305-11.
135. Xu, Y.X., et al., *Pin1 modulates the structure and function of human RNA polymerase II.* Genes Dev, 2003. **17**(22): p. 2765-76.
136. Warner, J.R., *The economics of ribosome biosynthesis in yeast.* Trends Biochem Sci, 1999. **24**(11): p. 437-40.
137. Lee, Y., et al., *MicroRNA genes are transcribed by RNA polymerase II.* EMBO J, 2004. **23**(20): p. 4051-60.
138. Huttenhofer, A., P. Schattner, and N. Polacek, *Non-coding RNAs: hope or hype?* Trends Genet, 2005. **21**(5): p. 289-97.
139. Smale, S.T. and J.T. Kadonaga, *The RNA polymerase II core promoter.* Annu Rev Biochem, 2003. **72**: p. 449-79.
140. Smale, S.T., *Transcription initiation from TATA-less promoters within eukaryotic protein-coding genes.* Biochim Biophys Acta, 1997. **1351**(1-2): p. 73-88.

141. Kadonaga, J.T., *The DPE, a core promoter element for transcription by RNA polymerase II.* Exp Mol Med, 2002. **34**(4): p. 259-64.
142. Pickart, C.M., *Mechanisms underlying ubiquitination.* Annu Rev Biochem, 2001. **70**: p. 503-33.
143. Blackwood, E.M. and J.T. Kadonaga, *Going the distance: a current view of enhancer action.* Science, 1998. **281**(5373): p. 60-3.
144. Hampsey, M., *Molecular genetics of the RNA polymerase II general transcriptional machinery.* Microbiol Mol Biol Rev, 1998. **62**(2): p. 465-503.
145. Burley, S.K. and R.G. Roeder, *Biochemistry and structural biology of transcription factor IID (TFIID).* Annu Rev Biochem, 1996. **65**: p. 769-99.
146. Svejstrup, J.Q., P. Vichi, and J.M. Egly, *The multiple roles of transcription/repair factor TFIIH.* Trends Biochem Sci, 1996. **21**(9): p. 346-50.
147. Saunders, A., L.J. Core, and J.T. Lis, *Breaking barriers to transcription elongation.* Nat Rev Mol Cell Biol, 2006. **7**(8): p. 557-67.
148. Bushnell, D.A., et al., *Structural basis of transcription: an RNA polymerase II-TFIIB cocrystal at 4.5 Angstroms.* Science, 2004. **303**(5660): p. 983-8.
149. Adelman, K., et al., *Efficient release from promoter-proximal stall sites requires transcript cleavage factor TFIIS.* Mol Cell, 2005. **17**(1): p. 103-12.
150. Rodriguez, C.R., et al., *Kin28, the TFIIH-associated carboxy-terminal domain kinase, facilitates the recruitment of mRNA processing machinery to RNA polymerase II.* Mol Cell Biol, 2000. **20**(1): p. 104-12.
151. Kim, D.K., et al., *Structure-function analysis of human Spt4: evidence that hSpt4 and hSpt5 exert their roles in transcriptional elongation as parts of the DSIF complex.* Genes Cells, 2003. **8**(4): p. 371-8.
152. Price, D.H., *P-TEFb, a cyclin-dependent kinase controlling elongation by RNA polymerase II.* Mol Cell Biol, 2000. **20**(8): p. 2629-34.
153. Conaway, J.W. and R.C. Conaway, *Transcription elongation and human disease.* Annu Rev Biochem, 1999. **68**: p. 301-19.
154. Zawel, L., K.P. Kumar, and D. Reinberg, *Recycling of the general transcription factors during RNA polymerase II transcription.* Genes Dev, 1995. **9**(12): p. 1479-90.
155. Moreland, R.J., et al., *Mechanism of action of RNA polymerase II elongation factor Elongin. Maximal stimulation of elongation requires conversion of the early elongation complex to an Elongin-activable form.* J Biol Chem, 1998. **273**(41): p. 26610-7.
156. Fleischmann, G., et al., *Drosophila DNA topoisomerase I is associated with transcriptionally active regions of the genome.* Proc Natl Acad Sci U S A, 1984. **81**(22): p. 6958-62.
157. Reines, D., et al., *Transcription elongation by RNA polymerase II: mechanism of SII activation.* Cell Mol Biol Res, 1993. **39**(4): p. 331-8.
158. Cheung, A.C. and P. Cramer, *Structural basis of RNA polymerase II backtracking, arrest and reactivation.* Nature, 2011. **471**(7337): p. 249-53.
159. Selth, L.A., S. Sigurdsson, and J.Q. Svejstrup, *Transcript Elongation by RNA Polymerase II.* Annu Rev Biochem, 2010. **79**: p. 271-93.
160. Clapier, C.R. and B.R. Cairns, *The biology of chromatin remodeling complexes.* Annu Rev Biochem, 2009. **78**: p. 273-304.

161. Whitehouse, I., et al., *Nucleosome mobilization catalysed by the yeast SWI/SNF complex.* Nature, 1999. **400**(6746): p. 784-7.
162. Sudarsanam, P., et al., *Whole-genome expression analysis of snf/swi mutants of Saccharomyces cerevisiae.* Proc Natl Acad Sci U S A, 2000. **97**(7): p. 3364-9.
163. Orphanides, G., et al., *FACT, a factor that facilitates transcript elongation through nucleosomes.* Cell, 1998. **92**(1): p. 105-16.
164. Bortvin, A. and F. Winston, *Evidence that Spt6p controls chromatin structure by a direct interaction with histones.* Science, 1996. **272**(5267): p. 1473-6.
165. Clayton, A.L., et al., *Histone acetylation and gene induction in human cells.* FEBS Lett, 1993. **336**(1): p. 23-6.
166. Liang, G., et al., *Distinct localization of histone H3 acetylation and H3-K4 methylation to the transcription start sites in the human genome.* Proc Natl Acad Sci U S A, 2004. **101**(19): p. 7357-62.
167. Li, B., et al., *Histone H3 lysine 36 dimethylation (H3K36me2) is sufficient to recruit the Rpd3s histone deacetylase complex and to repress spurious transcription.* J Biol Chem, 2009. **284**(12): p. 7970-6.
168. Krogan, N.J., et al., *Methylation of histone H3 by Set2 in Saccharomyces cerevisiae is linked to transcriptional elongation by RNA polymerase II.* Mol Cell Biol, 2003. **23**(12): p. 4207-18.
169. Schaft, D., et al., *The histone 3 lysine 36 methyltransferase, SET2, is involved in transcriptional elongation.* Nucleic Acids Res, 2003. **31**(10): p. 2475-82.
170. Xiao, T., et al., *Phosphorylation of RNA polymerase II CTD regulates H3 methylation in yeast.* Genes Dev, 2003. **17**(5): p. 654-63.
171. Xiao, T., et al., *Histone H2B ubiquitylation is associated with elongating RNA polymerase II.* Mol Cell Biol, 2005. **25**(2): p. 637-51.
172. Nowak, S.J., C.Y. Pai, and V.G. Corces, *Protein phosphatase 2A activity affects histone H3 phosphorylation and transcription in Drosophila melanogaster.* Mol Cell Biol, 2003. **23**(17): p. 6129-38.
173. Connelly, S. and J.L. Manley, *A functional mRNA polyadenylation signal is required for transcription termination by RNA polymerase II.* Genes Dev, 1988. **2**(4): p. 440-52.
174. Logan, J., et al., *A poly(A) addition site and a downstream termination region are required for efficient cessation of transcription by RNA polymerase II in the mouse beta maj-globin gene.* Proc Natl Acad Sci U S A, 1987. **84**(23): p. 8306-10.
175. Nag, A., et al., *The conserved AAUAAA hexamer of the poly(A) signal can act alone to trigger a stable decrease in RNA polymerase II transcription velocity.* RNA, 2006. **12**(8): p. 1534-44.
176. Nag, A., K. Narsinh, and H.G. Martinson, *The poly(A)-dependent transcriptional pause is mediated by CPSF acting on the body of the polymerase.* Nat Struct Mol Biol, 2007. **14**(7): p. 662-9.
177. Orozco, I.J., S.J. Kim, and H.G. Martinson, *The poly(A) signal, without the assistance of any downstream element, directs RNA polymerase II to pause in vivo and then to release stochastically from the template.* J Biol Chem, 2002. **277**(45): p. 42899-911.

178. Park, N.J., D.C. Tsao, and H.G. Martinson, *The two steps of poly(A)-dependent termination, pausing and release, can be uncoupled by truncation of the RNA polymerase II carboxyl-terminal repeat domain.* Mol Cell Biol, 2004. **24**(10): p. 4092-103.
179. Kim, M., et al., *The yeast Rat1 exonuclease promotes transcription termination by RNA polymerase II.* Nature, 2004. **432**(7016): p. 517-22.
180. West, S., N. Gromak, and N.J. Proudfoot, *Human 5' --> 3' exonuclease Xrn2 promotes transcription termination at co-transcriptional cleavage sites.* Nature, 2004. **432**(7016): p. 522-5.
181. Liang, S. and C.S. Lutz, *p54nrb is a component of the snRNP-free U1A (SF-A) complex that promotes pre-mRNA cleavage during polyadenylation.* RNA, 2006. **12**(1): p. 111-21.
182. Rosonina, E., et al., *Role for PSF in mediating transcriptional activator-dependent stimulation of pre-mRNA processing in vivo.* Mol Cell Biol, 2005. **25**(15): p. 6734-46.
183. West, S., N.J. Proudfoot, and M.J. Dye, *Molecular dissection of mammalian RNA polymerase II transcriptional termination.* Mol Cell, 2008. **29**(5): p. 600-10.
184. Cramer, P., *RNA polymerase II structure: from core to functional complexes.* Curr Opin Genet Dev, 2004. **14**(2): p. 218-26.
185. Young, R.A., *RNA polymerase II.* Annu Rev Biochem, 1991. **60**: p. 689-715.
186. Archambault, J. and J.D. Friesen, *Genetics of eukaryotic RNA polymerases I, II, and III.* Microbiol Rev, 1993. **57**(3): p. 703-24.
187. Nonet, M., D. Sweetser, and R.A. Young, *Functional redundancy and structural polymorphism in the large subunit of RNA polymerase II.* Cell, 1987. **50**(6): p. 909-15.
188. Allison, L.A., et al., *The C-terminal domain of the largest subunit of RNA polymerase II of Saccharomyces cerevisiae, Drosophila melanogaster, and mammals: a conserved structure with an essential function.* Mol Cell Biol, 1988. **8**(1): p. 321-9.
189. Egloff, S. and S. Murphy, *Cracking the RNA polymerase II CTD code.* Trends Genet, 2008. **24**(6): p. 280-8.
190. Payne, J.M. and M.E. Dahmus, *Partial purification and characterization of two distinct protein kinases that differentially phosphorylate the carboxyl-terminal domain of RNA polymerase subunit IIa.* J Biol Chem, 1993. **268**(1): p. 80-7.
191. Komarnitsky, P., E.J. Cho, and S. Buratowski, *Different phosphorylated forms of RNA polymerase II and associated mRNA processing factors during transcription.* Genes Dev, 2000. **14**(19): p. 2452-60.
192. McCracken, S., et al., *5'-Capping enzymes are targeted to pre-mRNA by binding to the phosphorylated carboxy-terminal domain of RNA polymerase II.* Genes Dev, 1997. **11**(24): p. 3306-18.
193. Max, T., M. Sogaard, and J.Q. Svejstrup, *Hyperphosphorylation of the C-terminal repeat domain of RNA polymerase II facilitates dissociation of its complex with mediator.* J Biol Chem, 2007. **282**(19): p. 14113-20.
194. Peterlin, B.M. and D.H. Price, *Controlling the elongation phase of transcription with P-TEFb.* Mol Cell, 2006. **23**(3): p. 297-305.

195. Cho, E.J., et al., *Opposing effects of Ctk1 kinase and Fcp1 phosphatase at Ser 2 of the RNA polymerase II C-terminal domain.* Genes Dev, 2001. **15**(24): p. 3319-29.
196. Egloff, S., et al., *Serine-7 of the RNA polymerase II CTD is specifically required for snRNA gene expression.* Science, 2007. **318**(5857): p. 1777-9.
197. Chapman, R.D., et al., *Transcribing RNA polymerase II is phosphorylated at CTD residue serine-7.* Science, 2007. **318**(5857): p. 1780-2.
198. Hampsey, M. and D. Reinberg, *Tails of intrigue: phosphorylation of RNA polymerase II mediates histone methylation.* Cell, 2003. **113**(4): p. 429-32.
199. Xu, Y.X. and J.L. Manley, *Pin1 modulates RNA polymerase II activity during the transcription cycle.* Genes Dev, 2007. **21**(22): p. 2950-62.
200. Verdecia, M.A., et al., *Structural basis for phosphoserine-proline recognition by group IV WW domains.* Nat Struct Biol, 2000. **7**(8): p. 639-43.
201. Kelly, W.G., M.E. Dahmus, and G.W. Hart, *RNA polymerase II is a glycoprotein. Modification of the COOH-terminal domain by O-GlcNAc.* J Biol Chem, 1993. **268**(14): p. 10416-24.
202. Buratowski, S., *The CTD code.* Nat Struct Biol, 2003. **10**(9): p. 679-80.
203. Exinger, F. and F. Lacroute, *6-Azauracil inhibition of GTP biosynthesis in Saccharomyces cerevisiae.* Curr Genet, 1992. **22**(1): p. 9-11.
204. Pokholok, D.K., N.M. Hannett, and R.A. Young, *Exchange of RNA polymerase II initiation and elongation factors during gene expression in vivo.* Mol Cell, 2002. **9**(4): p. 799-809.
205. van Hoffen, A., et al., *Transcription-coupled repair removes both cyclobutane pyrimidine dimers and 6-4 photoproducts with equal efficiency and in a sequential way from transcribed DNA in xeroderma pigmentosum group C fibroblasts.* EMBO J, 1995. **14**(2): p. 360-7.
206. Lee, S.K., et al., *Requirement for yeast RAD26, a homolog of the human CSB gene, in elongation by RNA polymerase II.* Mol Cell Biol, 2001. **21**(24): p. 8651-6.
207. Laine, J.P. and J.M. Egly, *When transcription and repair meet: a complex system.* Trends Genet, 2006. **22**(8): p. 430-6.
208. Allard, S., J.Y. Masson, and J. Cote, *Chromatin remodeling and the maintenance of genome integrity.* Biochim Biophys Acta, 2004. **1677**(1-3): p. 158-64.
209. Natale, V., *A comprehensive description of the severity groups in Cockayne syndrome.* Am J Med Genet A, 2011. **155A**(5): p. 1081-95.
210. Woudstra, E.C., et al., *A Rad26-Def1 complex coordinates repair and RNA pol II proteolysis in response to DNA damage.* Nature, 2002. **415**(6874): p. 929-33.
211. Hershko, A., et al., *Components of ubiquitin-protein ligase system. Resolution, affinity purification, and role in protein breakdown.* J Biol Chem, 1983. **258**(13): p. 8206-14.
212. Somesh, B.P., et al., *Communication between distant sites in RNA polymerase II through ubiquitylation factors and the polymerase CTD.* Cell, 2007. **129**(1): p. 57-68.

213. Somesh, B.P., et al., *Multiple mechanisms confining RNA polymerase II ubiquitylation to polymerases undergoing transcriptional arrest.* Cell, 2005. **121**(6): p. 913-23.
214. Harreman, M., et al., *Distinct ubiquitin ligases act sequentially for RNA polymerase II polyubiquitylation.* Proc Natl Acad Sci U S A, 2009. **106**(49): p. 20705-10.
215. Beaudenon, S.L., et al., *Rsp5 ubiquitin-protein ligase mediates DNA damage-induced degradation of the large subunit of RNA polymerase II in Saccharomyces cerevisiae.* Mol Cell Biol, 1999. **19**(10): p. 6972-9.
216. Ferdous, A., et al., *The 19S regulatory particle of the proteasome is required for efficient transcription elongation by RNA polymerase II.* Mol Cell, 2001. **7**(5): p. 981-91.
217. Bregman, D.B., et al., *UV-induced ubiquitination of RNA polymerase II: a novel modification deficient in Cockayne syndrome cells.* Proc Natl Acad Sci U S A, 1996. **93**(21): p. 11586-90.
218. Anindya, R., O. Aygun, and J.Q. Svejstrup, *Damage-induced ubiquitylation of human RNA polymerase II by the ubiquitin ligase Nedd4, but not Cockayne syndrome proteins or BRCA1.* Mol Cell, 2007. **28**(3): p. 386-97.
219. Chen, X., et al., *Rpb1 sumoylation in response to UV radiation or transcriptional impairment in yeast.* PLoS One, 2009. **4**(4): p. e5267.
220. Poschmann, J., et al., *The Peptidyl Prolyl Isomerase Rrd1 Regulates the Elongation of RNA Polymerase II during Transcriptional Stresses.* PLoS One, 2011. **6**(8): p. e23159.
221. Singh, N., et al., *The Ess1 prolyl isomerase is required for transcription termination of small noncoding RNAs via the Nrd1 pathway.* Mol Cell, 2009. **36**(2): p. 255-66.
222. Gasch, A.P., et al., *Genomic expression programs in the response of yeast cells to environmental changes.* Mol Biol Cell, 2000. **11**(12): p. 4241-57.
223. Neklesa, T.K. and R.W. Davis, *Superoxide anions regulate TORC1 and its ability to bind Fpr1:rapamycin complex.* Proc Natl Acad Sci U S A, 2008. **105**(39): p. 15166-71.
224. Ribar, B., L. Prakash, and S. Prakash, *Requirement of ELC1 for RNA polymerase II polyubiquitylation and degradation in response to DNA damage in Saccharomyces cerevisiae.* Mol Cell Biol, 2006. **26**(11): p. 3999-4005.
225. Crespo, J.L. and M.N. Hall, *Elucidating TOR signaling and rapamycin action: lessons from Saccharomyces cerevisiae.* Microbiol Mol Biol Rev, 2002. **66**(4): p. 579-91, table of contents.
226. Caldwell, R.B., et al., *Gene function analysis using the chicken B-cell line DT40.* Methods Mol Biol, 2007. **408**: p. 193-210.
227. Vaynberg, J. and J. Qin, *Weak protein-protein interactions as probed by NMR spectroscopy.* Trends Biotechnol, 2006. **24**(1): p. 22-7.
228. Sekar, R.B. and A. Periasamy, *Fluorescence resonance energy transfer (FRET) microscopy imaging of live cell protein localizations.* J Cell Biol, 2003. **160**(5): p. 629-33.
229. Phizicky, E.M. and S. Fields, *Protein-protein interactions: methods for detection and analysis.* Microbiol Rev, 1995. **59**(1): p. 94-123.

230. Nord, A.S., et al., *The International Gene Trap Consortium Website: a portal to all publicly available gene trap cell lines in mouse.* Nucleic Acids Res, 2006. **34**(Database issue): p. D642-8.
231. Nagy, A., *Cre recombinase: the universal reagent for genome tailoring.* Genesis, 2000. **26**(2): p. 99-109.