Répression de l'expression génique contrôlée par l'ARN et les histones chaperonnes chez la levure fissipare Schizosaccharomyces pombe

Matteo Cattaneo

▶ **To cite this version:**

Matteo Cattaneo. Répression de l'expression génique contrôlée par l'ARN et les histones chaperonnes chez la levure fissipare Schizosaccharomyces pombe. Biologie du développement. Université Grenoble Alpes, 2015. Français. NNT : 2015GREAV040 . tel-01539507

HAL Id: tel-01539507

https://theses.hal.science/tel-01539507

Submitted on 15 Jun 2017

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

UNIVERSITÉ GRENOBLE ALPES

THÈSE

Pour obtenir le grade de

DOCTEUR DE L'UNIVERSITÉ GRENOBLE ALPES

Spécialité : **CSV/ Biologie du développement - Oncogenèse**

Arrêté ministériel : 7 août 2006

Présentée par

Matteo CATTANEO

Thèse dirigée par **André VERDEL** et
codirigée par **Daniel PERAZZA**

préparée au sein de **l'Institut Albert Bonniot**
dans **l'École Doctorale Chimie et Sciences du Vivant**

RNA and histone chaperone -based gene silencing in the fission yeast *Schizosaccharomyces pombe*

Thèse soutenue publiquement le **14 décembre 2015**
devant le jury composé de :

Dr. Aline PROBST
Chargé de recherche, GReD - Clermont University (Aubière), Rapporteur
Dr. Benjamin LOPPIN
Directeur de recherche, CGφMC (Lyon), Rapporteur
Dr. Angela TADDEI, **Président du jury**
Directeur de recherche, Institut Curie (Paris), Membre
Dr. Saadi KHOCHBIN
Directeur de recherche, Institut Albert Bonniot (Grenoble), Membre
Dr. Daniel PERAZZA
Maître de conférences, Institut Albert Bonniot (Grenoble), Membre
Dr. André VERDEL
Chargé de recherche, Institut Albert Bonniot (Grenoble), Membre

THÈSE

Pour obtenir le grade de

DOCTEUR DE L'UNIVERSITÉ GRENOBLE ALPES

Spécialité : **CSV/ Biologie du développement - Oncogenèse**

Arrêté ministériel : 7 août 2006

Présentée par

Matteo CATTANEO

Thèse dirigée par **André VERDEL** et
codirigée par **Daniel PERAZZA**

préparée au sein de **l'Institut Albert Bonniot**
dans **l'École Doctorale Chimie et Sciences du Vivant**

RNA and histone chaperone -based gene silencing in the fission yeast *Schizosaccharomyces pombe*

Thèse soutenue publiquement le **14 décembre 2015**
devant le jury composé de :

Dr. Aline PROBST
Chargé de recherche, GReD - Clermont University (Aubière), Rapporteur
Dr. Benjamin LOPPIN
Directeur de recherche, CGφMC (Lyon), Rapporteur
Dr. Angela TADDEI, Président du jury
Directeur de recherche, Institut Curie (Paris), Membre
Dr. Saadi KHOCHBIN
Directeur de recherche, Institut Albert Bonniot (Grenoble), Membre
Dr. Daniel PERAZZA
Maître de conférences, Institut Albert Bonniot (Grenoble), Membre
Dr. André VERDEL
Chargé de recherche, Institut Albert Bonniot (Grenoble), Membre

Table of contents

Table of contents 3

Abstract 7

Résumé 7

List of main abbreviations 9

INTRODUCTION. 13

1. Chromatin, a dynamic structure 18

1.1. General features of chromatin 19

1.1.1. Histories of chromatin's investigation 19

1.1.2. Chromatin is a dynamic and highly regulated structure 20

1.1.3. Definition of Epigenetics 21

1.2. The dynamic nature of chromatin 21

1.2.1. Histone post-translational modifications 22

1.2.2. Histone variants 25

1.2.3. Histone Chaperones 26

1.2.4. ATP-dependent chromatin remodelers 29

1.2.5. RNA-mediated regulation of chromatin 30

1.3. Spatial and functional confinement of chromatin: euchromatin and heterochromatin 31

1.3.1 Two major distinct chromatin states: euchromatin and heterochromatin 31

1.3.2 Borders between euchromatin and heterochromatin and heterochromatin spreading 32

2. Heterochromatin and gene silencing 36

2.1. General features of heterochromatin in eukaryotes 37

2.1.1. Constitutive and facultative heterochromatin 37

2.1.2. Localization, structure and function of constitutive heterochromatin 37

2.1.3. Transcription of heterochromatin sequences 38

2.2. Heterochromatin in fission yeast 39

2.2.1. *Schizosaccharomyces pombe*: a reference model to study chromatin-based mechanisms and heterochromatin gene silencing 39

2.2.2. Constitutive and facultative heterochromatin 41

2.2.3. Location, function and structure of constitutive heterochromatin 41

2.2.4. Main proteins involved in heterochromatin assembly 46

2.3. Mechanisms of constitutive heterochromatin assembly and gene silencing in fission yeast ... 50
2.3.1. Essential role of transcription and RNAi in the context of constitutive heterochromatin . 51
2.3.2. RNA-independent mechanisms of constitutive heterochromatin formation 59
2.4. Mechanisms of facultative heterochromatin assembly and gene silencing in fission yeast 61
2.4.1 Different genomic sites of facultative heterochromatin..... 61
2.4.2. The RNA-binding protein Mmi1 mediates silencing at meiotic genes 62
2.4.3. The peculiar case of transposable element gene silencing in *S. pombe* 64
3. ATAD2-like proteins..... 68
3.1. The putative histone chaperone ATAD2 in mammals..... 71
3.1.1. ATAD2 is involved in transcriptional activation..... 71
3.1.2. Human ATAD2 in cancer..... 72
3.2. Yta7 in *Saccharomyces cerevisiae* 73
3.2.1. Yta7 is a probable histone chaperone regulating transcription..... 73
3.2.2. Yta7 and chromatin boundary elements..... 76
3.3. ATAD2-like proteins conservation through eukaryotes 78
3.4. ATAD2 from human to yeast: functional hortologs? 81
MATERIAL AND METHODS 85
1. Biological materials and culture conditions 85
1.1. Schizosaccharomyces pombe strains 85
1.2. Description of plasmids 87
1.3. Fission yeast culture conditions 88
1.4. Description of primers..... 88
2. Strains construction..... 90
2.1. Transformation with lithium acetate 90
2.2. Crosses and random spore analysis 91
3. Analysis of cells morphology and mortality 92
4. Growth assay 93
4.1. Growth assay on liquid medium..... 93
4.2. Growth assay on solid medium 93
4.3. Drug sensitivity and centromeric silencing assay..... 93
5. Analysis of the cell cycle (FACS)..... 94
6. Analysis of protein interactions..... 95
6.1. Protein extraction..... 95
6.2. Coimmunoprecipitation (CoIP)..... 96

6.3. Ago1 pull-down 96
6.4. Histone peptide pulldown H4/H4ac 97
6.5. Western blot 98
6.6. Protein complex purification (TAP purification) 99
7. Analysis of interaction between proteins and nucleic acids (ChIP) 102
7.1. Samples preparation 102
7.2. Immunoprecipitation 102
7.3. DNA purification and analysis 103
7.4. RNA immunoprecipitation (RIP) 103
8. Analysis of RNA expression 104
8.1. Extraction and purification of RNA 104
8.2. DNAse and RTqPCR 104
8.3. Transcriptomic sample preparation and analysis 105
9. Sporulation assay 106
10. Analysis of mating type identity (PCR) 107
11. Conditional Abo1 knockdown system 107
RESULTS 109
Overview of my PhD results 111
1. Role of Ccr4-Not complex in heterochromatin assembly and gene silencing 114
1.1. Context and main results 115
1.2. Purification of Mmi1 revealed its interaction with Ccr4-Not complex 116
1.3. Interaction between Mmi1 and Ccr4-Not is mediated by Rcd1 117
1.4. Ccr4-Not is required for Mmi1-mediated facultative heterochromatin assembly 119
1.5. Ccr4-Not promotes gene silencing at constitutive heterochromatin regions 122
1.6. General conclusion 125
2. RITS purification reveals a connection with histone chaperones 126
2.1. Context and main results 127
2.2. RITS purification identified new proteins possibly involved in constitutive heterochromatin silencing and/or formation 128
2.3. Spt6 has a role in heterochromatin formation and gene silencing 131
2.4. Abo1 is required for proper constitutive heterochromatin gene silencing 135
2.5. General conclusion 138

3. Characterization of *S. pombe* Abo1, a model to dissect the function of ATAD2 in cancer 140
3.1. Context and main results 141
3.2. Abo1, but not Abo2, is critical for proper cell growth in fission yeast 142
3.3. Abo1 and human ATAD2 share functional redundancy 149
3.4. Transcriptomic analysis of *abo1Δ* cells revealed a role for Abo1 in transcription regulation and gene silencing 151
3.5. Abo1 is connected to the chromatin- and transcription-linked protein Tfg3 157
3.6. Analysis of Abo1 purification by quantitative proteomics uncovered multiple physical links to chromatin 161
3.7. The critical function of Abo1 in cell growth is linked to histones 166
3.8. General conclusion 168
Additional results: 169
1. The bromodomain of Abo1 binds to histone H4 independently of its acetylation status 169
2. Functional similarities between Abo1 and Sap1/ CENPB proteins 170
DISCUSSION AND PERSPECTIVES 175
Mmi1 interacts with Ccr4-Not, a new regulator of heterochromatin formation and gene silencing 178
Ccr4-Not is required for facultative heterochromatin integrity 179
Ccr4-Not promotes constitutive heterochromatin gene silencing 180
Analysis of the RITS purification to identify new proteins involved in heterochromatin silencing and/or formation 181
RITS interacts with the transcription-coupled histone chaperone Spt6 183
Role of Spt6 in heterochromatin formation and gene silencing 184
Abo1 interacts with RITS and is a crucial regulator of gene expression in fission yeast 185
Abo1 is a chromatin-linked protein, possibly acting as histone chaperone 187
Abo1, possible orthologue of human ATAD2, is required for cell growth in *S. pombe* 188
No functional redundancy between Abo1 and Abo2 in fission yeast 190
A way to escape *abo1Δ* phenotype: mechanism of reversion 191
Conclusive remarks 193
REFERENCES 194
Acknowledgements 211

Abstract

RNA- and histone chaperone-based gene silencing in the fission yeast *Schizosaccharomyces pombe*

Proteins controlling chromatin dynamics and transcription are often conserved throughout eukaryotes and are deregulated in many diseases. In this study, we characterized three actors of RNA- and/or histone-based gene silencing in *Schizosaccharomyces pombe*: the 3' end RNA processing Ccr4-Not complex, the histone chaperone Spt6 and, in more details, Abo1. Abo1 is homologous to the human cancer-linked protein and putative histone chaperone ATAD2. We found that both Ccr4-Not and Spt6 directly contribute to RNA-based heterochromatin formation and gene silencing. In the case of Abo1, thanks to a combination of yeast genetics with transcriptomics and proteomics approaches, we showed that Abo1 is likely to be an orthologue of human ATAD2. Moreover, Abo1 is critical for proper cell growth and for the silencing of several hundreds of protein-coding and non-coding genes. Finally, Abo1 physically interacts with multiple histone- and transcription-linked proteins. Altogether, these findings provide new insights into how RNA- and histone-based processes act together to silence gene expression in *S. pombe*, and may also contribute to elucidate the functions of ATAD2 in cancer.

Chromatin, gene silencing, histone chaperone, *Schizosaccharomyces pombe*, RNA, ATAD2

Résumé

Répression de l'expression génique contrôlée par l'ARN et les histones chaperonnes chez la levure fissipare *Schizosaccharomyces pombe*

Les protéines qui contrôlent la dynamique de la chromatine et la transcription sont généralement conservées chez les eucaryotes, et dérégulées dans de nombreuses maladies. Dans cette étude, nous avons caractérisé trois acteurs de la répression transcriptionelle dépendante de l'ARN et/ou des histones chez *S. pombe*: le complexe de maturation de l'extrémité 3' des ARN Ccr4-Not, la chaperonne d'histone Spt6 et enfin, de manière plus approfondie, la protéine Abo1. Abo1 est l'homologue de la protéine humaine ATAD2 qui est une probable chaperonne d'histone impliquée dans de nombreux cancers. Nous avons trouvé que Ccr4-Not et Spt6 contribuent chacune directement à la formation de l'hétérochromatine, selon un processus dépendant de l'ARN, ainsi qu'à la répression de l'expression génique. En combinant des approches de génétique de levure à des approches de transcriptomique et de protéomique, nous avons montré qu'Abo1 est vraisemblablement un orthologue d'ATAD2 humaine, qu'elle joue un rôle critique pour la croissance normale des cellules et la répression de centaines de gènes codants et non-codants, et enfin qu'elle interagit physiquement avec de multiples protéines en lien avec les histones et la transcription. Ces résultats fournissent de précieuses indications sur la façon dont certains processus dépendants de l'ARN ou des histones agissent ensemble pour réduire au silence l'expression génique chez *S. pombe*. Nos données pourraient également contribuer à mieux comprendre les fonctions de la protéine ATAD2 dans le cancer.

Chromatine, silencing des gènes, histones chaperonnes, *Schizosaccharomyces pombe*, ARN, ATAD2

List of main abbreviations

5FOA: 5 FluoroOrotic Acid

Abo1: ATPase with bromodomain protein

Abp1: Actin-binding protein 1

Ago1: Argonaute protein 1

AID: Auxin Inducible Degron

ANCCA: AAA Nuclear Coregulator Cancer-Associated

ARC: Argonaute siRNA Chaperone

Asf1: Anti-silencing factor 1

ATAD2: ATPase family AAA+ Domain-containing protein 2

ATF: Activating Transcription Factor

ATP: Adenosine Tri-Phosphate

BD: BromoDomain

bp: base pair

CAF1: Chromatin Assembly Factor-1

Ccr4-Not: Carbon catabolite repression 4-Negative on TATA

Cdk1: Cyclin-dependent kinase 1

CENPA: CENtromere Protein A

CHD: Chromodomain Helicase DNA binding

ChIP: Chromatin ImmunoPrecipitation

Chp1: Chromodomain protein 1

CK2: Casein Kinase 2

Clr4: Cryptic loci regulator gene 4

CLRC: CLr4 methyltransferase-Containing Complex

cnt: *central core region*

CoIP: Co-ImmunoPrecipitation

CpG: Cytosine-phosphate-Guanine

CPT: CamPtoThecin

CREB: cAMP Response Element Binding

CTCF: CCCTC-binding Factor

Dcr1: Dicer1

DMSO: DiMethyl SulfOxide

DNA: DeoxyriboNucleic Acid

DNMT: DNA MethylTransferases

DSB: Double-Strand Break

DSR: Determinant Selective Removal

dsRNA: double stranded RNA

EMMc: Edinburgh Minimum Medium complete

FACS: Fluorescence Activated Cell Sorting

FACT: FAcilitates Chromatin Transcription

FC: Fold Change

H3K9me: methylation on the lysine 9 of histone H3

HAATI: Heterochromatin Amplification-mediated And Telomerase-Independent

HAT: Histone AcetylTransferase

HDAC: Histone DeACetylases

HDM: Histone DeMethylases

HIRA: HIstone cell cycle Regulation defective homolog A

HJURP: Holliday JUnction Recognition Protein

HMT: Histone MethyltTransferases

HOOD: HeterOchrOmatin Domain

HP1: Heterochromatin Protein 1

HAS: Helicase-SANT

HU: HydroxyUrea

imr: *innermost repeats*

INO80: INOsitol requiring 80

IP: ImmunoPrecipitation

IRC: *Inverted Repeat Centromere*

ISWI: Imitation SWItch

LEX-1: Lin-48 EXpression abnormal protein 1

lncRNA: long non-coding RNA

LTR: Long Terminal Repeat

Mei4: Meiosis inducing gene 4

miRNA: microRNA

MLLT3: Myeloid/Lymphoid or mixed-Lineage Leukemia Translocated to 3 chromosome

Mmi1: Meiotic mRNA interception 1

MMS : Methyl Methane Sulfonate

MNase: Micrococcal Nuclease digestion

MS: Mass Spectrometry

NAA: 1-NaphthaleneAcetic Acid

NAP: Nucleosome Assembly Protein

NASP: Nuclear Autoantigenic Sperm Protein

ncRNA: non-coding RNA

NURD: NUcleosome Remodelling and Deacetylase

NURS: NUclear RNA Silencing

OD: Optical Density

OE: OverExpression

otr: *outer repeats*

PCR: Polymerase Chain Reaction

PEV: Position Effect Variegation

piRNA: piwi-interacting RNA

PK: Protein Kinase

PP: Protein Phosphatase

priRNA: primal RNA

PCG: Protein Coding-Gene

PTGS: Post-Transcriptional Gene Silencing

PTM: Post-Translational Modification

Rcd1: Required for cell differentiation 1

RdDM: RNA directed DNA Methylation

rDNA: ribosomal DNA

RDRC: RNA-Directed RNA polymerase Complex

RdRP: RNA dependent RNA Polymerase

RIP: RNA ImmunoPrecipitation

RITS: RNAi-Induced Transcriptional Silencing

RNA pol II: RNA polymerase II

RNA: RiboNucleic Acid

RNAi: RNA interference

RPC: Recombination Promoting Complex

RTqPCR: Reverse Transcription quantitative PCR

Rtt109: Regulator of Ty transposition 109

Sap1: Switch-activating protein 1

s.d.: standard deviation

seq: sequencing

SHREC: Snf2/Hdac-containing REpressor Complex

siRNA: small interfering RNA

Spt6: Suppressor of Ty6

Ssm4: Suppressor of *sme2* gene

ssRNAs: single stranded RNA

Ste11: Sterility protein 11

Swi6: Switching gene 6

SWR: SWi2 and Snf2-Related ATPase

TAP: Tandem Affinity Purification

Tas3: Targeting complex subunit 3

TBZ: ThiaBendaZole

Taf14: TATA binding protein-associated factor 4

TCA : TriChloroacetic Acid

TE: Transposable Element

TERA : Transitional Endoplasmic Reticulum ATPase

TERRA: *Telomere Repeat-containing RNA*

Tfg3: Transcription factor g 3

TGS: Transcriptional Gene Silencing

ts: temperature-sensitive

VCP: Valosin-Containing Protein

WB: Western Blot

wt: *wild type*

wtf: with Tf

Xist: X inactive-specific transcript

YEA: Yeast Extract supplemented with Adenine

Yta7: Yeast Tat-binding Analog 7

YTH: YT521-B Homology

INTRODUCTION

This Introduction section, divided in 3 Chapters, is an overview of the field of research of my PhD thesis project, which mainly aimed at investigating the role of RNA- and/or transcription-linked proteins in silencing gene expression at the chromatin level in the fission yeast *Schizosaccharomyces pombe.*

Chromatin, the nucleoproteic entity containing the genomes of eukaryotes, consists of DNA molecules wrapped around an elementary and highly repeated particle formed by a core of proteins called histones. It is a highly dynamic structure and the mechanisms that regulate such dynamics implicate biochemical and structural modifications of histones that are in general well conserved from the unicellular yeasts to humans. There are two distinct states of chromatin: euchromatin, which is transcriptionally active or competent, and heterochromatin, which is more transcriptionally silenced and compact.

Chapter 1 presents an overview on histones, chromatin and its dynamic nature, and on euchromatin versus heterochromatin.

Chapter 2 describes in more details heterochromatin in the yeast *S. pombe*, which is the model organism used in this study. In this organism, heterochromatin formation and gene silencing both involves RNA and transcription. The process of RNA interference (RNAi), which produces and uses small RNAs as guides to recruit RNAi effector complexes to complementary RNAs to silence gene expression, plays a central role in heterochromatin formation and gene silencing. In addition, a process implicating a specific RNA-binding protein that recognizes nascent transcripts can also induce the formation of heterochromatin, especially at protein-coding genes. These two RNA- and transcription-dependent processes are presented in this Chapter, as well as the different regions of the genome where heterochromatin formation takes place in *S. pombe*.

Chapter 3 reports the current knowledge on the ATAD2-like family of proteins, which are believed to be histone chaperones involved in regulating gene expression. In my hosting team, a *S. pombe* ATAD2-like protein was identified as a potential partner of the RNAi effector complex that can trigger heterochromatin formation and gene silencing. The characterization of this *S. pombe* ATAD2-like protein has represented a major part of my PhD thesis project, and the results show that this putative histone chaperone may indeed work in the process of transcription, and is important for silencing gene expression both at euchromatin and heterochromatin regions.

1.

Chromatin, a dynamic structure

1.1. General features of chromatin

1.1.1. Histories of chromatin's investigation

The biological information in living organisms, transmitted from one generation to another, is stored as a sequence of nucleotides that compose DeoxyriboNucleic Acid (DNA). This macromolecule was first isolated by Friedrich Miescher in 1869, and its double-helix structure was first determined by James Watson and Francis Crick in 1953. Although DNA stores the genetic information, two other macromolecules carry out its instructions in cells: ribonucleic acid molecules (RNAs), which are obtained from DNA through a process called transcription, and proteins, which are composed of amino acids chains that are obtained from RNAs through a process called translation.

In the nuclei of eukaryotic cells, from yeast to humans, DNA is packaged with an equal mass of basic globular proteins called histones to form a structure named chromatin (Van Holde 1989). This association is mostly due to the electrostatic interactions between negatively charged DNA and positively charged histone proteins (Kornberg 1977). The repeating unit of chromatin is the nucleosome, which is formed by wrapping ~145–147 bp of DNA around the histone octamer that contains two copies of each histone: H2A, H2B, H3 and H4 (Luger et al. 1997; Richmond and Davey 2003). Adjacent nucleosomes are connected by short DNA segments called linker DNA that, in association with histone H1, hold the cores together and facilitate packaging of the 10 nm "beads on the string" nucleosomal chain (also called nucleofilament) into a more condensed 30 nm fiber (Kepper et al. 2008). During cell division, chromatin compaction increases even more to reach the most compacted state of organization, corresponding to the metaphasic chromosome (Van Holde 1989) (Figure 1).

A main function of chromatin is to condense a long molecule of DNA, which in human cells is around 2 meters long; into the small volume of the nucleus of a eukaryotic cell that has a diameter of some micrometers. Chromatin also protects the genetic information contained in DNA, for instance by preventing DNA damage events, and it ensures an important structural function in reinforcing DNA to allow cell division.

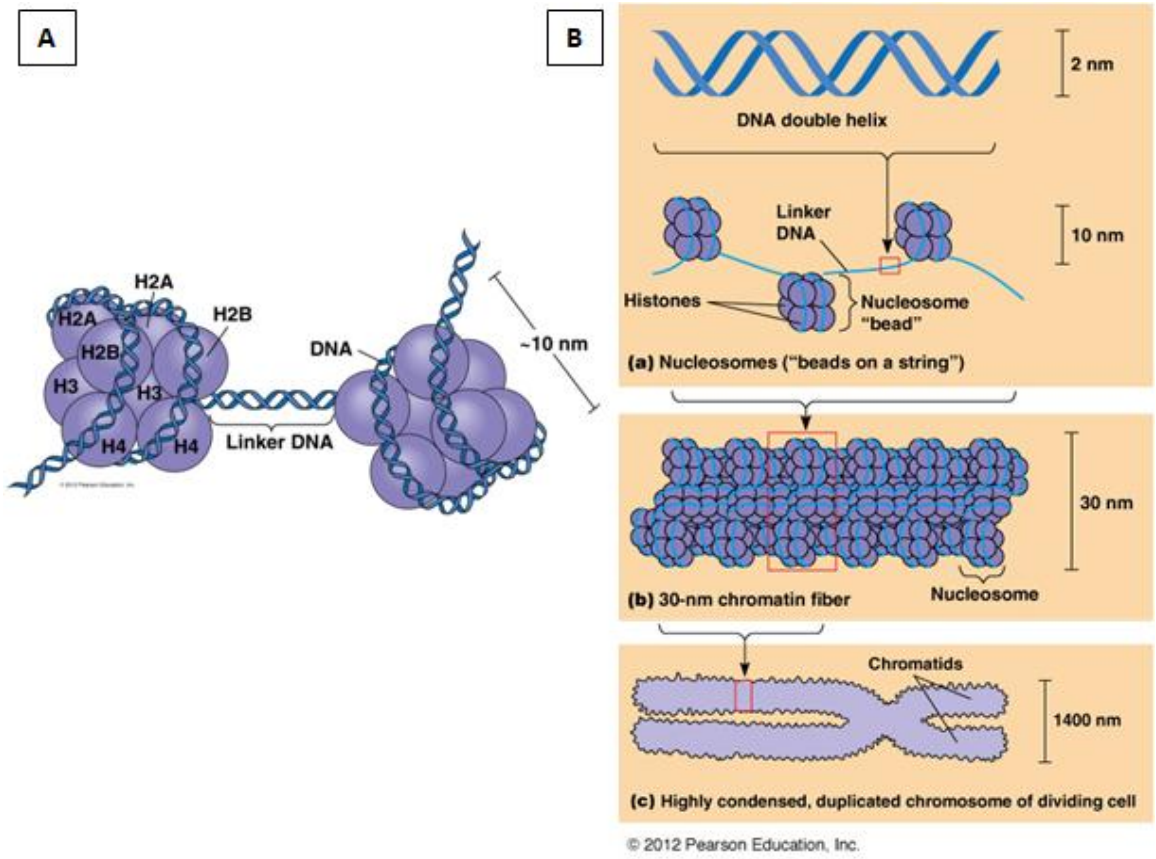


Figure1. Chromatin organization. A) Schematic representation of nucleosomes: ~145–147 bp of DNA are wrapped around the histone octamer that contains two copies of each histone: H2A, H2B, H3 and H4. **B)** Level of chromatin compaction from nucleosome to metaphasic chromosome: 10 nm "beads on a string" nucleosome and 30 nm chromatin fiber. Figure is adapted from Pearson Education 2012.

1.1.2. Chromatin is a dynamic and highly regulated structure

The most peculiar and fascinating aspect of chromatin resides in its dynamic regulation and organization. Nucleosome assembly is the first level of gene regulation, acting on the ability of machineries involved in replication, transcription and repair to access DNA. At a large scale, chromatin is organized in functional regions in the nucleus that have different levels of compaction, from easily accessible euchromatin to tightly packed heterochromatin. The transition between different states of chromatin is precisely controlled, for example during cell division or differentiation, or in response to external signals. The dynamic state of chromatin is achieved by modifications of histones and, as detailed in the following section, many proteins are involved in these processes, including histone post-translational modifiers, histone chaperones and chromatin remodelers.

1.1.3. Definition of Epigenetics

In a very broad sense, mechanisms that regulate chromatin dynamics can be defined as epigenetic mechanisms. The term Epigenetics (the Greek prefix *epi*- means "on top of" or "in addition to" genetics) was first coined by Conrad Hal Waddington in 1942 and, since then, its definition has evolved and its precise meaning is still under debate.

The term Epigenetics in its contemporary usage and to which we refer in this manuscript has emerged in the 1990s as 'the study of mitotically and/or meiotically heritable changes in gene function that cannot be explained by changes in DNA sequence' (Smit and Riggs 1996).

Of note, in addition to histones, the DNA sequence can be modified by the addition of a methyl group onto cytosine by a family of enzymes called DNA-methyltransferases (DNMT). This DNA modification can play a major role in epigenetic mechanisms. For example, in mammals, DNA methylation takes place mainly on the carbon 5 of cytosine in CpG dinucleotides and plays an important role in X chromosome inactivation, genomic imprinting, genome stability and transcriptional silencing (Sasai and Defossez 2009). However, this modification does not occur in certain eukaryotes, in which other epigenetic mechanisms have been identified. For example, it is absent in *Schizosaccharomyces pombe, Saccharomyces cerevisiae* and *Caenorhabditis elegans.*

1.2. The dynamic nature of chromatin

Several proteins are required to ensure the dynamic nature of chromatin, weakening histone-DNA interactions by adding post-translational modifications (PTMs) onto histones, by altering nucleosome composition with the help of histone chaperones or by incorporating histone variants mediated by ATP-dependent chromatin remodelers (Venkatesh and Workman 2015).

1.2.1. Histone post-translational modifications

Histone acetylation was the first discovered histone modification in 1961 (Phillips 1963). Since then, at least 15 histone modifications have been described and with the advent of genome-wide chromatin immunoprecipitation techniques, mapping of the global pattern of histone modifications has been performed in many organisms. This has allowed observation of a specific pattern of epigenetic modifications called histone code, whose clear biological function is still under investigation.

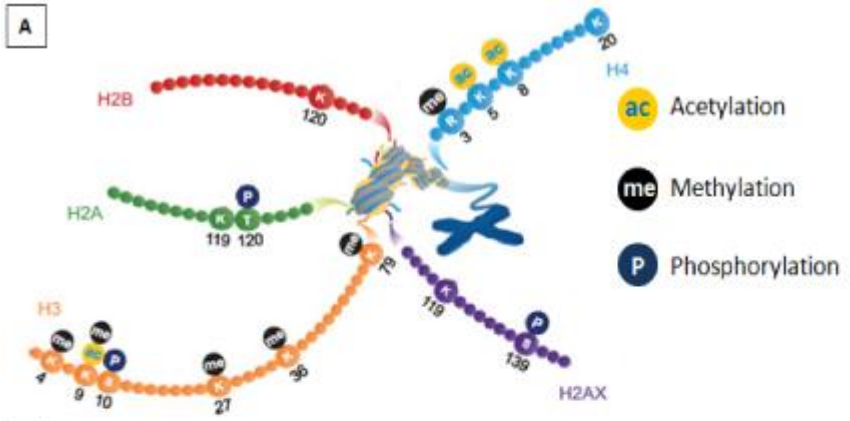
Histones are modified mostly on their N-terminal flexible tails; these modifications located at the periphery of the nucleosome mainly have the function of recruiting proteins to chromatin (Bannister and Kouzarides 2011). In contrast, modifications occurring at the C-terminal or at level of the histone core mainly have a structural role (Ye et al. 2005).

Most histone PTMs are reversible, as cells contain separate enzymes to add these marks (writers), and remove them (erasers). For instance, writers and erasers of acetylation are histone acetyltransferases (HAT) and deacetylases (HDAC), whereas for methylation there are histone methyltransferases (HMT) and demethylases (HDM). PTMs can recruit chromatin proteins that are called 'readers' since they contain domains able to recognize specific PTMs, and these factors in turn can modify directly or indirectly chromatin. Examples of reader domains include bromodomains, which recognize acetylated lysines on histones, or chromodomains which recognize methylated lysines.

A PTM on a specific histone could mediate the deposition of other PTMs in *cis* (on the same histone) or in *trans* (another histone) (Latham and Dent 2007). One of the first described mechanisms of *cis* regulation was mutual exclusion between methylation and acetylation on the lysine 9 of histone H3 (H3K9) (Turner 2005).

The most known histone modifications are histone methylation, acetylation and phosphorylation (Figure 2). These modifications and their impact on chromatin structure and transcription activation will be further described in the next sections.

A



B

Histone PTM	Residues	Effect on transcription	Writer	Reader	Eraser
Acetylation	K	activation	HAT	bromodomain proteins	HDAC
Methylation	K or R	H3K4 and H3K36: activation H3K9 and H3K27: repression	HMT	chromodomain proteins	HDM
Phosphorylation	S or T	H3S10: activation (H2AX: DNA repair)	PK	14-3-3 proteins	PP

Figure 2. Main Histones PTM. **A)** Scheme of main histone PTMs on histones H2A, H2B, H3, H4 and H2Ax: acetylation, methylation, phosphorylation. Adapted from Abcam.com. **B)** Table showing main features of histone acetylation, methylation and phosphorylation. K: lysine; R: arginine; S: serine; T: threonine; HAT: histone acetyltransferase, HDAC: histone deacetylase; HMT: histone methyltransferase; HDM: histone demethylase; PK: protein kinase; PP: protein phosphatase

1.2.1.1. Histone acetylation

Lysine acetylation is commonly associated with transcription activation. This modification, which mostly occurs on histones H3 and H4, neutralizes the positive charge of lysine residues, weakening interactions between histones and nucleosomal DNA, linker DNA or adjacent histones, thus increasing access of the transcription machinery to DNA (Zentner and Henikoff 2013).

Similarly, a variety of types of lysine acylation (crotonylation, formylation, succinylation, malonylation, propionylation and butyrylation) are known to neutralize the positive charge of lysines, weakening DNA-histones contacts, although their biological relevance is still under investigation (Olsen 2012).

1.2.1.2. Histone methylation

Histone lysines can also be mono-, di- or trimethylated, and these can be transcription repressive or activating marks (Bannister and Kouzarides 2011). Methylated H3K4 and H3K36 are normally associated with transcription activation, probably acting as regulatory modules that influence the deposition of other histone marks (Zentner and Henikoff 2013). H3K9 and H3K27 methylation is associated with transcription repression, mostly due to the recruitment of proteins involved in heterochromatin formation and gene silencing. For instance, the chromodomain of the HP1 (Heterochromatin Protein 1) family members binds H3K9me, while the chromodomain of Polycomb repressive complex proteins binds H3K27me (Fischle et al. 2003). Histone methylation can also increase nucleosome stability; for example, Swi6 proteins (HP1 ortholog in fission yeast) bind H3K9me and dimerize via their chromodomains to recognize pairs of H3K9me-modified tails in a single nucleosome. These dimers can then bridge the adjacent ones via their chromo shadow domains to stabilize nucleosomes and promote heterochromatin spreading (Canzio et al. 2011). Histones can also be mono- or dimethylated on arginines (Bedford and Clarke 2009). The role of arginine methylation has been less investigated, however growing evidence indicates that this modification could also affect chromatin structure, leading to the recruitment of proteins that regulate transcription and chromatin insulator activity (Litt, Qiu, and Huang 2009)

1.2.1.3. Histone phosphorylation

Histone phosphorylation, which occurs at serines or threonines, imparts a negative charge that weakens the association between DNA and histones, similarly to acetylation (Banerjee and Chakravarti 2011). A known example is phosphorylation of the histone variant H2A.X, known as γH2A.X, which facilitates DNA accessibility in order to permit the repair process after DNA double-strand breaks (Paull et al. 2000). Histone phosphorylation also alters the affinity of chromatin-binding proteins for their targets. For instance, phosphorylation of serine 10 in histone H3 (H3S10) destabilizes the interaction between HP1 and H3K9me

during mitosis, and this leads to chromatin relaxation and gene expression (Fischle et al. 2005).

1.2.1.4. Other Histone post-translational modifications

Other identified histone modifications are ADP-ribosylation of glutamic acids, which has been associated with more relaxed chromatin structure to facilitate DNA repair, threonine/serine glycosylation, which might be involved in transcriptional repression, and lysine ubiquitylation or sumoylation, which have been shown to have many context-dependent effects on chromatin (Messner and Hottiger 2011; Sakabe, Wang, and Hart 2010).

1.2.2. Histone variants

Beside histone modifications, a second strategy to regulate chromatin dynamics is to incorporate into nucleosomes some unconventional histones, which are called histone variants. There are variants for histones H3, H2A, H2B and for the linker histone H1. These variants are non-allelic isoforms that differ from canonical histones at the level of their primary sequence, with alterations ranging from a few amino acids to larger domains (Venkatesh and Workman 2015).

The substitution of canonical histones with their variants alters the biochemical properties of the nucleosome, affecting PTMs and protein recruitment, and therefore influencing chromatin structure (Talbert and Henikoff 2010).

Histone variants can be of the replicative type, if they have higher expression during S-phase, or of the replacement type, if they are incorporated into the genome in a replication-independent manner (Marzluff et al. 2002; Ahmad and Henikoff 2002). Each histone variant localizes to specific chromatin domains. For instance, centromeric histone H3 is replaced by CENPA, which is essential for kinetochore attachment and chromosomes segregation (Verdaasdonk and Bloom 2011). Another well-known example is H2A.Z. This variant, which is conserved across species, has a considerable amino acid sequence variation compared to the canonical histone H2A. This correlates with a subtle structural change that decreases the

stability of H2A.Z-containing nucleosomes, thus contributing to transcription activation, DNA repair and chromosome domain confinement (Meneghini, Wu, and Madhani 2003).

The dynamic exchange, incorporation and eviction of canonical histones and histone variants are regulated by two families of proteins that will be detailed in the next sections: histone chaperones and ATP-dependent chromatin remodelers.

1.2.3. Histone Chaperones

The term histone chaperone was coined by Ron Laskey in 1978 to identify histone-interacting proteins that are involved in histone storage (mostly in th cytoplasm) and transport, and nucleosome assembly and disassembly (Venkatesh and Workman 2015) (Figure 3A).

The role of histone chaperones is mainly to ensure the maintenance of chromatin in the genome, even during replication and transcription, in which the structure of chromatin needs to be transiently destabilized. In addition, histone chaperones protect cells from toxic soluble histones that, due to their basicity, may interact with acidic proteins and potentially lead to protein aggregation (Gurard-Levin, Quivy, and Almouzni 2014).

Histone chaperones can be classified on the basis of the histone substrates to which they bind. Interestingly, most of them bind either to H3-H4 or to H2A-H2B dimers. This specificity is mostly due to a combination of structural features and to the presence of histone chaperone-binding factors (Burgess and Zhang 2013). Some histone chaperones, like FACT (FAcilitates Chromatin Transcription), bind both H3–H4 and H2A–H2B (Winkler et al. 2011). A few histone chaperones can bind to specific canonical histones or histone variants, and often their function is connected to a specific genomic location. For example, HJURP (Holliday JUnction Recognition Protein) is in charge of incorporating the histone variant CENPA into centromeric chromatin (Foltz et al. 2009; Dunleavy et al. 2009); HIRA (HIstone Regulator A) and DAXX (Death Associated proteins) are mostly in charge of replacing canonical histone H3 with the H3.3 variant, while CAF1 (Chromatin Assembly Factor-1) with the histone variant H3.1 (Goldberg et al. 2010; Pchelintsev et al. 2013; Lewis et al. 2010; Tagami et al. 2004).

Histone chaperones participate in distinct steps of nucleosome assembly. For instance, NAPs (Nucleosome Assembly Proteins) help to shuttle newly synthesized histones from the cytoplasm to the nucleus (Mosammaparast, Ewart, and Pemberton 2002). Once in the nucleus, histones can also be stored, instead of being immediately incorporated into chromatin. Several histone chaperones are known to regulate the storage of histones, for instance the histone chaperone NASP (Nuclear Autoantigenic Sperm Protein) (Cook et al. 2011).

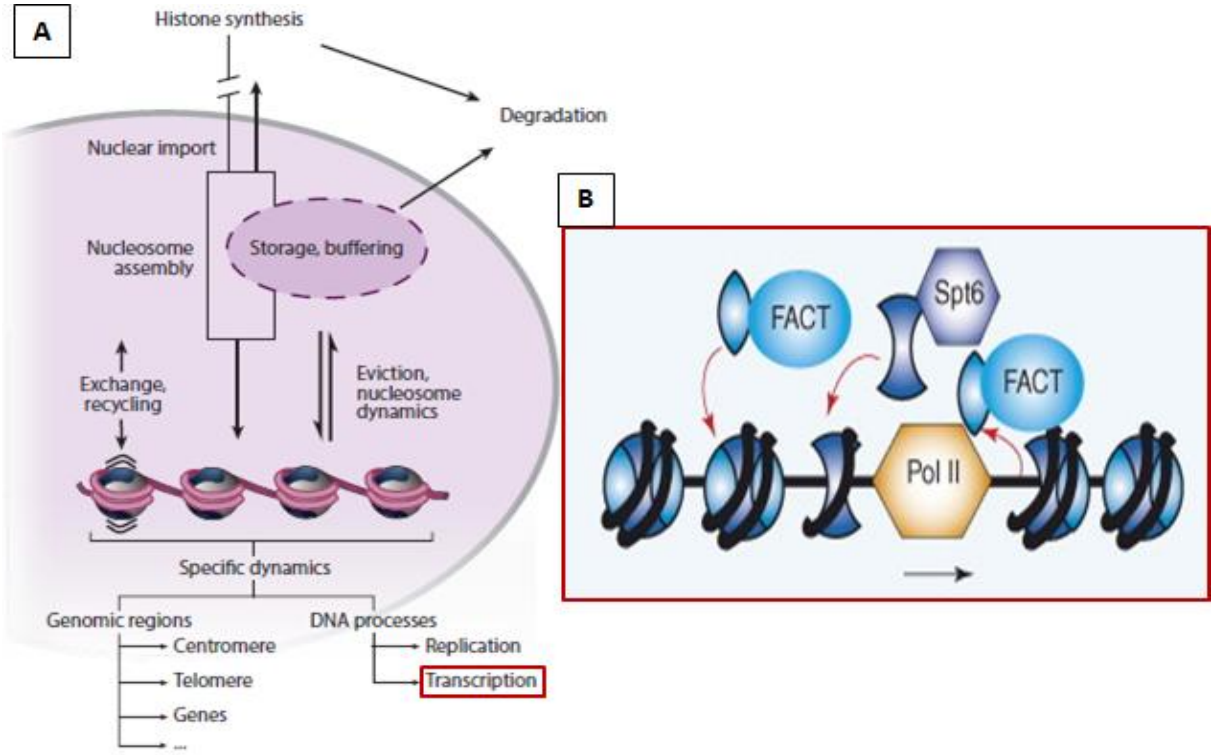


Figure 3. Main functions of histone chaperones. A) Histone chaperones are involved in transport, storage, degradation, assembly and dynamic regulation of chromatin, and they also play a crucial role during DNA replication and transcription. Adapted from Gurard-Levin et al. 2014. **B)** Scheme to present the main role of the histone chaperones FACT and Spt6 during transcription. Adapted from Williams and Tyler 2007.

Histone chaperones have a key function in mediating chromatin organization in the context of DNA replication. Indeed, histone chaperones play a crucial role in the transient disruption of chromatin organization during replication and its restoration after the passage of the replication fork (Gurard-Levin, Quivy, and Almouzni 2014). Histones chaperones can be divided in replication-dependent or -independent, considering their possible role during DNA

synthesis (Orsi, Couble, and Loppin 2009). For instance, CAF1 and HIRA promote histone deposition and nucleosome assembly dependently or independently of DNA replication, respectively, whereas Asf1 (Anti-Silencing Factor 1) may play a role in both pathways (Tagami et al. 2004; Ray-Gallet et al. 2002; Quivy, Grandi, and Almouzni 2001). The deposition of both parental recycled and newly synthetized histones need to be highly regulated to ensure the maintenance of chromatin marks during replication (Alabert and Groth 2012).

During transcription, histone chaperones mediate changes in chromatin structure connected to the progression of RNA polymerase II (RNA pol II). Indeed, they induce a local rearrangement of chromatin to permit the passage of RNA pol II and to regulate histone exchange ensuring the maintenance of chromatin integrity during transcription (Gurard-Levin, Quivy, and Almouzni 2014). Two crucial histone chaperones in this process are FACT and Spt6 (Figure 3B). FACT facilitates transcription elongation, removing histones and replacing them after the passage of RNA polymerase to reset chromatin (Belotserkovskaya et al. 2003). Spt6 interacts with RNA pol II and has a function in nucleosome reassembly after transcription, ensuring also the maintenance of chromatin state (Williams and Tyler 2007; Kato et al. 2013). Thus, histone chaperones play a major role in preserving or modifying the chromatin state in relation to transcription.

Of note, part of my PhD thesis project presented in Chapter 2 and 3 of the results section are linked to this connection between histone chaperones, chromatin modification and regulation of gene expression (mostly gene silencing).

In addition, there are many interplays between histone chaperones and histone PTMs. For example, in *S. cerevisiae*, acetylation of H3K56 by Rtt109 (Regulator of Ty Transposition 109) depends on the histone chaperone Asf1 for effective presentation of histones (Recht et al. 2006), and this PTM in turns modulates the interaction between H3-H4 and the histone chaperone Rtt106 (Fazly et al. 2012). Another example is the trimethylation of H3K36 by the histone methyltransferase Set2, which requires the histone chaperone Spt6 (Du and Briggs 2010).

1.2.4. ATP-dependent chromatin remodelers

ATP-dependent chromatin remodelers are conserved in eukaryotes and mainly present in multiprotein complexes that contain an ATPase subunit of the Snf2 subfamily. This subunit uses the energy of ATP hydrolysis to alter the interactions between DNA and histones, resulting in sliding or eviction of nucleosomes from specific regions of the genome (Venkatesh and Workman 2015). Chromatin remodelers can create open DNA regions that are then targeted by histone chaperones for nucleosome assembly.

In addition to their ATPase domain, chromatin remodelers contain reader domains that are required to target these complexes to specific regions of the genome by recognizing histone modifications, DNA sequences/structures or specific RNAs (Langst and Manelyte 2015).

The chromatin remodelers can be grouped into four families (Figure 4), which will be briefly described below.

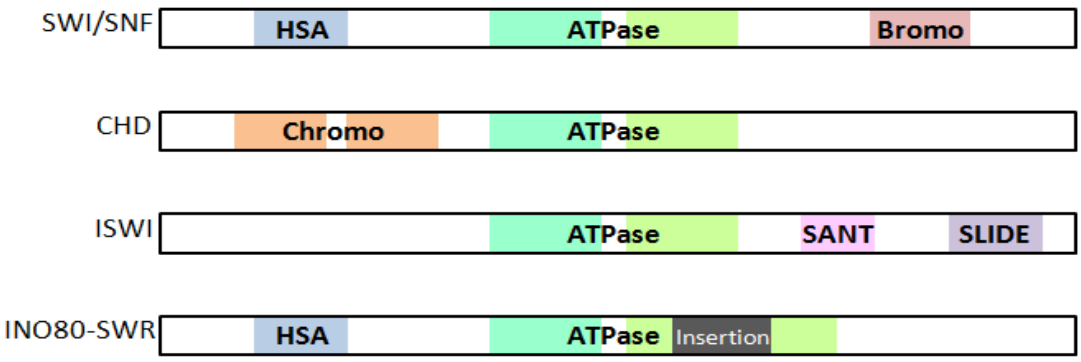


Figure 4. ATP-dependent chromatin remodellers. Schematic organization of the 4 ATP-dependent chromatin remodelers families (SWI/SNF, CHD, ISWI and INO80-SWR), and their functional domains. HSA: Helicase-SANT; Bromo: bromodomain; Chromo: chromodomain. Concept of the figure adapted from Längst and Manelyte 2015.

Members of SWI/SNF (SWItch/Sucrose Non-Fermentable) family are defined by an N-terminal HSA (Helicase-SANT) domain that recruits actin and actin-related proteins, and a bromodomain in the C-terminal known to bind acetylated lysines on histones

(Filippakopoulos and Knapp 2012). This family has been shown to slide and evict nucleosomes from DNA, but lacks assembly activities.

The CHD (Chromodomain-Helicase-DNA binding) family is defined by the presence of two chromodomains in the N-terminal portion that are known to bind methylated histones. CHD members can exist as monomers or as multi-subunit complexes, such as the NURD (NUcleosome Remodelling and Deacetylase) complex. CHD members have been shown to modulate nucleosome organization and to control gene expression in many eukaryotes (Murawska and Brehm 2011).

Members of the ISWI (Imitation SWItch) family harbor two domains called SANT and SLIDE, which together form a nucleosome recognition module that binds to DNA and unmodified H4 tails (Clapier and Cairns 2009). Many ISWI family complexes catalyze nucleosome spacing, and promote chromatin assembly and compaction, mainly after transcription or replication (Langst and Manelyte 2015).

INO80 and SWR (INOsitol requiring 80-SWi2 and Snf2-Related ATPase) family members share the same organization: a split ATPase domain that is also a scaffold for the binding of RuvB-like proteins Rvb1 and Rvb2 that have a DNA helicase activity. Members of this family have been implicated in transcription, DNA replication and repair, mostly having a role in histone variant exchange. For instance, INO80-SWR controls genome-wide distribution and dynamics of the histone variant H2A.Z, although with two distinctly opposite roles. SWR is involved in replacing H2A with H2A.Z within a nucleosome (Mizuguchi et al. 2004). Conversely, INO80 complex has a role in the removal of the H2A.Z variant (Papamichos-Chronakis et al. 2011).

1.2.5. RNA-mediated regulation of chromatin

In recent decades, RNA has also emerged as a key regulator of chromatin structure in eukaryotes, via the formation of RNA scaffolds for chromatin-modifying complexes (Holoch and Moazed 2015). For instance, small RNAs are able to mediate, via the RNA interference pathway, changes in the chromatin state, by inducing histone modifications and/or DNA methylation. This mechanism has been observed in many eukaryotes, including fission yeast,

C. elegans, *A. thaliana* and also animal cells (Volpe et al. 2002; Fire et al. 1998; Zilberman, Cao, and Jacobsen 2003; Brennecke et al. 2008). In addition, long non-coding RNAs (lncRNAs), and even some mRNAs, have also been shown to regulate chromatin modification and structure, mainly by serving as a platform for the recruitment of RNA-binding proteins (Rinn and Chang 2012). A well-known example in mammals is *Xist* (*X inactive-specific transcript*), an RNA that coats the entire inactive X chromosome and recruits the Polycomb complex to induce heterochromatin silencing (Penny et al. 1996; Lee and Bartolomei 2013).

Of note, the role of RNA-mediated regulation of chromatin structure/function in the context of gene silencing is described in more details in the case of the fission yeast *S. pombe*, in the Chapter 2 of this introduction.

1.3. Spatial and functional confinement of chromatin: euchromatin and heterochromatin

1.3.1 Two major distinct chromatin states: euchromatin and heterochromatin

In 1928, Emil Heitz, observing the nucleus of the eukaryotic cells, identified two distinct chromatin states that appeared different in structure and localization: heterochromatin and euchromatin.

Heterochromatin corresponds to the condensed regions, mostly located close to the nuclear envelope and at the nucleolar periphery, and is often replicated in the late S-phase. Heterochromatin is rich in repeated sequences and poor in protein-coding genes; it is characterized mainly by hypoacetylated lysines on histones H3 and H4, and methylated histones H3K9, H3K27 and H4K20 (Kouzarides 2007). In addition, in eukaryotes in which DNA methylation is found, DNA of heterochromatin regions is mostly methylated (Kouzarides 2007).

Euchromatin has a less condensed structure and is therefore more accessible, and it is replicated in early S-phase. Euchromatin contains mostly coding genes and is characterized mainly by hypomethylated DNA sequences, lysines hyperacetylation on histones H3 and H4, and H3K4 trimethylation (Kouzarides 2007).

The differences at the molecular level between these two states of chromatin correlate with specific functions: euchromatin is transcriptionally active/ competent, whereas heterochromatin is mostly transcriptionally silent and plays a role in genome stability and control of gene expression (Grewal and Jia 2007) (Figure 5).

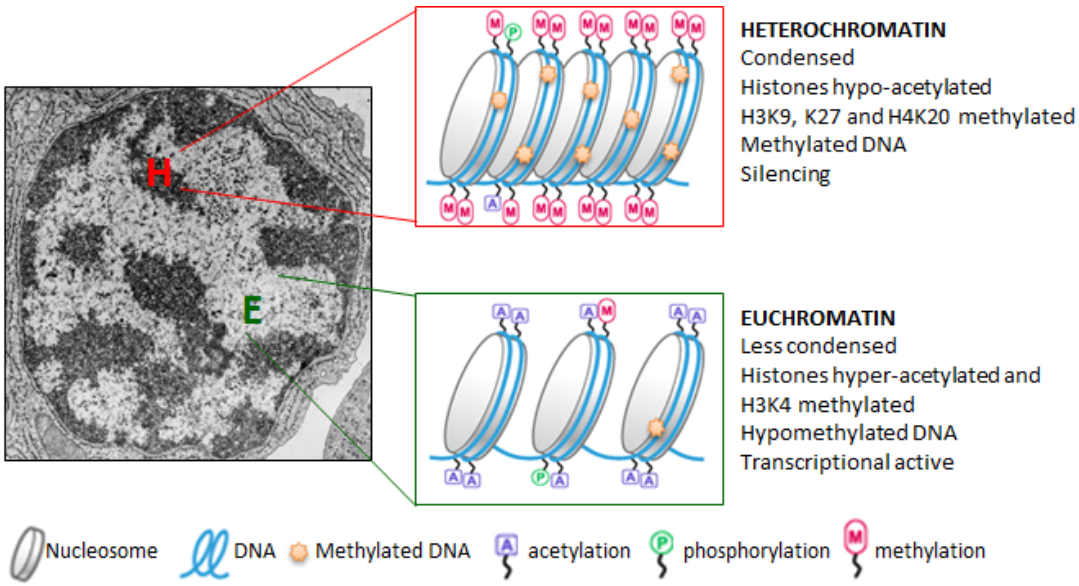


Figure 5. Heterochromatin and euchromatin. On the left, chromatin is organized in the nucleus in two distinct domains, which have two different levels of density: euchromatin (E) and heterochromatin (H). Electron Microscopy picture of a plasma cell from bone marrow (taken from PathologyOutlines.com). On the right, the main contrasting features of euchromatin and heterochromatin are summarized.

1.3.2. Borders between euchromatin and heterochromatin and heterochromatin spreading

The functional and structural differences between heterochromatin and euchromatin require highly regulated confinement of these two distinct states of chromatin. Heterochromatin is established at nucleation centers and then spreads to neighboring regions via a network of interactions among chromatin proteins (Wang et al. 2014). For

instance, in fission yeast, a reference model organism to study heterochromatin, the H3K9 methyltransferase Clr4 is both a reader and a writer, since it binds to H3K9me via its chromodomain and in turn methylates the adjacent nucleosome, thus permitting heterochromatin spreading (Zhang et al. 2008). The HP1 protein Swi6 is also recruited to H3K9me and promotes heterochromatin spreading. Indeed, the dimerization of Swi6 bridges each nucleosome with the adjacent one, where in turn Swi6 facilitates the recruitment of Clr4 and the histone deacetylase complex SHREC (Canzio et al. 2011; Fischer et al. 2009).

Heterochromatin spreading into surrounding regions can affect the expression of nearby genes. This phenomenon was first seen in 1930 in *Drosophila melanogaster* by Hermann Müller, who found that the *white* gene placed adjacent to pericentric heterochromatin was variably silenced. This phenomenon is thus called position effect variegation (PEV). Deregulated spreading of heterochromatin towards euchromatin can lead to inappropriate gene silencing, which has been implicated in a number of severe human diseases (Kleinjan and Lettice 2008). Therefore, in order to maintain a stable gene expression profile, it is very important that spreading is highly regulated. For this reason, heterochromatin regions are flanked by DNA sequences termed boundary elements or insulators. The presence of boundary elements and their associated proteins has now been identified in many eukaryotes (Wang et al. 2014). These boundary elements can act as barriers to prevent the spread of a chromatin state from one domain to an adjacent one, or they can prevent the communication between distant genetic elements to influence gene expression, a function known as enhancer-blocking activity (Wei, Liu, and Liang 2005).

The borders between two different chromatin states are called fixed if they are demarcated by specific DNA sequences and by a sharp change in chromatin profile. Conversely, they are called negotiable borders if located at transition regions, in which the chromatin state is defined by a balance of proteins and histone modifications associated either with heterochromatin or euchromatin (Kimura and Horikoshi 2004).

Many strategies are used to facilitate confinement of euchromatin and heterochromatin at the borders. For instance, one strategy involves recruitment of opposing histone-modifying enzymes to counteract the histone modifications of the adjacent domain, with simultaneous protection of local existing histone modifications (Wang et al. 2014). In fission yeast,

heterochromatin spreading is simultaneously blocked at pericentric boundary regions by the Lsd1 complex, which demethylates H3K9me (Lan et al. 2007), and by the bromodomain protein Bdf2, which protects acetylated H4K16 from Sir2-dependent deacetylation (Wang et al. 2013) (Figure 6).

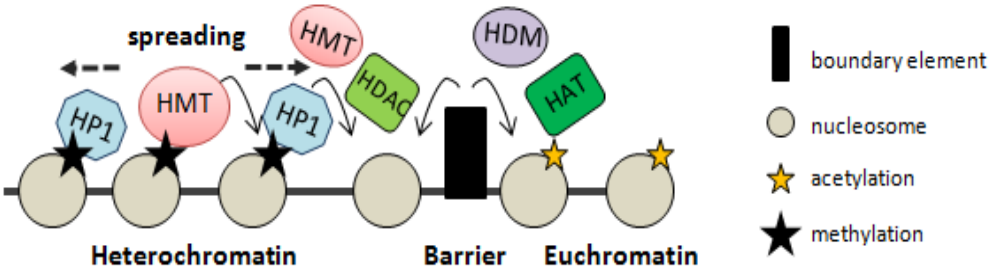


Figure 6. Heterochromatin spreading and boundaries with euchromatin. At heterochromatin, HMT mediates H3K9me, which is the binding site for HP1 proteins that permits heterochromatin spreading via recruitment of additional HMT and HDAC. At a barrier, a boundary element can recruit opposing histone-modifying enzymes (HMT and HDAC at heterochromatin; HDM and HAT at euchromatin) to counteract the histone modifications of the adjacent domain.

Another fascinating mechanism regulating chromatin spreading relies on nuclear spatial organization. Indeed, clustering of boundary elements and their interactions with nuclear structures facilitate the achievement of distinct chromatin domains. In addition, CTCF (CCCTC-Binding Factor) is a well-known boundary factor that regulates the 3D organization of chromatin (Ong and Corces 2014). A better understanding of the formation of these chromatin domains will help to uncover new mechanisms that regulate spreading and that are often perturbed in many pathologies, including cancer.

2.

Heterochromatin and gene silencing

2.1. General features of heterochromatin in eukaryotes

2.1.1. Constitutive and facultative heterochromatin

Heterochromatin was first defined as a highly condensed static structure and transcriptionally inert. Heterochromatin has since been further categorized as constitutive or facultative. Constitutive heterochromatin is defined as a stable compacted structure, found in every cell state and type, whereas facultative is a more flexible type of heterochromatin that can be either expressed or repressed depending on the cellular type, the stage of development and external stimuli (Saksouk, Simboeck, and Dejardin 2015). Facultative heterochromatin usually contains genes that need to be specifically expressed during cell differentiation (Grewal and Jia 2007). At the molecular level, these two types of heterochromatin present mostly the same features (histone hypoacetylation and hypermethylation, and DNA methylation). In higher eukaryotes, it has been suggested that constitutive heterochromatin harbors more methylated H3K9, whereas facultative heterochromatin harbors more methylated H3K27 (Trojer and Reinberg 2007).

2.1.2. Localization, structure and function of constitutive heterochromatin

In most eukaryotes, constitutive heterochromatin forms at centromeric and telomeric regions, which are both gene-poor areas of the genome, usually composed of tandem repetitions of non-coding DNA. Centromeres and pericentromeric heterochromatin are crucial for chromosome segregation during cell division, through interactions with cohesins and the formation of the kinetochore complex (Schueler and Sullivan 2006). Telomeres and subtelomeric heterochromatin protect chromosome ends (for example, by avoiding end-to-end joining events) and prevent telomeres from shortening at each cell division (Moser and Nakamura 2009).

Centromeric and pericentromeric DNA sequences are quite different among various eukaryotes, although the general organization and proteins that are associated with centromeres are mainly conserved (Buscaino, Allshire, and Pidoux 2010). In mammals, centromeres and pericentromeric regions aggregate in the nucleus, forming a densely staining mass of heterochromatin called the chromocenter (Taddei et al. 2004). In mammals and plants, centromeric repeat sequences are of the satellite type: non-coding repetitions of a tandem of a short DNA sequence modules, which can range from a few to more than 1000 base pairs long (Plohl et al. 2008). A notable exception is *S. cerevisiae*, whose centromeres consist of a single short non-coding sequence of 128 base pairs (Plohl et al. 2008).

Telomeres and subtelomeric heterochromatin are nucleoproteic structures that are located at the extreme ends of eukaryotic chromosomes and are composed of short tandemly repeated DNA sequences bound by conserved protective proteins. Telomeric repeated sequences, unlike centromeric ones, are composed of the short DNA motif TTAGGG, which is mostly conserved in eukaryotes (Saksouk, Simboeck, and Dejardin 2015). In human cells, telomeres localize at the nuclear periphery during post-mitotic nuclear assembly, whereas they are more internally localized during the rest of the cell cycle (Crabbe et al. 2012).

2.1.3. Transcription of heterochromatin sequences

Despite the first definition of heterochromatin as a static, condensed and transcriptional inert region, much evidence revealed that constitutive heterochromatin can be transcribed, at least at a basal level. For instance, transcription of pericentromeric repeats has been detected in many eukaryotes and has been associated with many biological processes, including cell proliferation and differentiation, senescence and stress response (Saksouk, Simboeck, and Dejardin 2015; Jolly et al. 2004). Telomeric sequences are also transcribed. For example, *TERRA* (*Telomere Repeat-containing RNA*) transcription has been found, from yeast to humans, and evidence indicates a role for this RNA in promoting heterochromatin formation and maintaining telomeric structural integrity (Wong 2010; Arnoult, Van Beneden, and Decottignies 2012).

In *S. pombe*, as described in the next sections, transcription of pericentromeric repeats is required for heterochromatin formation and maintenance through a pathway involving RNA

interference (RNAi) (Volpe et al. 2002; Lippman and Martienssen 2004). Similarly, there are many examples of transcription of pericentromeric heterochromatin in mammals. For instance, in early mouse development, pericentromeric satellites are transcribed, and this is required for the formation of chromocenters (Probst et al. 2010).

Transcription of constitutive heterochromatin must be highly controlled and misregulation of pericentromeric transcription has been observed in some pathologies, such as epidermal carcinoma or lung cancer (Enukashvily et al. 2007; Eymery et al. 2009), in which decondensation of these heterochromatin regions correlates with increased DNA breaks and genomic rearrangements.

2.2. Heterochromatin in fission yeast

2.2.1. *Schizosaccharomyces pombe*: a reference model to study chromatin-based mechanisms and heterochromatin gene silencing

The fission yeast *Schizosaccharomyces pombe* is a unicellular eukaryote broadly used as a model in studies concerning the cell cycle and regulation of gene expression and silencing by chromatin-based processes. The name *Schizosaccharomyces pombe* was coined in 1893 by Paul Lindner, who isolated it from East African millet beer; *Schizo* refers to its cell division by fission and *pombe* is the Swahili word for beer. It was first developed as an experimental model in the 1950s by Urs Leupold for studying its genetics, and by Murdoch Mitchison for studying its cell cycle.

Fission yeast has a rod shape with a diameter of 3-4 µm, but its length changes quite dramatically during the different phases of the cell cycle from 7 to 14 µm. The *S. pombe* genome, which was sequenced in 2002 (Wood et al. 2002), is 13.8 Mb in size, contains approximately 5000 open reading frames and 1500 non-coding genes on three chromosomes. In addition, the subcellular localization of the vast majority of its proteins

(more than 95%) is known (Matsuyama et al. 2006). The small genome size of fission yeast makes it a convenient model for genomic and epigenomic studies.

S. pombe is primarily a haploid organism, and this makes it a good model for studying the effect of gene deletions. Additionally, in comparison with other eukaryotes, it is an easy model for genetic manipulation by gene deletion, point mutation insertion or addition of tags (Bahler et al. 1998), mostly due to the high efficiency of homologous recombination. Another advantage is its rapid mitotic division of around 2 hours. When nitrogen and carbon sources become limiting, cells stop dividing, enter a quiescent phase (G0) and undergo a process of sexual differentiation, activating a set of meiotic genes in cascade that are required for conjugation, meiosis and sporulation (Hiriart and Verdel 2013) (Figure 7).

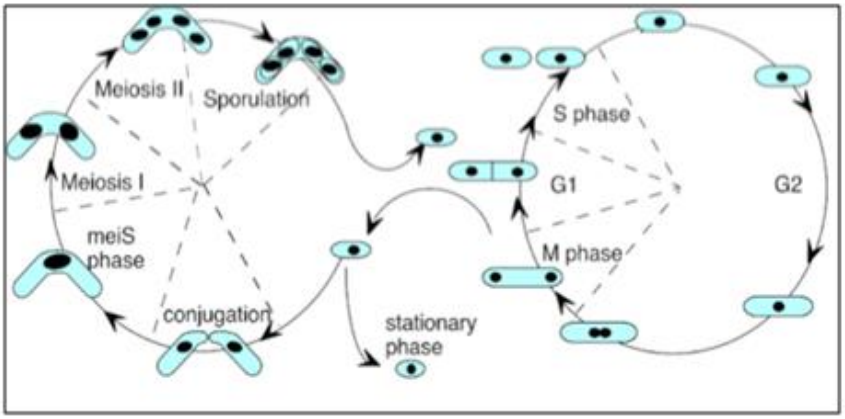


Figure 7. Scheme representing meiosis and the vegetative cycle in fission yeast. On the left, meiosis: two haploid cells conjugate forming a transient diploid, or zygote, that proceeds through meiosis to produce four haploid spores, which are packed into a tetrad ascus. On the right, the vegetative cycle: G1, S and M phases take only 10% of division time, whereas G2 is the longest phase. Mitosis generates two haploid daughter cells. Figure taken by PombeNet (Forsburg lab's website).

In the last decade, *S. pombe* has become a reference model for studying chromatin regulation, and particularly heterochromatin assembly and gene silencing. Indeed, unlike *S. cerevisiae*, fission yeast shares with higher eukaryotes many machineries and processes that are crucial for heterochromatin regulation and silencing, such as RNA interference (RNAi), the repressive histone modification mark H3K9me and many PTM readers, as chromodomain proteins (Martienssen, Zaratiegui, and Goto 2005).

In the next sections, mechanisms regulating gene silencing in *S. pombe* will be detailed, which are mainly based on the actions of histone modifiers, HP1 proteins and the RNAi pathway. Histone chaperones and ATP-dependent chromatin remodelers also have a crucial role in regulating heterochromatin formation and gene expression, although research on these proteins in *S. pombe* is still in its budding stages (Allshire and Ekwall 2015).

2.2.2. Constitutive and facultative heterochromatin

Interestingly, both facultative and constitutive heterochromatin regions are found in fission yeast. Facultative heterochromatin is mostly found at meiotic genes, which are silenced in vegetative cells, but need to be expressed during sexual differentiation (Cam et al. 2005; Zofall et al. 2012; Hiriart et al. 2012). Constitutive heterochromatin is found at four genomic regions: centromeres, telomeres, the mating type locus and rDNAs (Egel 2004). Each of these constitutive heterochromatin regions has essential functions: at centromeres, heterochromatin permits normal chromosome segregation (Allshire et al. 1995); at telomeres, it is important for meiotic chromosome segregation and to protect telomeric ends (Nimmo et al. 1998); and at the mating type locus, it facilitates the process of mating type switching (Jia, Yamada, and Grewal 2004). The function of heterochromatin at rDNA has not been understood yet, although it has been proposed that, like in *S. cerevisiae,* it could be involved in maintaining rDNA stability by preventing recombination between rDNA repeated sequences (Allshire and Ekwall 2015). A more detailed description of centromeric, telomeric and mating type heterochromatin and of the role of RNAi in all these regions will be provided in the next sections.

2.2.3. Location, function and structure of constitutive heterochromatin

2.2.3.1. Pericentromeric heterochromatin

The three centromeres of fission yeast differ in size, ranging from 35 to 110 Kbp (Wood et al. 2002), but they share similar structural organization: a central core region (*cnt*) is

surrounded by inverted centromeric repeats called innermost repeats (*imr*), which are flanked by tandem alternating copies of *dg* and *dh* centromeric repeats that form the outer repeats (o*tr*) (Takahashi et al. 1991) (Figure 8A). The central core region is the site of kinetochore assembly, which is the attachment site for spindle microtubules that separate duplicated chromosomes during mitosis and meiosis. This region is enriched with the histone variant of the CENPA family in *S. pombe,* which is called Cnp1 (Durand-Dubief and Ekwall 2008).

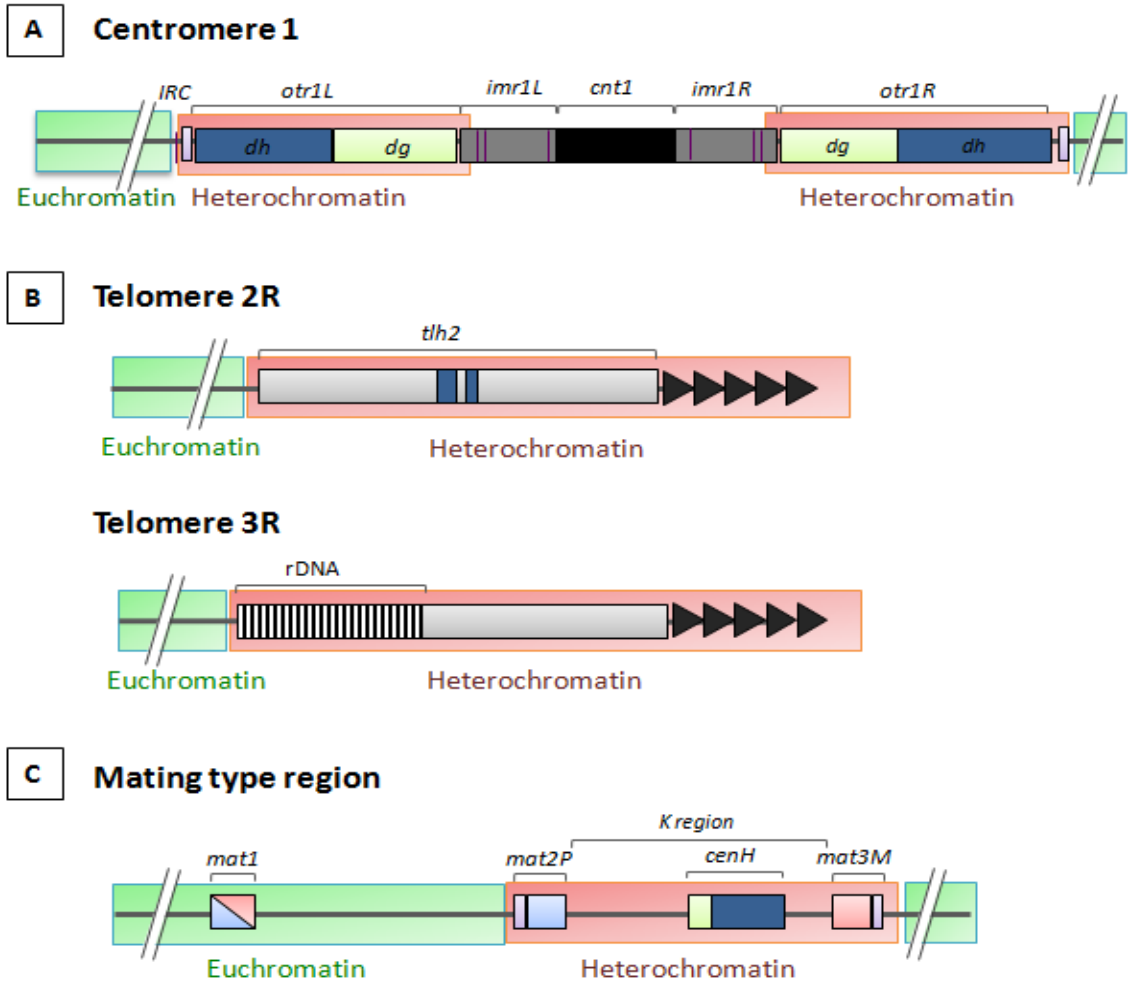


Figure 8. Structure of constitutive heterochromatin regions in fission yeast. A) Centromere I: *central core region* (*cnt*) surrounded by *innermost repeats* (*imr*) which are flanked by *dg* and *dh* repeats that form the *outer repeats* (otr). Barriers at chromosome I are provided by IRC (*Inverted Repeat Centromere*) sequences. **B)** Telomeres**:** terminal ends are composed by a repeated telomeric region and adjacent subtelomeric heterochromatin. Telomere 2R contains *tlh1* gene that contain *dh-like* sequences. Telomere 3R: contains clusters of rDNAs. **C)** Mating type region: subdivided in euchromatin, containing the expressed *mat1* gene; and heterochromatin, containing silenced *mat2P* and *mat3M* loci and the K region that contains *dg/dh-like cenH* sequence.

Barriers separating pericentromeric heterochromatin from adjacent euchromatin regions are contained in *IRC* (Inverted Repeat Centromere) sequences located on chromosome I and III and in transfer RNA genes, which are present on five out of six centromeric barriers (Cam et al. 2005).

In the nucleus, centromeres are located at the nuclear periphery close to the spindle pole body (a structure equivalent to the centrosome in higher eukaryotes) (Funabiki et al. 1993).

2.2.3.2. Subtelomeric heterochromatin

Chromosome ends in *S. pombe* are composed by single-stranded DNA at the extreme ends, followed by a double-stranded region of around 300 bp, which is composed of tandem repeats enriched for the consensus sequence GGTTAC. These two sequences form the telomeric region, which is bound by proteins involved in telomere protection and silencing (Dehe and Cooper 2010). Heterochromatin is mostly found at the subtelomere, which is a region of around 19 kb adjacent to each telomeric region. Interestingly, at subtelomeres of chromosome I and II there are two genes coding for two RecQ type DNA helicases, called *tlh1* and *tlh2*, which contain strong homologies with pericentromeric *dh* sequences, and are implicated in RNAi-mediated heterochromatin formation (Mandell et al. 2005). Conversely, each of the subtelomeric regions in the chromosome III contains a cluster of rDNA repeats (Wood et al. 2002) (Figure 8B).

In *S. pombe*, telomerase is active to maintain telomere sizes. However, in the absence of telomerase, subtelomeric heterochromatin is important in preserving telomeric length. Indeed, protection of chromosome ends can occur via a mechanism named HAATI (Heterochromatin Amplification-mediated And Telomerase-Independent) that consists of continual amplification and rearrangement of heterochromatic sequences mediated by the telomere end-protection proteins Pot1 and Ccq1, a component of the SHREC complex (Jain et al. 2010).

The borders between subtelomeric heterochromatin and euchromatin are not so well defined since there are no clear boundary elements identified so far (Cam et al. 2005). The

separation between euchromatin and heterochromatin at telomeres is probably achieved through a balance of several active and repressive histone marks, and/or recruitment of specific barrier proteins. For instance, an ATP-dependent chromatin remodeler called Fft3 has been shown to localize at *LTR* (*Long Terminal Repeats*) sequences located at telomeres and prevents euchromatin invasion of silenced subtelomeric regions (Stralfors et al. 2011). A recent study on chromatin compaction during interphase, using super-resolution fluorescence microscopy, surprisingly revealed that silent chromatin is less condensed than euchromatin, and that the most condensed regions in the genome are found between euchromatin and subtelomeric heterochromatin. These regions, called 'knobs', correspond to sequences of around 50 Kb, which are free of any heterochromatin marks and are therefore not silenced. Knob formation is independent of HP1 proteins and other gene silencing factors, whereas their condensation is regulated by H3K36 methylation. Disruption of H3K36me in this region eliminates knob formation and subtelomeric gene silencing (Matsuda et al. 2015).

Telomeres in fission yeast are clustered in 2-4 bodies at the periphery of the nuclear envelope (Funabiki et al. 1993) and interestingly, RNAi is required for this clustering (Hall, Noma, and Grewal 2003).

2.2.3.3. Heterochromatin at mating type locus

The third region of constitutive heterochromatin in fission yeast resides in a locus on chromosome II called mating type region, which is important for the process of sexual determination. This locus is subdivided into a euchromatin region, containing the expressed *mat1* gene, and a heterochromatin region, containing the silenced *mat2P* and *mat3M* loci and a region of about 11 Kb in between, known as the K region (Egel 1973) (Figure 8C). Interestingly, inside the K region, there is a sequence called *cenH* that shares homology with centromeric *dg* and *dh* repeats and is the nucleation site for RNAi-mediated heterochromatin formation in this region (Grewal and Klar 1997).

S. pombe cells can switch their mating type information by a process analogous to stem cell division in higher eukaryotes (Klar 1992; Klar, Ishikawa, and Moore 2014). Mating type

information is contained in *mat2P* and *mat3M* loci, but is expressed only after a long distance DNA recombination event with *mat1*, generating *mat1P* or *mat1M* (Arcangioli and Thon, 2004). Therefore, either *mat2P* or *mat3M* is expressed after translocation, whereas the other one remains silent, embedded into heterochromatin. The process of mating type switching starts with the introduction of a single-strand break imprint at *mat1*, which is converted to a double-strand break (DSB) in the next round of replication, and then healed by a translocation event using *mat2P* or *mat3M* as donors (Arcangioli and Thon, 2004).

Many proteins are involved in this first step; for instance, Sap1 (Switch activating protein 1) is a DNA-binding protein required for DSB generation (Arcangioli, Copeland, and Klar 1994). Directionality of switching depends both on the distance between *mat1* and the donor locus of recombination, and on the presence of Swi2/Swi5 RPC (Recombination Promoting Complex). In P cells, RPC is located at a sequence called SRE (recombination-enhancer element) close to *mat3,* which is thus used as a donor. Conversely, in M cells RPC spreads across the entire mating type region, also reaching the *mat2* locus (Jia, Yamada, and Grewal 2004) (Figure 9).

Heterochromatin is required for spreading of Swi2/Swi5, so mutations in many heterochromatin proteins affect the directionality of switching towards a preferential recombination event between *mat1* and *mat3* (Jia, Yamada, and Grewal 2004; Aguilar-Arnal, Marsellach, and Azorin 2008). Other proteins are also known to regulate the directionality of switching; for example Abp1, the fission yeast homologue of human CENPB. Removal of Abp1 impairs spreading of Swi2/Swi5, without disrupting heterochromatin, and this correlates with a preferential use of *mat3* as a donor (Aguilar-Arnal, Marsellach, and Azorin 2008) (Figure 9).

In addition, Abp1 has also been proposed to influence mating type identity by controlling both the total expression and preferential splicing variant of Swi2 (Matsuda et al. 2011; Yu, Bonaduce, and Klar 2012). Boundary elements at the mating type region consist of 2 Kb inverted repeats that flank silent heterochromatin on both sides (*IR-L*, *IR-R*) (Thon et al. 2002). The *IR* boundary elements contain B-boxes motifs, which are the binding sites for the Pol III transcription factor TFIIIC. TFIIIC binding, in the absence of RNA pol III, has been shown to mediate a specific tethering of boundary elements to the nuclear periphery, thus separating euchromatin from heterochromatin (Noma et al. 2006).

The mating type locus is found close to spindle pole bodies and the nuclear membrane. Interestingly, in strains lacking the HMT Clr4, the mating-type region presents a random localization in the nucleus (Alfredsson-Timmins, Henningson, and Bjerling 2007).

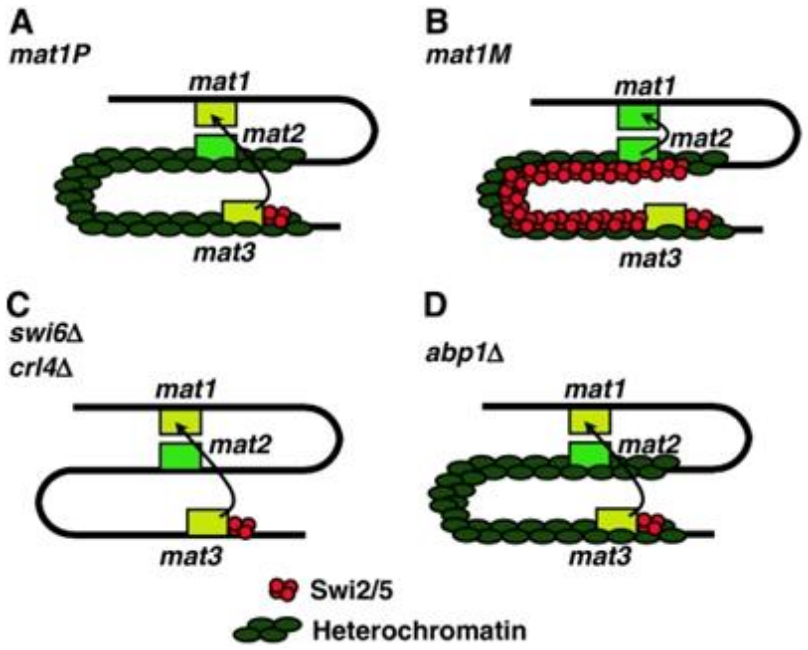


Figure 9. Normal and mutant mating type switching. Directionality of switching depends on the distance between *mat1* and either *mat2P* or *mat3M*, and on the presence of Swi2/Swi5 RPC (Recombination Promoting Complex). (**A**) *mat1P* cells: RPC is located close to *mat3M* (donor of recombination). (**B**) *mat1M cells:* RPC spreads across the entire mating type region, through heterochromatin, to reach *mat2P* locus (donor of recombination). (**C**) Heterochromatin protein mutants: defect in Swi2/Swi5 spreading, preferential recombination between *mat1* and *mat3M*. (**D**) *abp1Δ*: defect in Swi2/Swi5 spreading, without disrupting heterochromatin, preferential recombination between *mat1* and *mat3*. Figure taken from Aguillar-Arnal et al. 2008 (© 2008, European Molecular Biology).

2.2.4. Main proteins involved in heterochromatin assembly

The main factors involved in heterochromatin assembly, which are conserved between fission yeast and humans, are histone deacetylases, histone methyltransferases and chromodomain-containing proteins belonging to HP1 family (Table 1).

Fission yeast protein	Complex	Human Orthologs	Function
Clr4	CLRC	SUV39H1, SUV39H2	HMT (H3K9)
Set2		SETD2	HMT (H3K36)
Set9		SUV420H1, SUV420H2	HMT (H3K4, H4K20)
Set1		SETD1A, SETD1B	HMT (H3K4)
Clr3	SHREC	HDAC6, HDAC10	HDAC II
Clr6	Clr6 HDAC	HDAC1, HDAC2	HDAC I
Sir2		SIRT1	HDAC
Lsd1	Lsd1/2	KDM1B	HDM
Mst2	Mst2 HAT	KAT7, KAT6B, KAT8, KAT6A	HAT
Swi6		CBX1, CBX3, CBX5	HP1 family
Chp2		CBX1, CBX3, CBX5	HP1 family2
Chp1	RITS		Chromodomain protein
Tas3			RITS subunit 3
Ago1		PIWIL1, PIWIL2, PIWIL3, PIWIL4	Argonaute
Dcr1		DICER1	Dicer
Rdp1	RDRC		RNA-directed RNA pol
Rrp6	Exosome	EXOSC10	3'-5' exoribonuclease
Dis3		DIS3	3'-5' exoribonuclease
Pab2		PABPN1L, PABPN1	Poly(A) binding protein
Red1	NURS	ZFC3H1	RNA degradation, H3K9me
Mmi1		YTHDF1, YTHDF2, YTHDF3	Meiotic genes silencing
Ste11	Tor2-Mei2-Ste11		Transcription Factor Meiosis
Taz1		TERF1, TERF2	Telomere heterochromatin
Ccq1	SHREC	HDA1	Telomere maintenance
Atf1	Atf1-Pcr1		Mating type heterochromatin
Pcr1			Mating type heterochromatin
Abp1	CENPB	CENPB	Centromeric heterochromatin, transposon silencing and mating type identity
Cbh1			
Cbh2			
Sap1			DSB, DNA repair and replication
Epe1		KDM2A, PHF2, PHF8, KDM7A	Boundary element
Bdf2		BRD3, BRD4	Boundary element
Ccr4	CCR4-NOT	CNOT6, CNOT6L	poly(A) deadenylase
Caf1		CNOT8, CNOT7	poly(A) deadenylase
Asf1		ASF1A, ASF1B	Histone chaperone
Hip1	HIRA	HIRA	Histone chaperone
Spt16	FACT	SUPT6H	Histone chaperone
Pob3		SSRP1	Histone chaperone
Fft3		SMARCAD1	Chromatin remodeller
Hrp1	CHD	CHD1, CHD2	Chromatin remodeller
Hrp3		CHD2, CHD1	Chromatin remodeller
Spt6		SUPT6H	Chromatin remodeller
Mit1	SHREC	CHD3	Chromatin remodeller

Table 1. Main actors in heterochromatin regulation in fission yeast. Names and functions of main proteins in *S. pombe,* which are presented in the introduction, are listed together with their human orthologs (Pombase).

2.2.4.1. Histone deacetylases (HDACs)

Three families of HDACs are known in fission yeast: Clr3, Clr6 and Sir2. Each is part of a different protein complex that contributes to generate heterochromatin by removing the active acetyl group from histones.

Clr3 is a member of SHREC (Snf2/HDAC-containing Repressor Complex), which also contains the ATP-dependent chromatin remodeler Mit1. The deacetylase activity of Clr3 on histone H3K14, together with the function of Mit1, permit the assembly of higher-order chromatin structures critical for heterochromatin function (Yamada et al. 2005).

Clr6 deacetylates several residues on histones H3 and H4, and it can regulate heterochromatin assembly via its interaction with histone chaperones Asf1 and HIRA (Yamane et al. 2011).

The role of Sir2 in mediating heterochromatin assembly is mainly by deacetylating histone H3 on lysine 9, which is required for repressive H3K9 methylation (Shankaranarayana et al. 2003), and on lysine 14, which is critical for HMT Clr4 recruitment to centromeres (Alper et al. 2013). Additionally, Sir2 and Clr3 have been shown to be required for heterochromatin spreading from nucleation sites (Buscaino et al. 2013).

2.2.4.2. Histone methyltransferases (HMTs)

Clr4, a homologue of SUV39H in human and of Su(var)39 in Drosophila, is the only HMT known to methylate H3K9 in *S. pombe*. Therefore, it has an essential role in constitutive heterochromatin assembly, maintenance and propagation (Zhang et al. 2008). Interestingly, Clr4 has a C-terminal catalytic SET (Su(var)3-9 Enhancer of zeste, Trithorax) domain and also a chromodomain, which permits its binding to H3K9 methylation and propagation of heterochromatin in adjacent regions (Zhang et al. 2008). Clr4 is part of CLRC (Clr4 methyltransferase-Containing Complex), which also contains Rik1, Cul4, Raf1 and Raf2. CLRC also has an E3 ubiquitin ligase activity, which suggests that the ubiquitination of specific proteins may be required for heterochromatin assembly (Jia, Kobayashi, and Grewal 2005; Horn, Bastie, and Peterson 2005).

In *S. pombe***,** there are other HMTs that do not seem to regulate heterochromatin formation. Set2 methylates H3K36 and this modification has been associated with transcriptional elongation and double-strand break repair pathway choice (Morris et al. 2005; Pai et al. 2014). Set9 is known to methylate both H3K4, which facilitates transcription (Nishioka et al. 2002), and H4K20, which is important for activating a DNA damage response (Wang et al. 2009).

2.2.4.3. HP1-family proteins

In all eukaryotes, proteins of HP1 family contain a chromodomain, which permits their binding to H3K9me, and have a crucial role in heterochromatin gene silencing.

In *S. pombe,* there are four chromodomain proteins connected with heterochromatin: Clr4, which has already been described, Swi6, Chp2 and Chp1. Among them, Swi6 and Chp2 are HP1-like proteins and are involved in transcriptional silencing at heterochromatic regions, but they mainly play distinct roles, mostly interacting with different protein complexes.

Chp2 associates with SHREC, is required for H3K14 deacetylation, and mediates transcriptional repression by limiting RNA polymerase II access to heterochromatin (Sadaie et al. 2008).

Swi6 associates with chromatin and many nuclear proteins, and is required for efficient heterochromatin gene silencing (Motamedi et al. 2008; Fischer et al. 2009). In addition to its chromodomain, Swi6 also contains a chromo shadow domain permitting its dimerization, which is important to promote heterochromatin spreading (Cowieson et al. 2000; Canzio et al. 2011). Interestingly, Swi6 can also bind RNAs via its hinge domain, located between the chromodomain and the chromo shadow domain. The competition in Swi6 binding to chromatin or to RNAs plays a specific role in regulating degradation of transcripts that originate from heterochromatin (Keller et al. 2012).

Another chromodomain binding protein is Chp1, which is a member of the RITS (RNAi-Induced Transcriptional Silencing) complex. The role of RITS and RNAi machinery in the assembly of constitutive heterochromatin will be further discussed in the next section.

2.3. Mechanisms of constitutive heterochromatin assembly and gene silencing in fission yeast

In fission yeast, as in higher eukaryotes, heterochromatin assembly and gene silencing is mainly associated with the presence of repetitive DNA sequences that can be recognized by specific DNA-binding proteins that permit heterochromatin assembly (DNA-based mechanisms), or can be transcribed to non-coding RNAs, which in turn serve as platforms to recruit chromatin-related complexes to trigger heterochromatin formation (RNA-based mechanisms).

Currently, the RNA-based mechanisms of heterochromatin gene silencing mainly involve the RNAi pathway, as it will be detailed in the next section. In addition, there are other RNA-based mechanisms of gene silencing that are independent of the RNAi pathway. So far these mechanisms mainly involve the exosome, which is a multi-subunit protein complex conserved in all eukaryotes that controls degradation and metabolism of RNA (Milligan et al. 2008). The nuclear exosome contains two catalytic subunits called Rrp6 and Dis3 (Drazkowska et al. 2013; Briggs, Burkard, and Butler 1998). The exosome is known to interact with a complex called TRAMP, which is composed of a poly(A) polymerase named Cid14, and two other proteins called Mtr4 and Air1 (Buhler et al. 2007). In *S. pombe*, the nuclear exosome is involved in silencing constitutive heterochromatin regions in parallel of RNAi (Halic and Moazed 2010; Reyes-Turcu et al. 2011; Buhler et al. 2007), but its recruitment to heterochromatin is not understood yet. The exosome also has a crucial role in the formation of facultative heterochromatin at meiotic genes and in the silencing of retrotransponsable elements (Yamanaka et al. 2013), as will be further discussed later in this chapter (2.4.3.).

2.3.1. Essential role of transcription and RNAi in the context of constitutive heterochromatin

Before focusing on the key role of the RNA interference (RNAi) in heterochromatin formation and silencing in *S. pombe*, an overview of the RNAi pathway and its role in heterochromatin silencing in other eukaryotes will be provided in this section.

2.3.1.1. Generals features of RNAi

RNAi was first discovered in 1998 by Andrew Fire and Craig Mello, who introduced an exogenous double-stranded RNA (dsRNA) into *Caenorhabditis elegans*, which specifically abolished the expression of the homologous gene (Fire et al. 1998). This mechanism of silencing is found in almost all eukaryotes (with some exceptions like *S. cerevisiae* (Aravind et al. 2000)), from *S. pombe* to mammals. RNAi plays a crucial role in regulating many biological processes such as cell division, differentiation, apoptosis, protection against viruses, and maintenance of genome stability (Verdel and Moazed 2005). For instance, in mammals at least 30% of genes are controlled by RNAi (Hime and Somers 2009). Additionally, RNAi discovery also paved the way for the development of the 'knock down' technique, which is widely used in laboratories and consists of introducing small interfering RNAs (siRNAs) into cells to repress the expression of specific genes, as well as for the development of new therapeutic approaches (Vaishnaw et al. 2010; Wu, Lopez-Berestein, et al. 2014).

RNAi can only occur in regions that are transcriptionally active. Indeed, complementary transcripts, which are the "fuel" of the RNAi pathway, generate dsRNAs that are processed by Dicer into small RNA fragments (∼20−30 nucleotides) (Volpe and Martienssen 2011). Three main categories of small RNAs have been identified depending on their origins, structures, associated effector proteins, and biological roles: short interfering RNAs (siRNAs), microRNAs (miRNAs), and piwi-interacting RNAs (piRNAs) (Ghildiyal and Zamore 2009). These small RNAs are loaded into an effector complex mainly containing an Argonaute protein, which can target endogenous transcripts in a sequence specific manner. Argonaute cleaves targeted endogenous RNAs by its endonuclease domain (slicing activity), and silencing is achieved either post-transcriptionally (PTGS, Post-Transcriptional Gene Silencing), by transcript degradation or by translation inhibition, or transcriptionally (TGS), by

acting on the chromatin. A reinforcement positive loop within the RNAi pathway exists in many eukaryotes: RNA dependent RNA Polymerases (RdRPs) are known to generate additional dsRNAs from single-stranded RNAs (ssRNAs), and this permits further amplification of effective RNA fragments (Volpe and Martienssen 2011) (Figure 10).

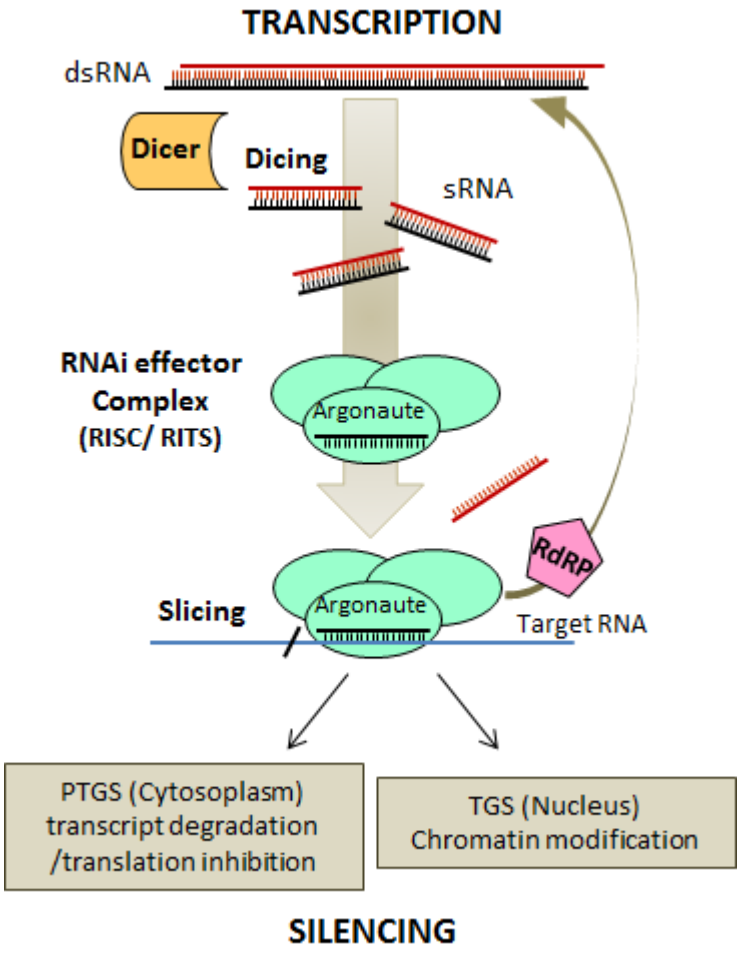


Figure 10. Scheme representing RNAi mechanisms of silencing. Transcription permits the production of dsRNAs that are processed by Dicer into small RNA fragments (sRNA). These are recognized by an effector complex, mainly containing an Argonaute protein, permitting its recruitment to newly transcribed RNAs in a sequence specific manner. Argonaute cleaves targeted RNAs (slicing), and silencing is achieved either post-transcriptionally (PTGS, Post-Transcriptional Gene Silencing), by transcript degradation or translation inhibition, or transcriptionally (TGS), by acting on the chromatin state. A reinforcement positive loop is mediated by an RNA dependent RNA Polymerases (RdRP).

In plants, RNAi mediates DNA methylation and heterochromatin modifications via a mechanism called RdDM (RNA directed DNA Methylation). This mechanism was mainly investigated in *Arabidopsis thaliana*, in which DNA repeated sequences are transcribed by RNA polymerase IV to produce ssRNAs. These transcripts are bound by an RdRP called RDR2 that generates dsRNAs, which are in turn recognized and cleaved by a ribonuclease of the Dicer family called DCL3, to produce siRNAs. The Argonaute protein AGO4 binds to these siRNAs, which permit its recruitment to repeated DNA sequences, where AGO4 recruits DNMT and HMT to mediate DNA methylation and heterochromatin silencing (Zilberman, Cao, and Jacobsen 2003; Verdel et al. 2009) (Figure 11).

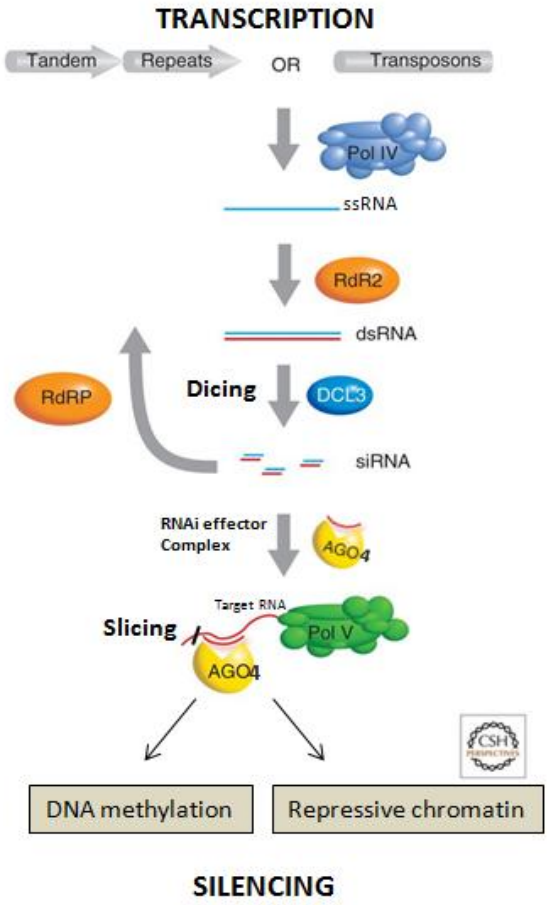


Figure 11. RNAi-mediated histone and DNA methylation in *Arabidopsis thaliana*. DNA repeated sequences are transcribed by RNA polymerase IV to produce ssRNAs, which are bound by an RdRP RDR2 to obtain dsRNAs. These are cleaved by the Dicer protein DCL3 to produce siRNAs. The argonaute protein AGO4, bound to siRNAs, permits the recruitment of DNMT and HMT to repeated DNA sequences.. This induces DNA methylation and heterochromatin silencing. Figure adapated from Martienssen and Moazed 2015 (©2015 Cold Spring Harbor Laboratory).

Evidence of RNAi-dependent heterochromatin silencing is also present in animals. For instance, in *Drosophila melanogaster,* RNAi mediates H3K9 methylation and transcriptional silencing (Pal-Bhadra et al. 2004; Fagegaltier et al. 2009). In mammals, RNAi operates mostly in the germline and has been implicated, for example, in DNA methylation and silencing of transposons (Aravin et al. 2008).

Fission yeast is a powerful model for studying the endogenous nuclear function of RNAi. Indeed, in *S. pombe* there is not the same redundancy exhibited in other organisms for proteins involved in RNAi, since its genome contains only a single homologue for Dicer, Argonaute and RdRP (Aravind et al. 2000). RNAi is a crucial mechanism contributing to heterochromatin formation in *S. pombe*, as will be presented in the following section.

2.3.1.2. Role of nuclear RNAi in *S. pombe*

In *S. pombe,* RNAi-based mechanisms permit the nucleation of heterochromatin via transcription of specific repeated sequences located in all main constitutive heterochromatin regions. At centromeres, transcription of o*tr* and part of the *imr* repeats is required for RNAi-dependent heterochromatin formation (Volpe et al. 2002). The other two main constitutive heterochromatin regions also contain centromeric *dh-like* repeats required for heterochromatin assembly, which are located at subtelomeric *tlh1/tlh2* genes and in the K region of the mating type (Noma et al. 2004). An important difference among constitutive heterochromatin regions is that, at centromeres, heterochromatin formation is uniquely dependent on RNAi-based mechanisms; in contrast, at telomeres and the mating type region, RNAi acts in parallel with DNA-based mechanisms (Allshire and Ekwall 2015).

<u>Transcription of repeated sequences is required to initiate heterochromatin assembly</u>

In fission yeast, transcription is required to trigger heterochromatin formation and transcriptional silencing. Indeed, proteins involved in the initiation of transcription, transcriptional elongation, and transcript processing are as important for heterochromatin assembly as they are for euchromatic gene expression (Allshire and Ekwall 2015). RNA polymerase II subunits and associated factors are required for heterochromatin assembly via the activation of the RNAi pathway. For instance, mutations in FACT, a histone chaperone

complex involved in transcriptional elongation, lead to defects in centromeric heterochromatin silencing (Lejeune et al. 2007); likewise the RNA pol II-interacting histone chaperone Spt6 is required for heterochromatin assembly and siRNA production (Kiely et al. 2011). Indeed, the first step of RNAi activation is the production of siRNAs. RNA pol II transcripts of repeated sequences can fold into hairpin-like double-stranded RNA structures that are then cleaved by Dicer1 (Dcr1) into siRNAs (Djupedal et al. 2009). Alternatively, similar siRNAs called priRNAs (primal RNAs) are produced, in a Dicer-independent manner, by bidirectional transcription of heterochromatin repeats, and then processed by a nuclease named Triman (Halic and Moazed 2010; Marasovic, Zocco, and Halic 2013).

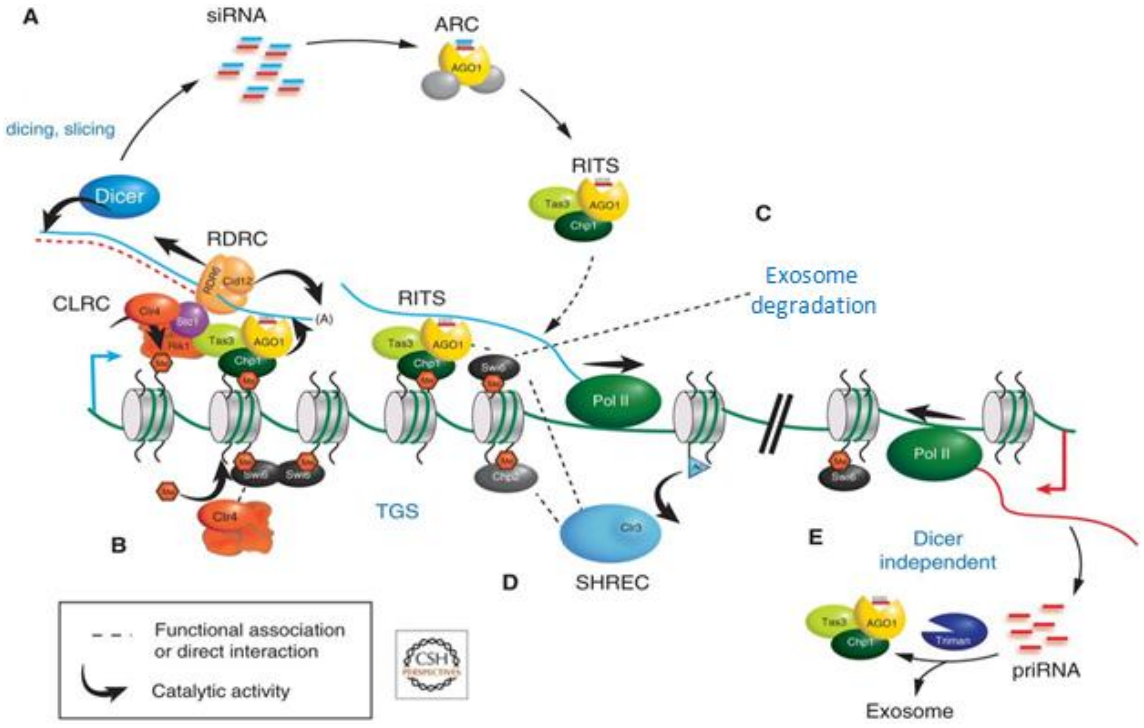


Figure 12. RNAi-mediated transcriptional assembly of heterochromatin in *S. pombe*. Transcription of heterochromatin repeats permits the production of small RNAs by Dicer-dependent and -independent pathways. (**A**) Processed small RNAs are first loaded onto ARC and then on the RITS complex, which targets the nascent noncoding RNA by base-pairing interactions and by its association to H3K9me. This leads to the recruitment of the RDRC to generate a positive feedback loop. The RITS complex also recruits the CLRC H3K9 methyltransferase complex to chromatin, via interactions with Stc1. (**B**) H3K9 methylation stabilizes RITS association with chromatin and also provides binding sites for HP1 proteins (Swi6 and Chp2). Swi6 facilitates the recruitment of RDRC and degradation by the exosome (**C**), whereas Chp2 recruits the SHREC complex (**D**). RNA degradation is permitted by RNAi-dependent (**A**, dicing and slicing) and RNAi-independent (**C**, Exosome degradation) mechanisms. Dicer-independent priRNAs, mediated by the Triman exonuclease (**E**) may trigger siRNA amplification (**A**). Figure adapted from Martienssen and Moazed 2015 (©2015 Cold Spring Harbor Laboratory).

The RNAi effector complex RITS is required for initiation of heterochromatin assembly

Processed siRNAs (or priRNAs) are then loaded onto Ago1, an Argonaute protein that constitutes the ARC (Argonaute siRNA Chaperone) complex, together with two chaperone proteins named Arb1 and Arb2. These two proteins are important for loading siRNAs onto Ago1 and for facilitating Ago1 'slicer' activity, which generates single stranded siRNAs (Buker et al. 2007). In addition, ARC acts to chaperone siRNAs to an RNAi effector complex called RITS (RNA-Induced Transcriptional gene Silencing). RITS is a trimeric complex composed by Ago1, which is bound to siRNAs, the chromodomain protein Chp1 and the GW-repeat protein Tas3. This complex is required for heterochromatin assembly in fission yeast (Verdel et al. 2004).

The RITS complex targets nascent heterochromatin transcripts through base-pairing interactions with Ago1-bound siRNAs, and by Chp1's association with H3K9me (Buhler, Verdel, and Moazed 2006; Partridge et al. 2002), which is crucial for establishing heterochromatin. Indeed, several mutations in the chromodomain of Chp1 reduce its binding affinity to H3K9me by 2 to 500-fold (e.g. Chp1V24R), and notably a 5-fold reduction in Chp1's affinity is sufficient to abolish the establishment of centromeric heterochromatin (Schalch et al. 2009). Interestingly, Chp1 can also bind to RNAs, via both its chromodomain and a central RRM (RNA Recognition Motif), and this binding is essential for heterochromatin gene silencing. In addition, the RNA-binding activity of Chp1 is strongly enhanced when it is also bound to H3K9me, suggesting that this can further stabilize RITS recruitment to heterochromatin (Ishida et al. 2012).

Once bound to heterochromatin, RITS recruits two protein complexes, which reinforce heterochromatin silencing via two positive feedback loops involving either H3K9 methylation, or siRNAs production. Firstly, RITS recruits the chromatin-modifying complex CLRC, which permits H3K9 methylation by Clr4 (Irvine et al. 2006). The bridge between RITS and CLRC is a protein called Stc1, which interacts with both Ago1 and Clr4 (Bayne et al. 2010). Thus, RITS binds to H3K9me and recruits Clr4, which is responsible for more H3K9 methylation. In turn, this modification stabilizes RITS binding to chromatin, generating a loop that reinforces heterochromatin silencing. More research is still needed to clearly understand which essential initiating steps versus reinforcing steps are required to generate

heterochromatin (Allshire and Ekwall 2015). Secondly, RITS recruits RDRC (RNA-Directed RNA polymerase Complex), which consists of the RdRP protein Rdp1, the RNA helicase Hrr1 and the Poly(A) polymerase Cid12 (Motamedi et al. 2004). RDRC uses single-stranded transcripts as templates to generate additional dsRNAs. Then, Dcr1 cuts these dsRNAs to produce new siRNAs to be loaded onto RITS, and this constitutes the second positive loop to reinforce heterochromatin silencing (Motamedi et al. 2004; Colmenares et al. 2007) (Figure 12).

Spreading and confinement of heterochromatin

The recruitment of Swi6 and Chp2 to these sites of heterochromatin nucleation permits the assembly of fully silenced heterochromatin and its spreading through neighboring regions. Swi6 interacts with the Ers1 protein, which in turn recruits RDRC, promoting additional dsRNA synthesis and efficient RNA degradation (Hayashi et al. 2012; Rougemaille et al. 2012). In contrast, Chp2 recruits the SHREC complex, which shuts down repeat transcription by deacetylating H3K14 (Motamedi et al. 2008; Fischer et al. 2009)

Many proteins that regulate confinement and spreading of heterochromatin have been investigated in *S. pombe*. The most studied is Epe1, which was first identified in a screen to find new proteins promoting heterochromatin spreading across barriers (Ayoub et al. 2003). Epe1 reduces siRNA production and promotes RNA pol II occupancy at heterochromatin regions. However, its mechanism of action is still not completely understood yet, and, despite its JmjC domain, no detectable demethylase activity has been revealed (Zofall and Grewal 2006; Isaac et al. 2007; Trewick et al. 2007). Interestingly, Swi6 recruits Epe1 to all heterochromatin regions, but Epe1 function is specific for hetero/euchromatin boundaries. Indeed, the Cul4-Ddb1 complex, whose activity is excluded from boundaries, ubiquitinates Epe1 to promote its degradation in all heterochromatin regions (Braun et al. 2011). Epe1 recruits to boundaries the bromodomain protein Bdf2, which recognizes acetylated histone H4 and antagonizes Sir2-mediated H4K16 deacetylation, thus facilitating the establishment of heterochromatin boundaries (Wang et al. 2013).
In addition, RNAi has also been reported to be involved in heterochromatin spreading and the slicing activity of Ago1 seems to be required for this process (Irvine et al. 2006).

Heterochromatin status through the cell cycle

Once heterochromatin is established, it must be maintained through cell division. During S-phase, H3K9me is highly reduced and HP1 proteins are released from chromatin due to phosphorylation of H3S10, which prevents chromodomain binding (Chen et al. 2008). The decondensation of heterochromatin during S-phase leads to RNA pol II-dependent transcription of heterochromatin repeats, which is followed by increases in siRNAs and RNAi-mediated heterochromatin maintenance via RITS, CLRC and RDRC (Kloc et al. 2008).

RNAi is also able to induce transitory cell cycle-regulated heterochromatin at some convergent genes. Indeed, in G1 phase, transcription of some convergent genes, including many genes coding for RNAi proteins, has been shown to produce dsRNAs that induce RNAi-dependent transitory heterochromatin structures. Then in G2, cohesins are recruited in these regions, and this avoids dsRNA formation by promoting gene-proximal transcription termination between convergent genes (Gullerova and Proudfoot 2008; Gullerova, Moazed, and Proudfoot 2011). In addition, the expression of convergent transcription on plasmids to produce dsRNAs results in TGS of endogenous genes, both in *S. pombe* and mammalian cells, thus suggesting that this mechanism may be conserved in higher eukaryotes (Gullerova and Proudfoot 2012).

RNAi promotes transcriptional termination at replication-transcription collision sites

The strong connection between RNAi machinery and transcription has been further supported by a model that proposes a role for RNAi in transcriptional termination. During S-phase, at the pericentromeric heterochromatin region, there is competition between transcription and replication machineries. Interestingly, RNAi avoids collisions by releasing RNA pol II, thus allowing DNA polymerase to complete DNA replication and associated histone modifiers to spread heterochromatin with the replication fork (Zaratiegui, Castel, et al. 2011) (Figure 13). Recently it was found that Dcr1, but not other components of RNAi, promotes the release of RNA Pol II at sites of replication-transcription collisions genome-wide, indicating a novel role for Dcr1-mediated transcription termination in genome

maintenance, and suggesting widespread regulation of genome stability by nuclear RNAi in higher eukaryotes (Castel et al. 2014).

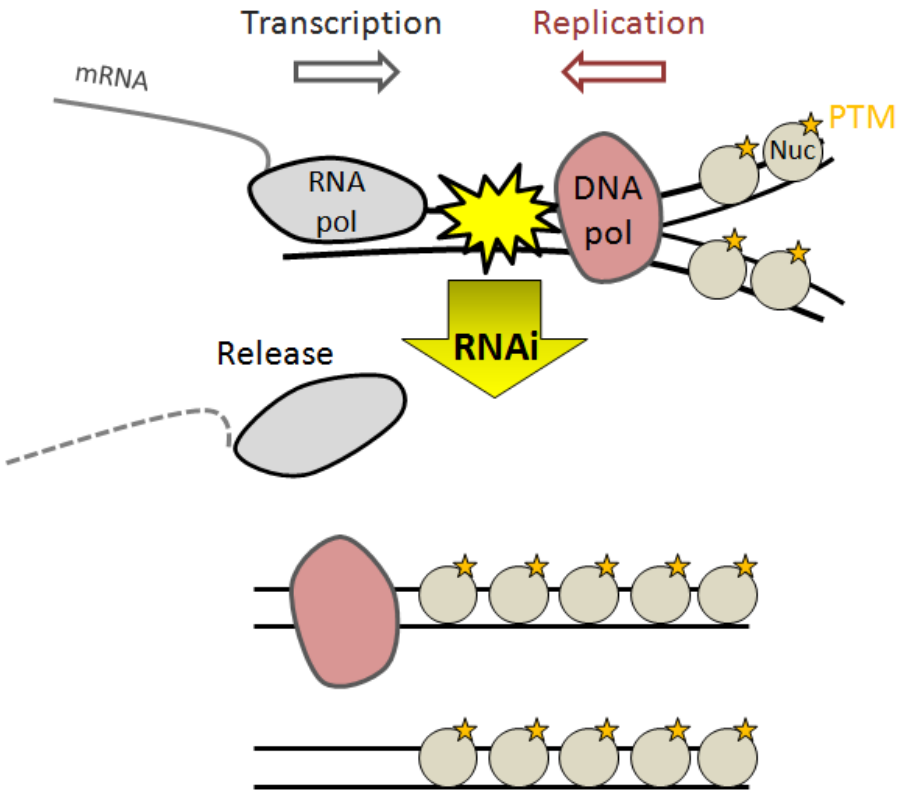


Figure 13. RNAi solves competition between transcription and replication machineries. During the S-phase of *S. pombe* cell cycle, RNAi avoids RNA pol II and DNA pol collisions by releasing RNA pol II, thus allowing DNA pol to complete DNA replication and associated histone modifications to spread.

2.3.2. RNA-independent mechanisms of constitutive heterochromatin formation

DNA-based mechanisms are known to act in parallel with RNAi for heterochromatin assembly at telomeres and the mating type region (Figure 14).

At telomeres, a protein called Taz1 (orthologue of human TRF1 and TRF2) binds directly to telomeric repeats and recruits the HMT Clr4 to establish heterochromatin (Kanoh et al. 2005; Hansen, Ibarra, and Thon 2006). In addition, Taz1 recruits two telomeric specific

proteins, Rap1 and Rif1, which are important for many telomeric functions: length control, clustering and heterochromatin maintenance (Kanoh and Ishikawa 2001; Cooper et al. 1997).

Similarly, at the mating type region, there are two DNA-binding proteins of the ATF/CREB (Activating Transcription Factor/cAMP Response Element Binding) family, which are named Atf1 and Pcr1. These proteins have a 'leucine zipper' domain that recognizes a specific heptameric sequence, called CRE (cAMP Response Element), located inside the mating type locus. The heterodimer Atf1-Pcr1 cooperates with the HDAC Clr3 and induces recruitment of the HDAC Clr6, the HMT Clr4 and the HP1 protein Swi6, which are required for heterochromatin formation and maintenance (Jia, Noma, and Grewal 2004; Kim et al. 2004; Yamada et al. 2005).

At the centromere, as previously explained, heterochromatin formation is uniquely dependent on RNAi-based mechanisms. Nevertheless, in this region there are also DNA-binding proteins (Abp1, Cbh1, and Cbh2), homologs to human CENPB that can facilitate centromeric heterochromatin assembly. These proteins bind to specific centromeric DNA sequences and induce H3K9 and K14 deacetylation, which are required for H3K9 methylation and Swi6 recruitment (Nakagawa et al. 2002).

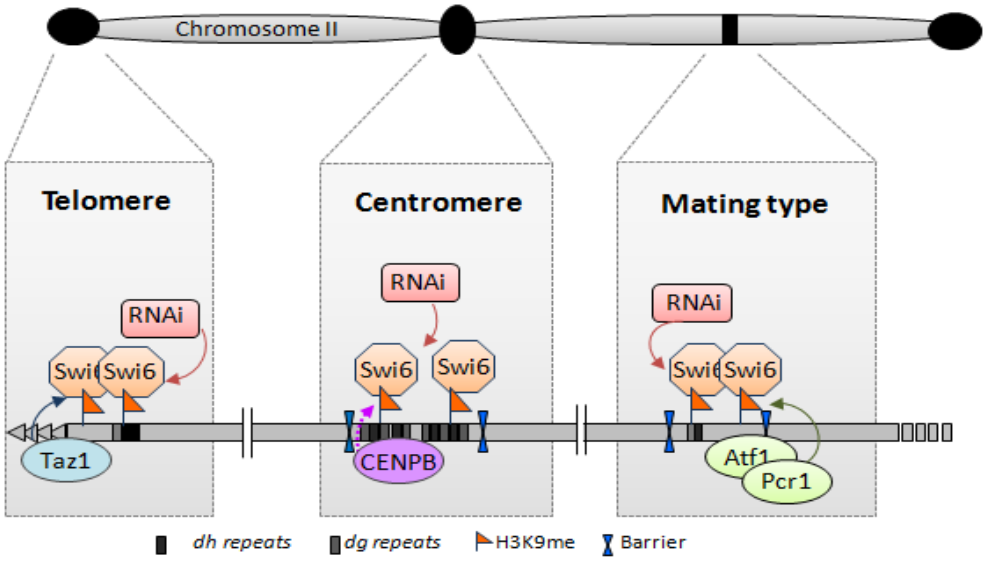


Figure 14. **DNA-based mechanisms act in parallel to RNAi for heterochromatin assembly.** Taz1 at telomeres and Atf1/Pcr1 at mating type region bind to DNA repeats and induce heterochromatin formation and maintenance in parallel with RNAi. At centromeres, heterochromatin formation is uniquely dependent on RNAi-based mechanisms, although CENPB proteins have been shown to facilitate the process.

2.4. Mechanisms of facultative heterochromatin assembly and gene silencing in fission yeast

2.4.1 Different genomic sites of facultative heterochromatin

Facultative heterochromatin in *S. pombe* is mainly found at meiotic genes, which are known to be specifically activated during sexual differentiation (Cam et al. 2005; Hiriart et al. 2012). Meiotic genes are normally found at the nuclear periphery during vegetative growth. Upon nitrogen starvation, a drastic change in their nuclear localization is observed, moving away from the nuclear membrane towards the interior, and this correlates with their activation (Alfredsson-Timmins et al. 2009).

S. pombe has developed a highly regulated mechanism to induce or repress sexual differentiation, acting at several levels. This process involves transcriptional regulation of meiotic genes in a cascade, which is mediated by the transcription factor Ste11 (Sterility protein 11); and post transcriptional regulation, which is mediated by the RNA binding protein Mmi1 (Meiotic mRNA interception protein 1), as will be further described in the next section. In addition, facultative heterochromatin islands can also facilitate the rapid modulation of meiotic gene expression during cell differentiation. Meiotic heterochromatin islands disassemble in response to nutritional signals that induce sexual differentiation and the antisilencing factor Epe1 is involved in this process (Zofall et al. 2012). Interestingly, as in the case of constitutive heterochromatin, both transcription and degradation of meiotic transcripts are required for facultative heterochromatin assembly (Zofall et al. 2012). The role of these meiotic heterochromatin islands is still unclear; indeed, it has been shown that H3K9 methylation at meiotic genes is not sufficient to restrict RNA polymerase II access or to repress gene expression during vegetative growth (Egan et al. 2014).

A recent study has revealed an important role for the Ccr4-Not complex in the integrity of facultative heterochromatin. Ccr4-Not is a conserved multiprotein complex that regulates gene expression at multiple levels (including RNA maturation, translation and degradation) (Collart and Panasenko 2012). Mmi1 recruits Ccr4-Not to its meiotic RNA targets. In addition,

mutations in genes encoding for the RNA deadenylase catalytic subunits of this complex (*ccr4, caf1*) do not affect the stability of meiotic transcripts, but lead to a loss of heterochromatin in these regions (Cotobal et al. 2015).

Interestingly, in the absence of the exosome, additional H3K9me islands are observed in the genome, which are called HOODs (HeterOchrOmatin Domains) (Yamanaka et al. 2013). These heterochromatin induced regions mainly correspond to retrotransposable elements, which normally must be silenced in cells; although their transitional expression is known to be crucial in other organisms, such as during embryo development in animals (Macfarlan et al., 2012). An overview of transposable elements, and of the different strategies used by fission yeast for their silencing, will be provided in the next section (2.4.3).

2.4.2. The RNA-binding protein Mmi1 mediates silencing at meiotic genes

Mmi1 contains a YTH (YT521-B Homology) RNA-binding domain that is conserved in eukaryotes (Stoilov, Rafalska, and Stamm 2002). This domain permits Mmi1 binding to target RNAs in a region called DSR (Determinant Selective Removal) (Harigaya et al. 2006), which is enriched for repeats of a specific hexanucleotide UNAAAC (Chen, Futcher, and Leatherwood 2011).

Mmi1 binds to meiotic RNAs that contain a high density of this motif and recruits several proteins that are required for its functions (Yamashita et al. 2012), such as Pab2, which binds to polyadenylated meiotic RNAs and facilitates their exosome-mediated degradation (Yamanaka et al. 2010). Mmi1 also recruits the zinc finger protein Red1 (Sugiyama and Sugioka-Sugiyama 2011), which in turn recruits the HMT Clr4 to establish facultative heterochromatin at meiotic genes (Zofall et al. 2012). Red1 is a component of the NURS (Nuclear RNA Silencing) complex, which is also conserved in higher eukaryotes, and is involved in RNA degradation and H3K9 methylation at meiotic genes (Egan et al. 2014).

Mmi1 is therefore a crucial protein to repress meiotic genes during vegetative growth. Conversely, Mmi1 must be inactivated during meiosis to permit the progression of the sexual differentiation program. Indeed, at the onset of meiosis, Mmi1 is sequestered and

inactivated into the Mei2 dot, a meiosis-specific subnuclear structure comprising the Mei2 protein and the non-coding *meiRNA* (Watanabe and Yamamoto 1994).

RNAi has also been shown to participate in the control of sexual differentiation. Indeed, the RITS complex binds to meiotic RNAs and genes and is important for their silencing. The recruitment of RITS is mediated by Mmi1 and its RNA surveillance machinery. In particular, Pab2 assists RITS binding to meiotic RNAs, whereas Red1, by mediating Clr4-dependent H3K9 methylation, permits RITS anchoring to chromatin via the chromodomain of Chp1 (Hiriart et al. 2012) (Figure 15).

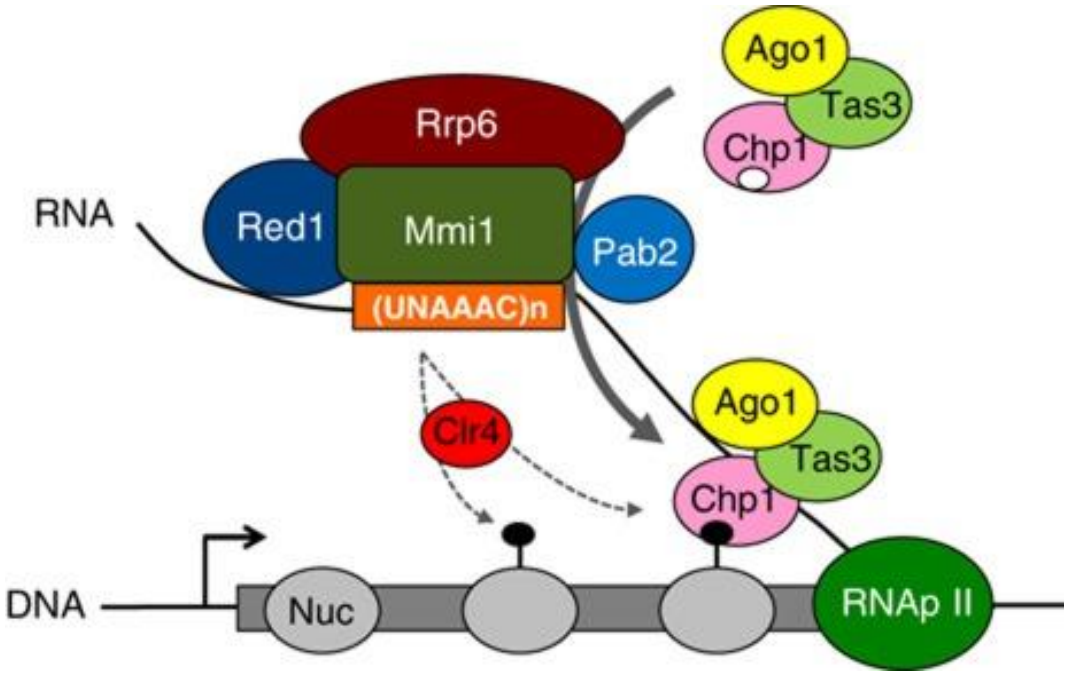


Figure 15. Mmi-mediated silencing at meiotic genes. Mmi1 binds to meiotic RNAs and recruits Pab2, which binds to poly(A) transcripts and facilitates their exosome-mediated degradation; and Red1, which is involved in RNA degradation and Clr4-mediated H3K9 methylation at meiotic genes. The RITS complex also binds to meiotic RNAs and genes and is important for their silencing. The recruitment of RITS is mediated by Pab2, which assists its binding to meiotic RNAs, whereas Red1 helps RITS anchoring to chromatin via H3K9me. Figure taken from Hiriart et al. 2012.

2.4.3. The peculiar case of transposable element gene silencing in *S. pombe*

2.4.3.1. General features of transposable elements

A transposable element (TE, transposon) is a DNA sequence that can change its position within the genome. The first transposable element was discovered by Barbara McClintock in 1944 using *Zea mays* (McClintock 1953); later investigations on model organisms such as *D. melanogaster*, *S. cerevisiae* and *C. elegans* permitted better understanding of the functions of these elements. Transposable elements are present in organisms ranging from bacteria to humans, even though they appear to be more common in higher eukaryotes. For instance, TEs constitute at least 45% of human and up to 50–90% of some plant genomes (Kazazian 2004).

According to their mechanism of transposition, TEs can be divided in two classes. Class I TEs, also called retrotransposons, act with a 'copy and paste' mechanism: DNA is transcribed to RNA, which is then reverse transcribed to DNA to be inserted at a new position into the genome. Class II TEs act with a 'cut and paste' mechanism, in which DNA transposition occurs without any RNA intermediate.

The mobility of transposable elements is considered a driving force for evolution and organism adaptation (Pritham and Feschotte 2007), but on the other hand it can also be a dangerous source of genome instability. For example, a transposition event can influence the expression of a neighboring gene, possibly leading to disease (Belancio, Deininger, and Roy-Engel 2009).

2.4.3.2. Transposable elements in *S. pombe*

In *S. pombe*, there are two families of transposable elements, *Tf1* and *Tf2*, which both belong to the Ty3/Gypsy group of LTR retrotransposons. LTRs (Long Terminal Repeats) are repeated DNA sequences located at each end of a fragment synthesized by a reverse transcriptase, which are required for transposon integration into host genomes (Bowen et al. 2003). No full length copy of *Tf1* has been found in *S. pombe*, but 13 full-length copies of *Tf2* have been identified (Bowen et al. 2003). A full-length LTR retrotransposon is highly similar to a retrovirus and contains genes coding for all the proteins required for transposition: a caspid-

like protein Gag, a protease, a reverse transcriptase and an integrase (Teysset et al. 2003). Five *Tf* fragments are also present in the *S. pombe* genome. They have lengths ranging between 233 and 2414 bp and it is largely uncertain if they originate from *Tf1* or *Tf2* elements. In addition, more than 75 shorter LTRs fragments have been identified, which are smaller than 200 bp. In fission yeast there are also solo-LTRs, which are mainly generated by LTR-LTR recombination event of previously existing transposons (Bowen et al. 2003). Interestingly, many LTR elements are present at subtelomeric regions; this may be explained by duplications of subtelomeric sequences occurring during meiotic prophase, when all the telomeres cluster in the nucleus (Bowen et al. 2003).

Analysis of solo-LTRs serves as a footprint to understand the mechanism of transposition events in fission yeast. LTR retrotransposon insertion occurs in close association with RNA polymerase II promoters. Indeed, they integrate preferentially in promoter proximal regions of genes, around 100-400 bp upstream of their open reading frame (Behrens et al., 2000; Bowen et al., 2003). In addition, there is a strong preference for promoters of genes that are induced in stress conditions, suggesting a role of transposons in regulating genes required to promote cells survival under environmental stress (Guo and Levin 2010). Interestingly, chromosome III has twice the density of insertion events compared to the other two chromosomes. This bias is due to the presence of 23 members of a family of genes called *wtf* (which stands for "with *Tf*") on chromosome III. Of the 25 members of this family, 21 are flanked by solo LTRs or LTR fragments (Bowen et al., 2003). These proteins are highly expressed during meiosis, but their function and their link with transposable elements are still unknown (Watanabe et al., 2001).

2.4.3.3. Silencing of transposable elements in higher eukaryotes versus fission yeast

TEs need to be silenced and eukaryotes have developed several mechanisms to repress their expression and inhibit their integration in the genome (Zamudio and Bourc'his 2010).

In higher eukaryotes, several epigenetic mechanisms are required for the silencing of transposons; involving DNA methylation, histone modifications and the RNAi pathway. DNA

methylation is required for TEs transcriptional silencing, inducing a local repressive chromatin state. Indeed, mutations in genes encoding for proteins of the DNA methylation machinery cause an increase in TEs expression in germ cells that correlates with reduced fertility (Bourc'his and Bestor 2004; Zamudio and Bourc'his 2010). Histone repressive marks (H3K9, H3K27 and H4K20 methylation) are present at TE promoters and seem also to have a role in TEs silencing (Matsui et al. 2010; Leeb et al. 2010). The role of RNAi in the silencing of TEs has been shown in many higher eukaryotes (Brodersen and Voinnet 2006). In particular, piRNAs (PIWI-interacting small RNAs) have been shown to mediate RNAi-based TE repression in germ cells (Klattenhoff and Theurkauf 2008).

On the contrary, RNAi seems to play just minor role in the silencing of transposons in *S. pombe*. Indeed, there is no or very little enrichment of heterochromatin and RNAi components (H3K9me, Clr4, HP1 and RITS) at *Tf2* retrotransposons, and transposons are only weakly activated in RNAi or Clr4 mutants (Cam et al. 2005; Hansen et al. 2005). However, in the absence of the exosome subunit Rrp6, as previously explained, siRNA clusters and H3K9me clearly accumulate at *Tf2* sequences and this requires Ago1, Dcr1, Clr4 and Rdp1. Therefore, there is a competition between the exosome and the RNAi to target transposable RNAs; so that they are normally targeted by the exosome, whereas the role of RNAi and heterochromatin on these regions is visible only after exosome removal (Yamanaka et al. 2013).

Other proteins are involved in the silencing of TEs in *S. pombe*, such as the CENPB homologue Abp1. This protein is an example of a domesticated transposable element; in fact, a part of its sequence derives from transposases of pogo DNA TEs (Smit and Riggs 1996). Abp1 binds to transposon sequences, specifically to a certain motif present inside LTR elements (TAATATAATA), and permits their silencing (Cam et al. 2008; Lorenz et al. 2012). Removal of the Abp1 dimerization domain has little effect on *Tf2* expression; in contrast, removal of its DNA-binding domain or its transposase domain results in an increase in *Tf2* transcripts, although still less than what is seen with complete deletion of *abp1* (Lorenz et al. 2012). Abp1 regulates TE silencing by recruiting several histone modifiers, such as the HDACs Clr3 and Clr6, and the H3K4 HMT Set1 (Lorenz et al. 2012; Cam et al. 2008). In addition, *Tf2*

elements are known to cluster in the nucleus into *Tf* bodies, and Abp1 is also required for the organization of these nuclear structures (Cam et al. 2008).

Strains deleted for *abp1* have a growth defect, which correlates with morphological abnormalities, increased mortality and abnormal mitosis (Zaratiegui, Vaughn, et al. 2011). This phenotype is even worse if *abp1* is deleted together with one of the other two CENPB homologues, *cbh1* or *cbh2*, and the triple deletion mutants are not viable (Baum and Clarke 2000). Interestingly, the severe phenotype of CENPB deletion mutants is spontaneously rescued by a mutation in the DNA binding factor Sap1. During replication of LTR elements, CENPB promotes replication fork progression; in contrast, Sap1, which is also recruited on LTRs, is important for fork pausing and determination of replication polarity. Thus, in mutants of CENPB there is a Sap1-dependent replication fork blockage. The loss of Sap1 allows replication fork progression, but results in increased rearrangements of transposable elements (Zaratiegui, Vaughn, et al. 2011).

Another protein involved in the silencing of full length TEs is the histone chaperone HIRA, which also suppresses transcripts generated by solo-LTR or *Tf* fragments (Anderson et al., 2009). Interestingly, HIRA and CENPB have been shown to silence TEs through distinct pathways (Cam et al. 2008).

3.

ATAD2-like proteins

In my host team, preliminary results suggested a possible role in heterochromatin formation and gene silencing for an uncharacterized protein in *S. pombe* called Abo1. Characterization of this protein was a major part of my PhD thesis project.

In this chapter, data on Abo1 homologues in human (ATAD2, ATPase family AAA+ Domain-containing protein 2) and *S. cerevisiae* (Yta7) will be presented. Studies on these proteins have suggested that they may act as putative histone chaperones in regulating transcription, although their precise molecular function is unknown. Furthermore, the conservation of this family of proteins in other eukaryotes will be discussed.

Of note, the main reference for this chapter is a review that we have recently published: 'Lessons from yeast on emerging roles of the ATAD2 protein family in gene regulation and genome organization' (Cattaneo et al. 2014).

In addition, numerous studies in eukaryotic cells, ranging from yeast to humans, indicate that mutations in factors involved in nucleosome assembly result in defects in genome stability, regulation of telomeres, gene transcription and heterochromatin assembly, and this may promote development of human diseases (Burgess and Zhang 2013). In particular, the crucial role of ATP-dependent chromatin remodelers and histone chaperones in chromatin organization and gene expression indicates that mutations or aberrant expressions of these proteins can trigger tumorigenesis (Gonzalez-Perez, Jene-Sanz, and Lopez-Bigas 2013). For instance, members of SWI/SNF family are predicted to have driver functions in various cancers, and 20% of all human tumors contain mutations in at least one member of this complex (Shain and Pollack 2013). In addition, increased protein levels of both histone chaperones Asf1b and CAF-1 correlate with the enhanced proliferation status of cancer cells (Corpet et al. 2011; Polo et al. 2010). Therefore, alterations in the expression of chromatin-related proteins may lead to ectopic gene activation, which can be responsible for malignant cell transformation. Indeed, out-context expression of tissue-restricted genes is found in many cancer types (Boussouar et al. 2013), opening a new field in cancer biology that connects oncogenic mechanisms to aberrant gene expression programs.

One example of out-context expression of a potential chromatin regulator concerns ATAD2, which, besides its expression in early prostate development and male germ cells, is also ectopically expressed in several types of tumors (Caron et al. 2010). In addition to studies

conducted on ATAD2 in mammals, there is a relatively rich literature on Yta7 (Yeast Tat-binding Analog 7), which is the unique ATAD2 homologue expressed in *S. cerevisiae*. These two proteins are highly similar, in particular for their functional domains (Figure 16).

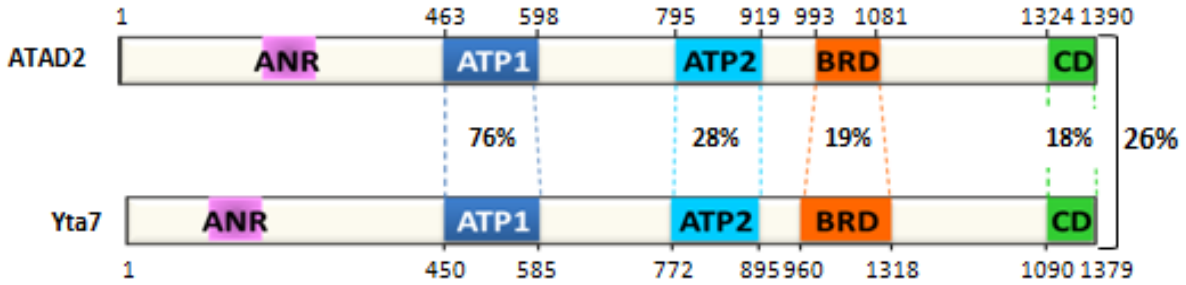


Figure 16. Human ATAD2 and *S. cerevisiae* Yta7. Schematic representation of human ATAD2 and *S. cerevisiae* Yta7 drawn to scale. The percentages of identity are indicated both for the overall length of the two proteins (on the right side of the panel) and for each conserved domain (alignment done with CLUSTALO). ANR, Acid N-terminal Region; ATP1 and ATP2, AAA+ ATPase domain 1 and 2; BRD, BRomoDomain; CD, C-terminal Domain. Figure taken from Cattaneo et al. 2014.

3.1. The putative histone chaperone ATAD2 in mammals

3.1.1. ATAD2 is involved in transcriptional activation

ATAD2, also known as ANCCA (AAA Nuclear Coregulator Cancer-Associated protein), contains two main functional domains: an AAA+ ATPase domain and a bromodomain. The AAA+ ATPase domain is important for protein multimerization and for ATAD2 function (Caron et al. 2010; Zou et al. 2007). The bromodomain is known to preferentially bind acetylated histones H3 and H4 (Caron et al. 2010; Revenko et al. 2010). The presence of these two domains suggests that ATAD2 is a factor that could potentially act as a histone chaperone.

Beside its possible role in chromatin regulation, ATAD2 has also been strongly associated with transcriptional activation. The first characterization of ATAD2 showed that this protein is an estrogen responsive gene and that it is also required for transcriptional activity of the estrogen receptor (Zou et al. 2007). Furthermore, the additional role of ATAD2 as a co-activator of androgen receptor (Zou et al. 2009) places ATAD2 at the heart of the sex hormone response system (Hsia et al. 2010).

Later studies have revealed a more general role of ATAD2 in transcription regulation of genes that are mainly involved in cell proliferation and survival. For instance, it is an E2F and MLL cofactor, stimulating E2F-dependent cell proliferation (Revenko et al. 2010), and also a MYC partner (Ciro et al. 2009). These transcriptional activities of ATAD2 have been at least partly attributed to its two conserved domains (Zou et al. 2009; Revenko et al. 2010), although the mechanism of action of ATAD2 in controlling transcription is still unclear.

3.1.2. Human ATAD2 in cancer

Analysis of ATAD2 expression in many different cancer types revealed a striking ATAD2 upregulation in many tumors. For instance, ATAD2 is highly expressed in breast, ovarian, endometrial, lung and cervical cancer, hepatocellular carcinoma and large B-cell lymphoma (Hsia et al. 2010; Wan et al. 2014; Raeder et al. 2013; Zhang et al. 2013; Wu, Liu, et al. 2014; Alizadeh et al. 2000; Zheng et al. 2015). In addition, overexpression of ATAD2 has been linked to poor prognosis in malignancies such as prostate, lung, triple-negative breast cancer and hepatocellular carcinoma (Caron et al. 2010; Hsia et al. 2010; Kalashnikova et al. 2010; Revenko et al. 2010; Yang et al. 2014; Zou et al. 2009).

In mammals, there is a paralogue of ATAD2 known as ATAD2B that does not seem to be associated with cancer (Caron et al. 2010). Therefore, although these two paralogues share their functional domains, they seem to be functionally divergent (Leachman et al. 2010).

Despite the strong association between ATAD2 expression and cancer, the molecular function of this protein in the process of carcinogenesis is still unclear. The involvement of ATAD2 in transcription activation suggests that once this protein is activated in cancer (for example, after a deregulation of E2F), it can initiate a loop of transcription amplification leading to high levels of ATAD2 and its co-regulators (Boussouar et al. 2013). The over-expression of ATAD2 in somatic tissues could thus start a transcriptional program that provides a benefit to cells and induces their transformation. Conversely, a knock-down of ATAD2 in cancerous cells lines, which normally overexpress ATAD2, inhibits invasion, migration and cell proliferation, leading to G1 phase cell cycle arrest (Zheng et al. 2015). It also induces an increase in spontaneous cell apoptosis, particularly in response to genotoxic treatments (Caron et al. 2010).

These data suggest that inactivation of ATAD2's contribution to tumorigenesis could constitute an effective anti-cancer therapy. In addition, both the bromodomain and the AAA+ ATPase domain appear to be possible druggable targets. Inhibitors have been discovered against several bromodomains, in particular for the BET family of bromodomain-containing factors, which show very promising anti-cancer activities (Muller, Filippakopoulos, and Knapp 2011). Nevertheless, computational studies have classified the ATAD2

bromodomain as 'difficult to target' since its acetyl-lysine binding site is quite dissimilar from other druggable bromodomains (Vidler et al. 2012). The development of ATAD2 bromodomain-specific inhibitors could be challenging and, despite the recent use of fragment-based approaches to discover new small molecules (Harner et al. 2014; Demont et al. 2015), specific and efficient ATAD2 bromodomain inhibitors have yet to be developed. The AAA+ ATPase domain of ATAD2 is also a good druggable target, but the main challenge may be the specificity and selectivity of these drugs (Boussouar et al. 2013). Indeed, the AAA+ ATPase domain of ATAD2 is highly similar to that of other ATAD-like proteins that are involved in many biological functions, such as ATAD2B, ATAD3, ATAD4 and ATAD5. For instance, ATAD5 has been reported to have tumor suppressor activity (Bell et al. 2011), so simultaneous inhibition of this protein could inadvertently favor cancer progression. Overall, inhibition of ATAD2 domains appears to be a promising cancer therapy, but further studies are necessary to understand the role of each of these domains in cancer progression.

3.2. Yta7 in *Saccharomyces cerevisiae*

3.2.1. Yta7 is a probable histone chaperone regulating transcription

Significant advancement in understanding the function of ATAD2-like proteins has been achieved thanks to numerous studies conducted on the unique ATAD2 homologue in *S. cerevisiae* called Yta7. Yta7, as well as ATAD2 in mammals, is implicated in control of gene expression and in regulation of transcription. In addition, Yta7 has been proposed to regulate chromatin dynamics and gene expression as a possible histone chaperone acting at boundaries sites.

A genome-wide chromatin localization analysis demonstrated that Yta7 binds to all histone genes (Gradolatto et al. 2008). Initial studies proposed that Yta7 could be an activator of histone gene transcription, since deletion of the *YTA7* gene correlates with a decrease in *HTA1* (*H2A*) transcripts concomitant with decreased recruitment of RNA polymerase II to both the promoter region and ORF of *HTA1* (Kurat et al. 2011; Fillingham et al. 2009). More

recent studies have shown that the effects of Yta7 in regulating histone transcription may in fact differ depending on the analyzed histone gene. Indeed, in *yta7Δ* cells *HTA1* transcripts are significantly reduced, while in these same cells *HHT1* (*H3*), *HHF1* (*H4*) and *HTA2* (*H2B*) transcript levels remain unchanged, despite the fact that Yta7 binds to all of these histone loci (Lombardi, Ellahi, and Rine 2011; Zunder and Rine 2012).

Transcription of histone genes is tightly regulated throughout the cell cycle in order to provide the histone supply required for replication of chromatin during S-phase, while avoiding inappropriate and toxic accumulation of neosynthesized histones during other phases of the cell cycle (Gunjan and Verreault 2003). Interestingly, Yta7 binding to the histone gene *HTA1* is precisely regulated during the cell cycle (Kurat et al. 2011). After loading of RNA polymerase II to histone genes in G1/S, Yta7 is phosphorylated at multiple sites in its N-terminus by at least two different kinases, named Cdk1 (Cyclin-Dependent Kinase 1) and CK2 (Casein Kinase 2). Phosphorylation of Yta7 causes its release from *HTA1* gene and correlates with its efficient transcription (Kurat et al. 2011). The molecular mechanism underlying Yta7-dependent transcriptional gene activation is not completely understood, but it has been proposed that phosphorylation-mediated removal of Yta7 from a given histone gene during S-phase could be an important step in allowing efficient transcriptional elongation along the histone gene. In agreement with this hypothesis, with a mutant of Yta7 that cannot be phosphorylated, recruitment of RNA polymerase II is markedly decreased within the ORF of the *HTA1* gene, but not at the promoter region (Kurat et al. 2011) (Figure 17A).

In addition to the role of Yta7 in regulating transcription of histone genes, a transcriptome analysis of *yta7Δ* cells has revealed that the expression of inducible genes is also deregulated in the absence of Yta7 (Lombardi, Ellahi, and Rine 2011). Yta7 localizes to the promoters and 5'ORF of early meiotic genes and galactose genes when they are induced. Moreover, the presence of Yta7 limits nucleosome density in these genic regions, possibly by promoting histone H3/H4 eviction. In accordance with this hypothesis, the deficiency in inducing gene expression in *yta7Δ* cells is rescued by a decrease in dosage of histones H3 and H4, caused by the deletion of a pair of histone genes (*hht1-hhf1Δ*). Interestingly, the AAA+ ATPase domain is required for this potential chaperone activity of Yta7, indicating that ATP

hydrolysis may be important for eviction or degradation of histones (Lombardi, Ellahi, and Rine 2011) (Figure 17B).

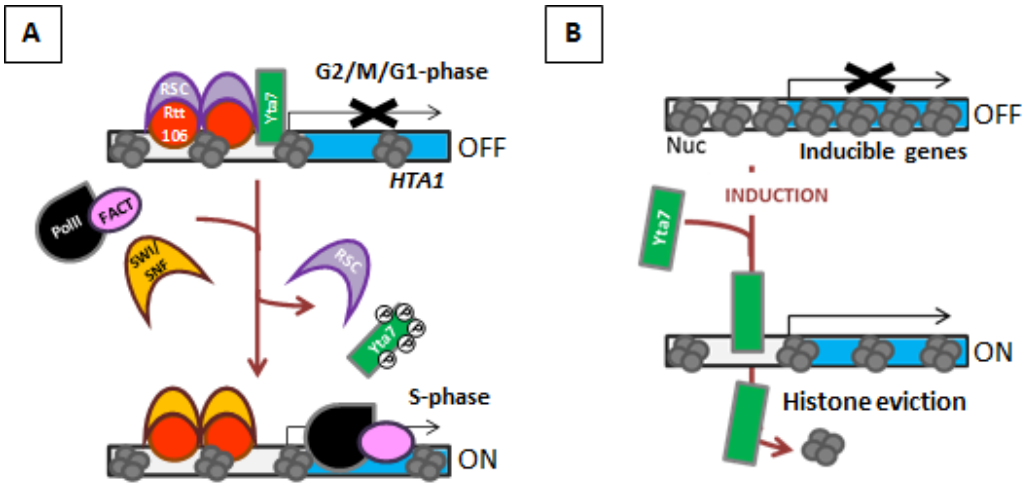


Figure 17. Yta7 is a probable histone chaperone regulating transcription of histone and inducible genes. A) Yta7 is directly involved in regulating S-phase specific transcription of *HTA1* gene. After binding of RNA polymerase II, phosphorylation and removal of Yta7 allows efficient transcriptional elongation along *HTA1*. S-phase specific removal of RSC and recruitment of SWI/SNF by Rtt106 to histone genes accompany Yta7 departure and *HTA1* transcriptional activation. **B)** Yta7 is required for transcription of inducible genes. Yta7 binds to the promoter region of these genes and is believed to promote the eviction or degradation of histones. Figures taken from Cattaneo et al. 2014.

In support of a possible role of Yta7 as a histone chaperone, a recent study showed that cells lacking Yta7 exhibited increased nucleosome density within genes downstream of the +1 nucleosome. In contrast, cells overexpressing Yta7 displayed reduced nucleosomes within genes. Importantly, Yta7-bound regions were enriched for this nucleosome shift, indicating that Yta7 acts locally to modulate nucleosome spacing (Lombardi, Davis, and Rine 2015). Yta7 was co-purified with several subunits of the RNA polymerase II (Rpb2, Rpb5 and Rpb10), as well as with several histone chaperones, the FACT complex and Rtt106 protein (Kurat et al. 2011; Tackett et al. 2005), and with the chromatin remodeler Chd1 (Lambert et al. 2010). These results further reinforce the connection between Yta7 transcriptional regulation and chromatin dynamics. Nonetheless, it should also be pointed out that so far, there is no *in vitro* evidence that Yta7 is a *bona fide* histone chaperone. Moreover, since *in vivo* experiments do not exclude the possibility that the change in histone density observed in *yta7Δ* cells is due to the activity of one of the histone chaperones interacting with Yta7, these findings await further experiments to conclude that Yta7 (and, by extension, other ATAD2-like proteins) are indeed histone chaperones.

3.2.2. Yta7 and chromatin boundary elements

The role of Yta7 in regulating the expression of histone genes has also been linked to boundary activity at their promoters (Figure 18A). Indeed, at these boundaries, Yta7 prevents the spreading of the histone chaperone Rtt106 from the promoters to their respective coding regions (Fillingham et al. 2009; Kurat et al. 2011; Zunder and Rine 2012). The recruitment of Rtt106 to histone gene promoters depends on histone chaperones Asf1 and the HIR complex (Fillingham et al. 2009). Rtt106, once bound to these regions, can both positively and negatively regulate the expression of histone genes during the cell cycle, depending on its interactors. Outside of S-phase, when histone genes are repressed, Rtt106 recruits the ATP-dependent remodeling complex RSC to mediate gene repression. In contrast, during histone gene activation in S-phase, Rtt106 recruits the SWI/SNF complex to activate their transcription (Ferreira, Flaherty, and Prochasson 2011) (Figure 18A). In *yta7Δ* cells, Rtt106 spreads within the coding sequence of histone genes together with the RSC complex (Fillingham et al. 2009) (Figure 18A). This spreading is associated with a clear decrease in *HTA1* transcripts, suggesting that Rtt106 and RSC may be responsible for the transcriptional repression of *HTA1* gene. However, the potential repressive action of Rtt106 requires further investigation; in fact, spreading of Rtt106 and RSC occurs at all histone genes, even though it is not always associated with a decrease in histone mRNA levels (Zunder and Rine 2012).

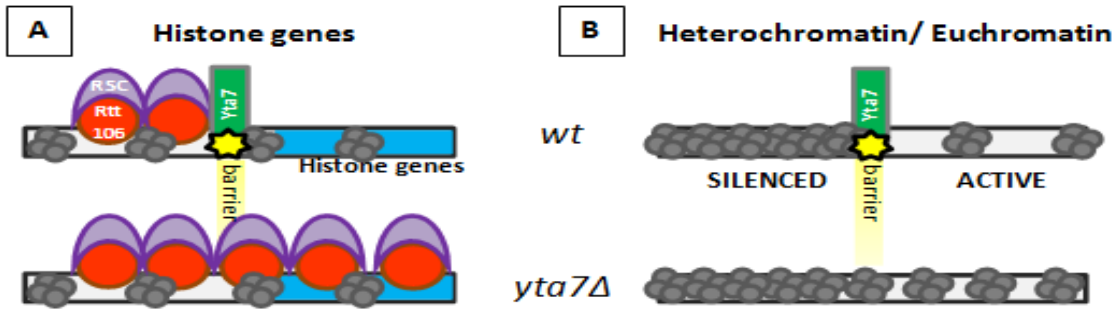


Figure 18. Yta7 acts as a boundary element. Yta7 is a boundary element acting both at the promoter of histone genes (left) and at the border between heterochromatin and euchromatin (right). Figure taken from Cattaneo et al. 2014.

Yta7 boundary function is not limited to histone promoters. Earlier evidence has suggested Yta7 activity at barriers that demarcate euchromatin from heterochromatin regions (Jambunathan et al. 2005; Tackett et al. 2005) (Figure 18B). *S. cerevisiae* contains heterochromatin-like regions located at the mating type, telomeres and rRNA-encoding DNA loci. The involvement of Yta7 in such barrier function was first recognized in a genetic screen aimed at identifying new genes that, when mutated, allow inappropriate spreading of silent chromatin from the mating type locus (*HMR*) over the adjacent tRNA gene into the neighboring genomic region (Jambunathan et al. 2005). The boundary activity of Yta7 has also been investigated by using silencing assays to test sensitivity to the drug 5 FluoroOrotic Acid (5FOA) in yeast cells in which the *URA3* reporter gene was inserted within either the silenced *HMR* or the transcriptionally competent adjacent region (Tackett et al. 2005). In *yta7Δ* cells, the silent transcriptional state spreads to neighboring regions. Furthermore, both the bromodomain and the AAA+ ATPase domains are required for Yta7 barrier function (Gradolatto et al. 2009; Lombardi, Ellahi, and Rine 2011). In agreement with the direct involvement of Yta7 in such barrier function, it has been found to co-purify with several proteins known to localize at the boundaries between euchromatin and heterochromatin-like regions in *S. cerevisiae* (Tackett et al. 2005). Nonetheless, the exact role of Yta7 at these chromatin barriers remains to be determined. For example, it would be interesting to know if the probable histone chaperone activity of Yta7 plays any important role at these barriers and if this function is conserved in other ATAD2-like proteins in eukaryotes.

3.3. ATAD2-like proteins conservation through eukaryotes

ATAD2 is conserved in many eukaryotes, although its function remains mostly obscure. Aside from the literature on mammal ATAD2 and budding yeast Yta7, almost nothing is known concerning the function of this protein family in other eukaryotes. One exception is the ATAD2 homologue in *Caenorhabditis elegans* LEX-1 (Lin-48 EXpression abnormal protein 1), which was found in a screen to control the expression of repeated transgenes, suggesting that repeated sequences may also be genomic targets for ATAD2 proteins (Tseng et al. 2007).

In this section, an overview of ATAD2-like proteins in eukaryotes will be presented; their strong sequence conservation indicates possible shared functions.

A phylogenetic tree of ATAD2-like proteins illustrates the strong conservation of this protein family within the eukaryotic kingdom (Figure 19). All ATAD2-like proteins share a bipartite AAA+ ATPase domain, which is found N-terminal to a bromodomain. This tree also shows that in several organisms the genome encodes two ATAD2 paralogues. These include Chordata and the fission yeast *Schizosaccharomyces pombe,* which possess two ATAD2-like proteins known as ATAD2 and ATAD2B, and Abo1 and 2, respectively. It is of note that the high sequence similarity between these paralogues argues in favor of a possible functional redundancy.

Other eukaryotes such as *Arabidopsis thaliana*, *Zea mays*, *Caenorhabditis elegans* and *S. cerevisiae* possess only one gene encoding an ATAD2-like protein. *Xenopus laevis* is quite peculiar, since it possesses a gene expressing a shorter version of an ATAD2-like protein. This protein contains only the first AAA+ ATPase domain and the N-terminal part of the second one. Intriguingly, in *Drosophila melanogaster* and *Tetrahymena thermophila* no gene encoding a homologue of ATAD2 has been identified. In these two organisms, the closest relative proteins belong to VCP/TERA (Valosin-Containing Protein/Transitional Endoplasmic Reticulum ATPase), which is also a conserved protein family with a domain organization

similar to that of ATAD2 family members, although the amino acid sequence is poorly conserved aside from the AAA+ ATPase domain. The existence of organisms that have lost part or the totality of ATAD2-like proteins might indicate that a concomitant functional adaptation has occurred to compensate for the lack of ATAD2 activity in these organisms. This observation also points to the existence of possible redundant functional pathways in various eukaryotic cells that express ATAD2 family members.

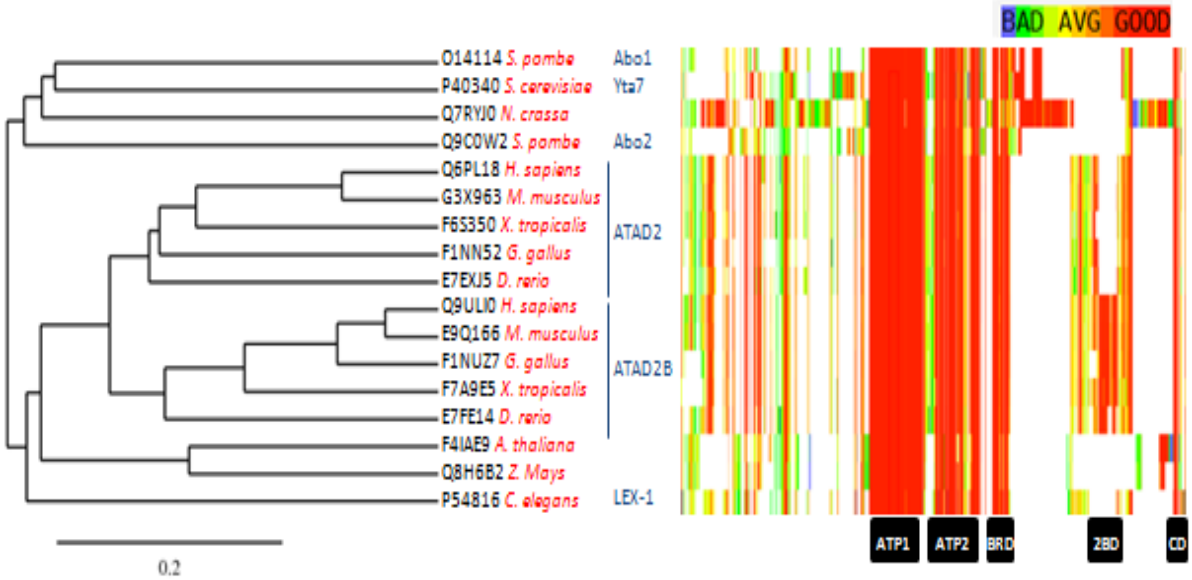


Figure 19. Members of the ATAD2 protein family are conserved between yeast and human. Left part of the panel is a graphical representation of a phylogenetic tree performed with TreeDyn (v198.3) (Dereeper et al. 2008; Dereeper et al. 2010) from the amino acid sequences of ATAD2-like proteins (UniProt 2014) aligned by CLUSTALO (McWilliam et al. 2013). Scale bar: 0.2 changes per site. Right part of the panel shows a multiple sequence alignment performed using PSI-coffee (Di Tommaso et al. 2011; Kemena and Notredame 2009). Figure taken from Cattaneo et al. 2014.

ATAD2-like proteins have their highest sequence similarity within the two AAA+ ATPase domains and the bromodomain. The AAA+ ATPase domain is found in all kingdoms of living organisms, in proteins possessing many different cellular functions. ATAD2-like proteins contain two AAA+ ATPase domains both located in their N-terminal part, although the first domain appears to be more conserved across evolution. ATP binding and ATPase activities have been demonstrated for human ATAD2 (Zou et al. 2007). Additionally, mutations in the

first AAA+ ATPase domain that affect ATP binding and hydrolysis impact the ability of mouse Atad2 and human ATAD2 to oligomerize, to bind to acetylated histone H4 (Caron et al. 2010) and to co-activate transcription (Zou et al. 2007), indicating that this domain is critical for ATAD2 functions. Since the amino acid sequence of the AAA+ ATPase domain is highly similar among ATAD2-like proteins, it is likely that the activity and function of this domain are also conserved in other eukaryotes. In accordance with this possibility, mutations in the first AAA+ ATPase domain of the *S. cerevisiae* Yta7 protein also affect many of its functions (Kurat et al. 2011; Lombardi, Ellahi, and Rine 2011).

ATAD2-like proteins also contain a putative bromodomain, a module known to bind acetylated lysine in histones and other proteins (Filippakopoulos and Knapp 2012). Both mouse Atad2 and human ATAD2 show preferential binding to acetylated histones H3 and H4 (Caron et al. 2010; Revenko et al. 2010). The overall conservation of the bromodomain amino acid sequence in ATAD2-like proteins suggests that its global architecture and its capacity to bind to histones may be conserved. Nonetheless, alignment of the Yta7 bromodomain with other yeast bromodomains has revealed that residues critical for binding acetylated histones are missing, indicating that a preferential binding to acetylated histones is probably not the case for all ATAD2-like proteins (Jambunathan et al. 2005). *In vitro* histone pull-down experiments have indeed shown that Yta7 binds histones, but in an acetylation-independent manner (Gradolatto et al. 2009; Jambunathan et al. 2005). Interestingly, similar pull-down experiments, using truncated forms of Yta7, revealed a second region that binds histones in the N-terminal part of Yta7, upstream of the AAA+ ATPase domains. This region contains a stretch of acidic residues that may be responsible for electrostatic interactions with charged and unmodified lysine and arginine residues in histones (Gradolatto et al. 2009). Remarkably, a patch of acidic residues in the N-terminal part of the protein seems to be a common feature of all ATAD2-like proteins, suggesting that the function of this negatively charged region in binding histones may be conserved.

Finally, members of the ATAD2 family also have in common a fourth region of around 60 amino-acids located at the extreme C-terminal part of the protein. This domain, which is highly conserved among ATAD2-like proteins, does not correspond to any annotated domain and is not found in any other type of protein. Interestingly, upstream of this C-terminal domain, there is another region conserved in ATAD2B proteins, but not in the ATAD2

paralogues, indicating that this domain may play an important role in attributing a specific function to ATAD2B proteins. The analysis of these two newly identified domains could provide some clues to the function of ATAD2-like proteins.

3.4. ATAD2 from human to yeast: functional orthologs?

The high degree of similarity between Yta7 and ATAD2, and more generally among all ATAD2-like proteins, in terms of their sequence and domain organization, supports the hypothesis that at least part of their function is shared.

There is clearly involvement of both proteins in transcriptional regulation. However, although data on Yta7 suggest a role for this factor in the organization of the genome and chromatin dynamics, such evidence is scarce for ATAD2. Indeed, only one experiment, which measured histone H2A mobility in a lung cancer cell line, has shown that a decrease in ATAD2 level modifies H2A turnover (Caron et al. 2010). Even in this case, there is no indication of the mechanism that links ATAD2 and histone mobility. Considering the importance of Yta7 in regulating nucleosome density, it is tempting to propose that ATAD2 can also act as a histone chaperone, evicting histones from chromatin to avoid potentially deleterious effects associated with an increase in histone density. Therefore, ATAD2 and Yta7 could primarily be chromatin-related proteins, and effects on gene expression regulation could be a mere consequence of their actions on chromatin organization. Alternatively, these proteins could be dual factors, playing a role both in transcriptional regulation as scaffolds or as co-activators, and in chromatin remodeling as histone chaperones.

A systemic upregulation of ATAD2 in the vast majority of cancer types, together with experiments where ATAD2 expression has been reduced in cancer cell lines, indicates a critical role for this protein in the regulation of cell proliferation and cell death. On the other

hand, there are no indication of such a role for Yta7, indicating that some functions and mechanisms of action could be different between mammals and *S. cerevisiae*.

The investigation of additional ATAD2-like proteins in other model organisms could therefore be important in order to better understand the mechanism of action of the proto-oncogene ATAD2 and its role as a chromatin-related regulator of transcription and cell growth. In this regard, characterizing the function of ATAD2 proteins using the fission yeast *S. pombe*, another yeast model distantly related to budding yeast and often used for studying chromatin biology, could also be informative. This yeast may be a better model of investigation for comparative studies with mammals; it possesses the RNAi pathway and the H3K9me heterochromatin mark, which are both present in mammals but absent in *S. cerevisiae*.

MATERIAL AND METHODS

1. Biological materials and culture conditions

1.1. *Schizosaccharomyces pombe* strains

All fission yeast cells used in this study are listed in the following table. These strains are obtained either by transformation by lithium acetate or by cross and random spore analysis.

Strain	Genotype	Origin
SPV 8	*h+ leu1-32 ori1 ade6-M216 ura4-D18 imr::ura4+*	This study
SPV 1733	*h+ leu1-32 ori1 ade6-M216 ura4-D18 imr::ura4+ Δclr4::NAT*	This study
SPV 874	*h+ leu1-32 ori1 ade6-M216 ura4-D18 imr::ura4+ Δchp1::HPH*	This study
SPV 2893	*h+ leu1-32 ura4 mut ade6-M210 cdc25-22 ts*	P. Bernard
SPV 13	*h+ leu1-32 ori1 ade6-M216 ura4-D18 imr::ura4+ chp1::TAP-KAN*	This study
SPV 1889	*h+ leu1-32 ori1 ade6-M216 ura4-D18 imr::ura4+ chp1::TAP-KAN Δago1::HPH*	This study
SPV 894	*h+ leu1-32 ori1 ade6-M216 ura4-D18 imr::ura4+ chp1::TAP-KAN Δtas3::HPH*	This study
SPV 2086	*h+ leu1-32 ori1 ade6-M216 ura4-D18 imr::ura4+ not1::HA-NAT*	This study
SPV 3824	*h+ leu1-32 ori1 ade6-M216 ura4-D18 imr::ura4+ Δnot2::KAN not1::3HA-NAT*	This study
SPV 3822	*h+ leu1-32 ori1 ade6-M216 ura4-D18 imr::ura4+ Δnot3::KAN not1::3HA-NAT*	This study
SPV 3830	*h+ leu1-32 ori1 ade6-M216 ura4-D18 imr::ura4+ Δnot4::KAN not1::3HA-NAT*	This study
SPV 3827	*h+ leu1-32 ori1 ade6-M216 ura4-D18 imr::ura4+ Δcaf1::HPh not1::3HA-NAT*	This study
SPV 4278/4279	*h+ leu1-32 ori1 ade6-M216 ura4-D18 imr::ura4+ not1::3HA-NAT Δrcd1::KAN*	This study
SPV 2169	*h+ leu1-32 ori1 ade6-M216 ura4-D18 imr::ura4+ Δrcd1::KAN*	This study
SPV 3180	*h+ leu1-32 ori1 ade6-M216 ura4-D18 imr::ura4+ Δcaf1::HPH*	This study
SPV 3688	*h+ leu1-32 ori1 ade6-M216 ura4-D18 imr::ura4+ Δnot4::KAN*	This study
SPV 2774	*h+ leu1-32 ori1 ade6-M216 ura4-D18 imr::ura4+ Δnot3::KAN*	This study
SPV 1785/1786/1787	*h+ leu1-32 ori1 ade6-M216 ura4-D18 imr::ura4+ ::GFP-HPH after DSR mei4*	This study
SPV 1817/1818	*h+ leu1-32 ori1 ade6-M216 ura4-D18 imr::ura4+ ::GFP-HPH after DSR mei4 Δmmi1::NAT*	This study
SPV 3690/3691	*h+ leu1-32 ori1 ade6-M216 ura4-D18 imr::ura4+ ::GFP-HPH after DSR mei4 Δnot4::KAN*	This study
SPV 4135/4136	*h+ leu1-32 ori1 ade6-M216 chp1::TAP-KAN Δ(828-1554)mei4::GFP-HPH Δcaf1::NAT*	This study
SPV 3733	*h- leu1-32 ade6-M210 ura4-D18*	F. Winston
SPV 3734	*h- leu1-32 ade6-M210 ura4-D18 spt6-1::NAT*	F. Winston
SPV 3735	*h- leu1-32 ade6-M210 ura4-D18 spt6::3HA-TAP-KAN*	F. Winston
SPV 3736	*h- leu1-32 ade6-M210 ura4-D18 spt6::3HA-TAP-KAN Δclr4::KAN*	F. Winston
SPV 3774/3775/3776	*h+ leu1-32 ori1 ade6 mut ura4-D18 imr::ura4+ Δchp1::HPH spt6::3HA-TAP-KAN*	This study

Strain	Genotype	Origin
SPV 3737	*h+ leu1-32 ori1 ade6-M216 ura4-D18 imr::ura4+ abo1::TAP-KAN*	This study
SPV 3937/3938	*h+ ade6 mut ura4-D18 leu1-32 Δabo1::NAT*	This study
SPV 3772/3773	*h+ leu1-32 ori1 ade6-M216 ura4-D18 imr::ura4+ Δabo1::NAT*	This study
SPV 3789/3790/3791	*h+ leu1-32 ori1 ade6-M216 ura4-D18 imr::ura4+ Δabo1::NAT*	This study
SPV 4664/4665	*h+ leu1-32 ori1 ade6-M216 ura4-D18 imr::ura4+ Δabo2::NAT*	This study
SPV 3940	*h+ ade6-Mut ura4-D18 leu1-32 Δabo2::KAN Δabo1::NAT*	This study
SPV 3941	*h90 leu1-32 ade6-M216 ura4::fbp1-lacZ Δabo1::NAT Δabo2::KAN*	This study
SPV 3942	*h+ leu1-32 ori1 ade6-M216 ura4-D18 imr::ura4+ Δabo1::NAT Δabo2::KAN*	This study
SPV 3740	*h+ leu1-32 ori1 ade6-M216 ura4-D18 imr::ura4+ abo1::13myc-HPH*	This study
SPV 4758	*h- ade6-M216 ura4-D18 leu1 FLAG-tfg3*	A. Ishihama
SPV 4789	*h- ade6-M216 ura4-D18 leu1 FLAG-tfg3 abo1::13myc-HPH*	This study
SPV 705	*h90 leu1-32::nmt1-spt16-YFP-flag-his-leu1+*	This study
SPV 4509	*h90 leu1-32::nmt1-spt16-YFP-flag-his-leu1+ abo1::13myc-HPH*	This study
SPV 4350	*h+ leu1-32 ura4-D18 sap1::3FLAG-KAN*	E. Noguchi
SPV 4607	*h+ leu1-32 ori1 ade6-M216 ura4-D18 imr::ura4+ abo1::3myc-HPH sap1::3Flag-KAN*	This study
SPV 730	*h90 leu1-32::nmt1-abp1-YFP-flag-his-leu1+*	This study
SPV 4642/ 4643	*h90 leu1-32::nmt1-abp1-YFP-flag-his-leu1+ abo1::13myc-HPH*	This study
SPV 4351	*h+ leu1-32 ura4-D18 sap1-1ts::3FLAG-KAN*	E. Noguchi
SPV 4352	*h+ leu1-32 ura4-D18 sap1-48ts::3FLAG-KAN*	E. Noguchi
SPV 4566/4567/4568	*h+ leu1-32 ura4-D18 sap1-1ts::3FLAG-KAN Δabo1::NAT*	This study
SPV 4569/4570	*h+ leu1-32 ura4-D18 sap1-48ts::3FLAG-KAN Δabo1::NAT*	This study
SPV 4451/4452	*h- ura4-D18 ade6::ade6+-Padh15-skp1-OsTIR1-NAT-Padh15-skp1-AtTIR1-2NLS*	D. Moazed
SPV 4530/4531/ 4532	*h- ura4-D18 ade6::ade6+-Padh15-skp1-OsTIR1-NAT-Padh15-skp1-AtTIR1-2NLS abo1::2HA-AID-KAN*	This study
SPV 4794/ 4795/4796	*h- ura4-D18 ade6::ade6+-Padh15-skp1-OsTIR1-NAT-Padh15-skp1-AtTIR1-2NLS Δtfg3*	This study
SPV 4797/4798/ 4799	*h- ura4-D18 ade6::ade6+-Padh15-skp1-OsTIR1-NAT-Padh15-skp1-AtTIR1-2NLS abo1::2HA-AID-KAN Δtfg3*	This study
SPV 4814/4815/ 4816	*h- ura4-D18 ade6::ade6+-Padh15-skp1-OsTIR1-NAT-Padh15-skp1-AtTIR1-2NLS Δhht1-hhf1:::HPH*	This study
SPV 4817/4818/ 4819	*h- ura4-D18 ade6::ade6+-Padh15-skp1-OsTIR1-NAT-Padh15-skp1-AtTIR1-2NLS abo1::2HA-AID-KAN Δhht1-hhf1:::HPH*	This study
SPV 4820/4821/ 4822	*h- ura4-D18 ade6::ade6+-Padh15-skp1-OsTIR1-NAT-Padh15-skp1-AtTIR1-2NLS Δhht2-hhf2::HPH*	This study
SPV 4823/4824	*h- ura4-D18 ade6::ade6+-Padh15-skp1-OsTIR1-NAT-Padh15-skp1-AtTIR1-2NLS abo1::2HA-AID-KAN Δhht2-hhf2::HPH*	This study
SPV 1600	*h90 leu1-32 ade6-M216 ura4::fbp1-lacZ*	This study
SPV 3904	*h90 leu1-32 ade6-M216 ura4::fbp1-lacZ Δabo1::NAT*	This study
SPV 4580/4581	*h90 leu1-32 ade6-M216 Δabo1::NAT*	This study
SPV 4514	*h90 leu1-32 ura4D18 Δhis3 Δabp1::KAN*	F. Azorin

Table 2. List of strains used in this study. Name of each strain (SPV: *Schizosaccharomyces pombe* Verdel), genotype and origin (this study or the laboratory of origin) are indicated.

1.2. Description of plasmids

Main plasmids used in this study are listed in the next table. Plasmids are used for the following experiments: pFA6 plasmids were used for gene deletion or tag insertion; pJR plasmids for complementation experiment of *abo1Δ* cells with Abo1/ ATAD2; and pGEX plasmids for pulldown experiments.

Plasmid	Description	Origin
pKG1810	FA6a kanMX6 CTAP2	L. Gould
PAN20	pFA6a natMX6 pGAL1 3HA	Euroscarf (P30422)
PAN27	pFA6a natMX6	Euroscarf (P30437)
PAN28	pFA6a hph	Euroscarf (P30438)
	pFA6a kanMX6	I. Hagan
PAN43	pFA6a 13myc hph	J. Bahler (PAV9)
PAN58	pJR1-3XL	J.C. Ribas
PAN61	pJR1-41XL	J.C. Ribas
PAN65	pJR1-81XL	J.C. Ribas
PAN105	pan58 HA hATAD2	This study
PAN106	pan61 HA hATAD2	This study
PAN107	pan65 HA hATAD2	This study
PAN110	pan58 HA abo1FL	This study
PAN103	pGEX 4T1 3HA natMX6	S. Khochbin
PAN104	pcDNA3.1 HA hATAD2	S. Khochbin
PAN108	pGEX 4T1 hATAD2 BD	This study
PAN113	pGEX 4T1 abo1 BD	This study
	pGEX-6P-1 GST	D. Moazed
	pGEX-6P-1 GST-Ago1	D. Moazed

Table 3. List of main plasmids used in this study. Name of each plasmid, description and origin (this study or the laboratory of origin) are indicated.

1.3. Fission yeast culture conditions

Fission yeast cells were normally cultivated at 30°C in rich medium YEA (Yeast Extract supplemented with Adenine). Liquid cultures were grown in an incubator rotating at 220rpm. The medium was autoclaved 20min at 120°C.

YEA medium contains 5g/l yeast extract (BD), 30g/l dextrose (BD) and 0.225g/l adenine (Sigma). In addition, solid medium contains 20g/l agar (Bacto Agar, BD). Selective medium contains 100-200µg/ml of antibiotics (Hygromycin B, Roche; Geneticin G418, Roche; Nourseothricin CloNat, Werner Bioagents).

For specific experiments, fission yeast cells were grown in minimal medium. EMMc medium was used to grow cells for co-immunoprecipitation experiments, or to investigate the growth of Abo1 knock-down cells. EMMc-Leu medium was used for complementation experiments of *abo1Δ* cells growth with Abo1/ATAD2. SPAS medium was used to induce sporulation. For solid minimal medium, 20g/l agar was autoclaved in ½ total volume of H_2O and then mixed with ½ total volume of 2X filtered medium (filter 0.22 µm ø).

EMMc (Edinburgh Minimum Medium complete): 12.4g/l EMM without dextrose (MP Biomedicals), 20g/l dextrose, 0.225g/l leucine, 0.225g/l adenine, 0.225g/l uracil (Sigma).

SPAS medium: 10g/l dextrose, 1g/l monopotassium phosphate (Sigma), vitamins (4.2µM pantothenic acid, 81.2µM nicotinic acid, 55µM myo-inositol, 40.8nM biotin), 45mg/l leucine, 45mg/l adenine, 45mg/l uracil, 45mg/l histidine, 45mg/l lysine (Sigma).

1.4. Description of primers

Main primers used in this study (for qPCR and analysis of mating type identity) are listed in the next table. Primers used for quantitative PCR are designed using Primer3.

Primer	Sequence 5'-3'	Description
PV_174	*TACCCCATTGAGCACGGTAT*	*act1* forward
PV_175	*CTTCTCACGGTTGGATTTGG*	*act1* reverse
PV_176	*GTACTGGCCCATACCGTGAT*	*tub1* forward
PV_177	*CGAATGGAAGACGAGAAAGC*	*tub1* reverse
PV_180	*AAAAGCGACCTTCAAGCAAA*	*mei4* forward
PV_181	*TTGCATCGTTTGAGACTTCG*	*mei4* reverse
PV_182	*TGCAAGAGGAAACTCAAAGG*	*ssm4* forward
PV_183	*TTCCTCCTCCACTTGTTTTGA*	*ssm4* reverse
PV_209	*TACCTTTGGGACGTGGTCTC*	*ura4* forward
PV_210	*CCCGTCTCCTTTAACATCCA*	*ura4* reverse
PV_238	*ATGGTCGTCGCTTCAGAAATTGC*	*tlh1* forward
PV_239	*CTCCTTGGAAGAATTGCAAGCCTC*	*tlh1* reverse
PV_240	*CTGCCAAGACCTCAACCAAT*	*mat3* forward
PV_241	*TCTCCACATCTCTCCAACCA*	*mat3* reverse
PV_256	*GGATACCGAGACGCAGGATA*	*cendh* forward
PV_257	*TGGCTTGTTGTACGTTGTTCA*	*cendh* reverse
PV_268	*CCACCAGACCATTACAAGCA*	*cendg* forward
PV_269	*CTCGCCTATTTACCGATCCA*	*cendg* reverse
PV_308	*GCTTCAAGGACGACAACACA*	*cenH* forward
PV_309	*GCGTCACACTTTGGAGCATA*	*cenH* reverse
PV_792	*TTTGCTCGTATGCAAGATCG*	*spo5* forward
PV_793	*TATTCACGAGCACGCCATAA*	*spo5 reverse*
PV_794	*GTTGAAGTTGGACGGGATGT*	*rec8* forward
PV_795	*TTCTACCCTACTCGGCATCG*	*rec8* reverse
PV_1080	*ACCATGTATGATACGATATGGAGA*	*5'LTR* forward
PV_1081	*GAGGAACGAGGTTCAGCAGT*	*5'LTR reverse*
PV_1084	*AAACTTGCTTTTCGCTGTCC*	*TE rt* forward
PV_1085	*CTACATGCGATTCTTTGACTTCAC*	*TE rt* reverse
PV_1117	*AGAAGAGAGAGTAGTTGAAG*	*MT1* mating type
PV_1118	*ACGGTAGTCATCGGTCTTCC*	*MP* mating type
PV_1119	*TACGTTCAGTAGACGTAGTG*	*MM* mating type
PV_1563	*AACTCGAAGCATTTTCGTCA*	*tel2R* forward
PV_1564	*AACCCAAAATTCAGCGTCAT*	*tel2R* reverse
PV_1577	*TTTTTGGACTGTCGCTTTCA*	*tRNA* forward
PV_1578	*ATTTCGCTAGGCAAGAACGA*	*tRNA* reverse
PV_1958	*AGGCTGTATTCCCAACATCC*	*gf2* forward
PV_1959	ATGCCTTCTTCTTCTTCTCAG	*gf2* reverse
Khochbin's lab	*TCCAACTGGAGAATTTGTATGC*	*ATAD2* forward
Khochbin's lab	*ACTGCTTGCTCCATTTTCTGA*	*ATAD2* reverse

Table 4. List of main primers used in this study. Name of each primer, sequence and description are indicated.

2. Strains construction

2.1. Transformation with lithium acetate

Transformations of *S. pombe* cells are performed using a method described by Bahler et al. in 1998. A DNA fragment is introduced in fission yeast by thermal shock in presence of lithium acetate, and then homologous recombination permits integration of this fragment into the genome. Positive clones are selected on selective medium and, in addition, presence of the new DNA fragment is verified by PCR.

2.1.1. DNA preparation

A plasmid can be directly transformed into fission yeast cells. The plasmid, which also contains a gene of selection, needs to be amplified by transformation in competent bacteria (TOP10 Chemically Competent E. coli, ThermoFisher). 25µl of bacterial competent cells were mixed with plasmid DNA (10-20ng) and incubated 20min on ice. After heat-shock at 42°C for 45sec, bacteria were then plated on LB medium (25g/l LB broth base, Life Technologies) containing resistance. Transformed bacterial colonies were then used for plasmid amplification. Plasmid extraction and purification was performed using NucleoSpin Plasmid Kit (Macherey–Nagel), following manufacturer's instructions.

Otherwise, DNA fragments used for transformation can be amplified from a plasmid by PCR, using a couple of specific primers of around 100bp (base pairs). These primers contain ~20bp homologous to the plasmid sequence (required for DNA amplification), and additional ~80bp homologous to the genomic insertion site (required for the homologous recombination process). For each DNA amplification, 8 PCR reactions of 50µl were performed using a high fidelity enzyme (Phusion high-fidelity DNA polymerase, NEB) in order to limit mutation rate. PCR reactions were conducted following manufacturer's instructions. Amplicons were verified using UV light after electrophoresis on an agarose gel containing ethidium bromide (0.2µg/ml, Sigma). PCR products were then purified using NucleoSpin Gel and PCR Clean-up Kit (Macherey–Nagel) following manufacturer's instructions. DNA concentration was then measured by NanoDrop spectrophotometer.

2.1.2. Transformation

S. pombe 100mL culture OD600 0.2-0.5 were harvested at 800g for 5 minutes at room temperature. Cells were first washed in 20ml sterile dH_2O, and then in 1ml lithium acetate buffer (TE1X buffer pH7.5, lithium acetate 0.1M pH7.0). Cells were then resuspended in 500µl lithium acetate. Salmon sperm DNA (~20µg, Sigma), previously fragmented by sonication and denaturated 10min at 100°C, functions as a DNA carrier and it was mixed with transformant DNA (~1-2µg of PCR-amplified DNA or ~100-200ng of plasmid DNA) and 100µl fission yeast cells. After an incubation of 10min at room temperature, 260µl of PEG buffer (TE1X buffer pH7.5, lithium acetate 0.1M pH7.0 and PEG 4000 40%) was added to samples, which were then incubated 45min at 30°C. After addition of 43 µl DMSO, cells were exposed to thermal shock 5min at 42°C. Cells were then centrifuged 2min at 1800g, washed in 1ml sterile dH_2O and resuspended in 500µl dH_2O. 250µl cells were plated on YEA medium using glass beads (0.4mm ø, Dutscher), incubated at 30°C for 1-2 days and then replicated on selective medium. For transformation with plasmid, 250µl cells were directly plated on selective medium and incubated at 30°C.

2.1.3. Selection of positive clones

After 2-3 days of growth at 30°C, colonies that appeared on selective plates were streaked in order to obtain isolated clones and DNA fragment insertion was verified by PCR using BioMix Red (Bioline; a reaction mix containing a stable Taq DNA polymerase) and electrophoresis analysis on agarose gel. For each transformation, 3 independent verified isolates were stocked at -80°C in 25% glycerol stock.

2.2. Crosses and random spore analysis

Cells to cross (*h+* and *h-*, or *h90*) were cultivated in YEA liquid medium until OD600 ~0.5. For each strain to cross, 10^6 cells were mixed together and washed 3 times in 1ml sterile dH_2O. Cells were then resuspended in 20µl sterile dH_2O, plated by 3µl drops on SPAS solid medium and incubated 2 days at 30°C to induce sporulation. Spore selection was made by random spore analysis: a tiny amount of cells/spores was inoculated in 200µl sterile dH_2O and mixed with 1.5µl of Cytohelicase (20mg/ml, Sigma). After ~5h of incubation at 32°C, 800µl of SDS 1% were added to each sample, which was then centrifuged 2min at 1800g and washed

twice in 800µl sterile dH_2O. Total number of spores was estimated using a Malassez slide and ~200-500 spores were plated on YEA medium containing resistance. After 2-3 days of growth at 30°C, colonies appearing on selective plates were streaked to have isolated clones that were then tested by PCR analysis. For each cross, 3 independent verified isolates were stocked at -80°C in 25% glycerol stock**.**

3. Analysis of cells morphology and mortality

Cells were cultivated in liquid YEA medium at 30°C until OD600 ~0.5. After 2 washes in 1ml sterile dH_2O, ~10^4 cells were resuspended in 20µl glycerol 25%. Slides were prepared using 1µl of each sample. Abnormal cells were counted compared to *wild type* strain using a Malassez slide (n=1000 for each isolate, experiment done in triplicate). Cell Mortality was tested by two complementary methods: trypan blue staning and plating assay.

Trypan blue staining

Cells were cultivated in liquid YEA medium at 30°C until OD600 ~0.5. After 2 washes in 1ml sterile dH_2O, ~10^4 cells were resuspended in 10µl glycerol 50%. 10µl cells were mixed with 10µl Trypan Blue (0.4%, Sigma). Trypan blue enters and stains exclusively dead cells. Dead cells were counted compared to *wild type* strain using a Malassez slide (n=1000 for each isolate, experiment done in triplicate).

Plating assay

Cells were cultivated in liquid YEA medium at 30°C until OD600 ~0.5, then counted using a Malassez slide. Serial dilutions were made in order to plate exactly 100 cells for each YEA plate. The experiment was done in triplicate and in addition, each time, 3 technical replicates were done for each dilution and plating process. Cells were spread using glass beads and then, after 6 days at 30°C, the number of isolated colonies was counted for each plate.

4. Growth assay

4.1. Growth assay on liquid medium

Cells were cultivated at 30°C in liquid YEA medium for ~6-8h, diluted at the same concentration and grown for ~14h to reach an OD600nm below 0.8-1. Then, a new dilution was done (normally OD600 0.05-0.1) and this corresponds to the first value of the kinetics. Cells concentration was then followed-up in time at least for 24h.

4.2. Growth assay on solid medium

Cells were cultivated at 30°C in liquid YEA medium (without reaching saturation). For each strain, 10^7 cells were washed in 500µl sterile dH_2O and then resuspended in 275µl adapt medium. A 96-wells plate was used to obtain 6 serial 10X dilutions for each strain (from ~10^7 to 10^2 cells) that were plated on solid medium and incubated 2-3 days. Depending on the experiment, growth of each mutant was compared to the one of a *wild type* control strain. Otherwise, growth at 37°C was compared to the one at standard condition (30°C). This assay was also used to test cell sensitivity to several drugs (see next section). In this case, cells growth profile in presence of the drug was compared to the one in control medium.

4.3. Drug sensitivity and centromeric silencing assay

4.3.1. Thiabendazole test

Thiabendazole (TBZ) is a drug that affects microtubule polymerization and that severely perturbs centromere integrity. Cells with defects at centromeric heterochromatin are known to be highly sensitive to TBZ. Therefore, this test is commonly used to suggest a defect in

centromeric heterochromatin for a specific strain. In this study, cell sensitivity to TBZ was shown for two concentration of this drug: 15 and 20 µg/ml (Sigma).

4.3.2. Silencing assay (FOA)

Silencing assay (5FOA) was used in this study to evaluate centromeric heterochromatin silencing. Strains *ura4::imr1R* were generated, in which *ura4* gene was deleted and an exogenous copy was inserted into centromeric heterochromatin (region *imr1R* of centromere 1). These cells were plated on solid medium containing 5-fluoroorotic acid (5-FOA, 1g/l, TRC). This compound, if Ura4 is expressed, is converted into 5-fluorouracil which is toxic for cells. Therefore, strains with defects in centromeric heterochromatin express Ura4 and present a high sensitivity to 5FOA, visible as a reduced growth compared to control.

4.3.3. Genotoxic agents: HU, CPT, MMS

Several assays were used to investigate the growth defect of specific strains in presence of compounds that affect genome stability.

- Hydroxyurea (HU) interferes with replication fork progression during S phase by reducing the pool of dNTPs. In this study, strains sensitivity to HU is shown at 6 and 9µM (Sigma).
- Methyl methanesulfonate (MMS) stalls replication forks, possibly by guanine alkylation. In this study, strains sensitivity to MMS is shown at 0.2‰ (Sigma).
- Camptothecin (CPT) inhibits DNA topoisomerase I, thus causing replication fork blockage. In this study, strains sensitivity to CPT is shown at 10 and 20µM (Sigma).

5. Analysis of the cell cycle (FACS)

Cells were cultivated in 25ml liquid YEA medium and harvested at OD600 0.1-0.2, washed once in dH_2O and fixed in 70% chilled ethanol at a density of 10^7 cells/ml. Fixed cells can be stored at 4°C indefinitely. For FACS analysis, $5x10^6$ cells were gently rehydrated in 50mM sodium citrate for 5 min and washed twice in 0.2ml of the same solution. RNAs were degraded 2h at 37°C adding 0.1 mg/mL RNAse (Roche) in 0.5 ml of 50mM sodium citrate and

DNA was subsequently stained by 2µg/ml of propidium iodide. Before FACS analysis, the suspensions were sonicated 30sec to dissociate cell aggregates.

Stained cells were analyzed by a LSRII model cytometer (Becton Dickinson, USA) with 488nm excitation, through forward (FSC) and side (SSC) light scattering (area) and fluorescence signal. DNA content was quantified from area (PI-A) and pulse-width (PI-W) of the propidium iodide signal collected through a 660/20 emission filter. Data analysis was done with FCSExpress v5 (DeNovo Software, USA). Characterization of cell cycle phases was performed following a method described for fission yeast by Knutsen et al 2011.

6. Analysis of protein interactions

6.1. Protein extraction

Proteins extracts were normally obtained from cells grown until saturation (OD600>8) at 30°C in 3ml liquid cultures. Otherwise, to perform a co-immunoprecipitation or an Ago1 pull-down experiment, cells were harvested at OD600 ~0.6 in 50 ml liquid cultures. Cells were washed in 1ml dH_2O and resuspended in 300µl lysis buffer (100mM HEPES pH 7.5, 20mM $MgCl_2$, gyclerol 10%, 10mM EGTA, 10mM EDTA, 0.4%NP40, 150mM NaCl, 1mM PMSF, 1mM Benzamidine, 1mM DTT, 10µg/ml LABP (protease inhibitor cocktail), 20mM β-Glycerophosphate and 11mM NaF). 1ml of glass beads (0.5mm ø, Retsch) were added to samples and then 2 cycles of 30sec agitation with a beadbeater (Biospec) were performed (samples incubation 2min on ice in between cycles). Samples were then centrifuged 5min at 12000g and supernatant was kept. Protein concentrations were measured by NanoDrop spectrophotometer to equilibrate all protein extracts.

6.2. Coimmunoprecipitation (CoIP)

A volume of 10µl of input was collected from protein samples after cell lysis to be used as loading control. The remaining part was used for the immunoprecipitation.

For Mmi1 IP, each sample was splitted into two parts: one incubated with 1µg specific rabbit antibody anti-Mmi1; and the other part was incubated with an unspecific irrelevant rabbit IgG, in order to control protein unspecific fixation to beads. After 2h incubation at 4°C, samples were incubated 1h at 4°C with 5µl prewashed dynabeads coupled with protein A (Fisher Scientific). After 3 washes in 500µl lysis buffer, proteins were eluted in 20µl laemmli buffer2X (10µl of laemmli 4X were added to input) and heated 10min at 65°C to get rid of magnetic beads.

For protein-Flag IP, samples were incubated 2h at 4°C with 20µl prewashed anti-Flag M2 agarose beads (Sigma). After 3 washes in 500µl lysis buffer, proteins were eluted in 20µl laemmli buffer2X (10µl of laemmli 4X were added to input).

6.3. Ago1 pull-down

6.3.1. GST/ GST-Ago1 production

BL21 strain (Life Technologies) was transformed with a plasmid containing GST/ GST-Ago1 (Buker et al. 2007) and grown at 37°C in LB liquid medium to stationary phase. Then, bacteria were diluted to OD600 ~0.2 and grown again to OD600 ~0.7. GST-Ago1 production was triggered by 0.1mM IPTG (Fluka) induction for ~20h at 20°C. GST production was triggered by 1mM IPTG induction for 3 hours at 37°C. Cells were then collected, washed in 10ml PBS and resuspended in 1/50 cold lysis buffer (100mM Hepes pH7.5, 500mM NaCl, 5mM ß-Mercaptoethanol, Triton 0.5%, 1mM PMSF, 1mM Benzamidine, 0.01mM LABP). Samples were sonicated for 8 cycles of 30sec (5sec ON/ 5sec OFF) (Sonic Dismembrator, FisherScientific). After centrifugation at 12000g for 30min at 4°C, supernatant was incubated for 1h30 with glutathione resin (Sigma). Resin was then washed 4 times in 10 volumes of

lysis buffer. Homogeneity of GST and GST-Argonaute purifications was assessed with both coomassie brilliant blue staining and Western blot against GST and Ago1.

6.3.2. Ago1 pull-down

For each sample, two protein extracts were mixed (600µl in total, corresponding to 100ml initial cell culture). Samples were treated 1h at 4°C with RNAse (5µl, Roche) and DNAse (2µl, Roche). A volume of 20µl of input was collected after RNAse/DNAse treatment to be used as loading control. The remaining part was used for the GST/GST-Ago1 pull-down and splitted into two parts: one incubated with 20µl GST resin, the other with GST-Ago1 resin. After 2h incubation at 4°C, samples were washed three times in 200µl lysis buffer and eluted in 20µl laemmli buffer2X (20µl of laemmli 4X were also added to input).

6.4. Histone peptide pulldown H4/H4ac

First, H4/H4ac biotinylated peptides (6nmol) were mixed with 40µl of Streptavidin Sepharose beads (adding 100ng/ml TSA for H4 tetra-acetylated peptides) and incubated 20min at 4°C. After two washes in 500µl PBS 1X, beads were washed once in 500µl wash buffer LSDB 1X (Glycerol 40%, $MgCl_2$ 6mM, Hepes 100mM, KCl_2 250mM, NP40 0.1%, DTT 1mM) and resuspended in 20µl of the same buffer. BL21 strain (Life Technologies) were transformed with 20ng of each plasmid expressing GST-Abo1BD (or GST-ATAD2BD WT, used as control). Protein production was triggered by 1mM IPTG (Fluka) induction for 1h30 at room temperature and it was controlled by coomassie brilliant blue staining. After cell lysis, histone pulldown was performed on the soluble fraction (10µl input was collected as loading control). Cells extracts were incubated with peptide-beads (H4, H4ac or only beads) in rotation 2h at 4°C. After two washes in 500µl wash buffer LSDB 1X and one wash in PBS 1X, beads were resuspended in 20µl laemmli buffer2X (10µl of laemmli 4X were also added to input). The binding to histone H4/H4ac of different bromodomains was tested by Western blot anti GST.

6.5. Western blot

All protein samples were denaturated 10min at 65°C, then loaded on an acrylamide gel and separated by electrophoresis ~1h at 160V in a TGS 1X migration buffer (TGS10X: 30.25g/l Tris-HCl, 144g/l glycine, 10g/l SDS). Proteins were then transferred to a nitrocellulose membrane (Hybond-C Extra, Amersham) for ~1h at 100V in TGS 0.8X buffer containing 20% ethanol. Membranes, after saturation in TBS 1X (20 mM Tris-HCl pH 7.6, 150mM NaCl) 0.1% Tween20 (Sigma) 10% milk for 2h at room temperature (or ~12-16h at 4°C), were incubated with a primary antibody dilution normally in TBS 1X 0.1% Tween20 1% milk (see table for antibodies and dilutions used in this study). Membranes were then washed 3 times for 5-10min in TBS-Tween20 0.1%, and then incubated for 45min with a secondary antibody anti-mouse or anti-rabbit Immunoglobulins/HRP (DACO) 1/5000 dilution in TBS 1X 0.1% Tween20 1% milk. After 3 washes of 5-10min in TBS-Tween20 0.1%, protein signal was revealed by HRP enzymatic activity using an ECL kit and autoradiography films (Amersham).

Antibody	Origin	Dilution used for WB
α-HA	Abcam Ab9110	1/1000 in TBS 1X 0.1% Tween20 1% milk
α-Mmi1	Eurogentec 556	1/5000 in PBS 1X 0.2% Tween20 10% FBS
α-GFP	Roche 11814460001	1/1000 in TBS 1X 0.1% Tween20 1% milk
α-Tubulin	Sigma T5168	1/5000 in TBS 1X 0.1% Tween20 1% milk
α-TAP	Thermo Scientific 10506450	1/5000 in TBS 1X 0.1% Tween20 1% milk
α-Chp1	Abcam Ab18191	1/500 in TBS 1X 0.1% Tween20 1% milk
α-ATAD2	Sigma HPA029424	1/1000 in TBS 1X 0.1% Tween20 1% milk
α-Myc	Eurogentec 9E10	1/1000 in TBS 1X 0.1% Tween20 1% milk
α-Flag	Sigma F3165	1/2000 in TBS 1X 0.1% Tween20 1% milk
α-GST	Santa Cruz SC-138	1/2000 in TBS 1X 0.1% Tween20 1% milk

Table 5. List of main primary antibodies used for Western blot (WB) in this study. Name of each antibody, origin and dilution used in this study are indicated.

6.6. Protein complex purification (TAP purification)

C-terminal TAP-tagged Abo1 expressed at the native locus was purified by Tandem Affinity Purification (TAP) (Rigaut et al. 1999). Cells were grown in fermenter (Labofors-3, Infors) and collected during G1-S phase. Abo1-TAP was purified following an optimized version of the original protocol. Indeed, samples were gently sonicated and treated with benzonase, in order to enrich TAP purification for chromatin-linked proteins. In parallel, TAP purification was also performed on an untagged *wild type* strain, which was used as control to evaluate the enrichment of copurified proteins (Figure 20).

Cell lysis was performed in 110ml lysis buffer (50mM HEPES-NaOH, 5mM $MgCl_2$, 20mM β-Glycerophosphate, 10% glycerol, 1mM EDTA, 1mM EGTA, 50mM NaF, 0.1mM Na_3VO_4, 0.2% NP-40, 150mM NaCl, 1µg/ml LABP, 1mM Benzamidine, PMSF 1mM) using glass beads (0.5mm ø, Retsch). Samples were then sonicated at 4°C 3 cycles of 1min (alternating 5sec ON/ 5sec OFF) (Sonic Dismembrator, FisherScientific) and treated 1h at 4°C with benzonase (250U, Millipore). Aliquots were collected at each step to verify DNA fragmentation by agarose gel (expected size of DNA fragments around 200bp).

Samples were then incubated 2h at 4°C with 500µl prewashed IgG Sepharose beads (6 Fast Flow, GE Healthcare), and then transferred to a 5ml column. Each column was washed 3 times with 1ml lyisis buffer and equilibrated with 1ml TEV-C buffer (20mM Tris-HCl pH8, 150mM NaCl, 0.1% NP-40, 1mM MgCl2, 0.5mM EDTA, 1mM DTT, 5% Glycerol). Columns were then incubated 1h at room temperature in 1ml TEV-C buffer containing 40µl TEV-his protease (see at the end of this section for TEV-his production). This cleavage step is necessary to elute Abo1-TAP protein complex which otherwise would remain bound to IgG beads via its protein A. Eluted material was then transferred in a 10ml column containing 6ml of CAM-B buffer (20mM Tris-HCl pH8, 150mM NaCl, 1mM $MgCl_2$, 1mM Imidazole, 3mM $CaCl_2$, 10mM ß-mercaptoethanol, 5% Glycerol). After 3 washes in 1ml TEV-C 300 (TEV-C buffer with 300mM NaCl), column were incubated at 4°C for ~14h with 400µl Calmodulin sepharose beads (GE Healthcare), which were prewashed and resupsended in CAM-B buffer.

After 3 washes in 1ml CAM-B NP40 0.05% buffer, 4 serial elution steps were performed using CAM-E buffers (10mM Tris-HCl pH8.0, 0.02% NP-40, 1mM $MgCl_2$, 1mM Imidazole, 10mM ß-

mercaptoethanol, 5% Glycerol) with increasing NaCl and EGTA concentrations. First, E1 elution was performed in 200µl CAM-B 150-4 (150 mM NaCl, 4mM EGTA). Then, E2-3 in 700µl of the same buffer, E4-5 in 700µl CAM-B 150-20 (150 mM NaCl, 20mM EGTA) and finally E6 in 350µl CAM-B 500-20 (500 mM NaCl, 20mM EGTA).

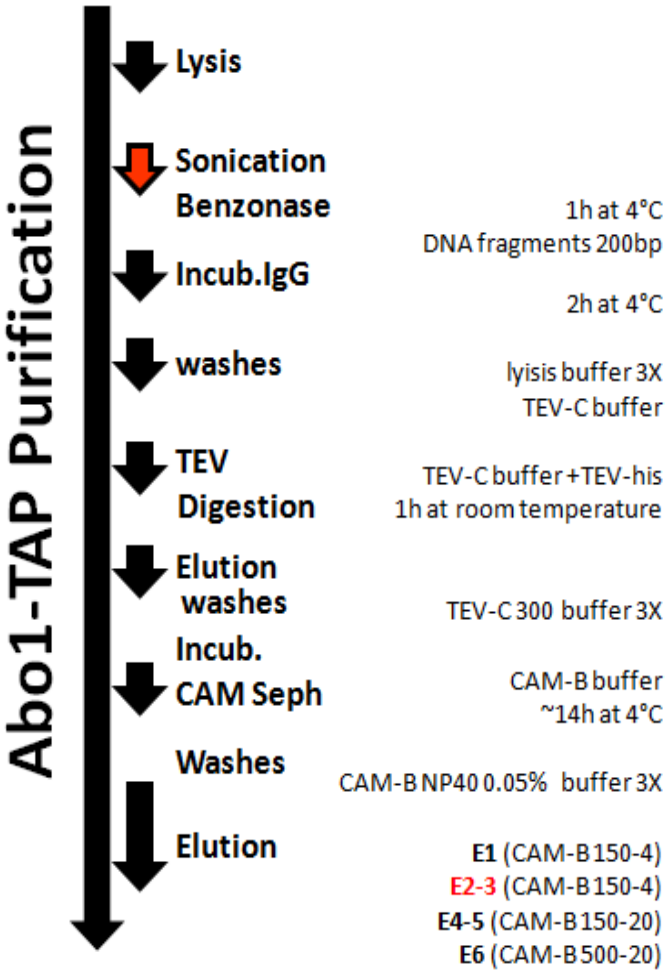


Figure 20. Abo1-TAP purification protocol. Schematic representation of the protocol used for Abo1-TAP purification. Indicated in red the step of sonication/ benzonase treatment to enrich the purification for chromatin-linked proteins and eluted fraction E2-3 (used for MS analysis).

Aliquots were collected at each step of the purification and tested by Western blot using an antibody anti-TAP (Thermo Scientific). Final eluted materials were separated by electrophoresis on 8% acrylamide gel that was silver-stained using the SilverQuest Silver Staining Kit (Life Technologies), following manufacturer's instructions. Fraction E2-3 was precipitated with 20% TCA (TriChloroacetic Acid, Sigma) at 4°C for 30min, and then

centrifuged at maximum speed for 20min at 4°C. Pellet was washed first in 600µl 10% TCA, and then in 1ml acetone (Sigma); centrifuging at maximum speed for 20min at 4°C after each wash. TCA precipitated pellet was then dried at room temperature. In order to improve the reliability of the purification experiment, mass spectrometry analysis was performed (each time twice) on 5 independent TAP purification for both Abo1-TAP and untagged strains. In addition, TCA precipitation material was resuspended in laemmli buffer containing 1mM PMSF, and separated in a silver-stained 10% acrylamide gel. Gel slices containing specific bands, which were highly enriched in Abo1-TAP purification compared to the untagged one, were collected for mass spectrometry analysis. The mass spectrometry analysis of our samples by using the LTQ-Orbitrap Velos Pro Mass Spectrometer (Thermo Scientifiv) and the statistical treatment of obtained data were performed by our collaborators (EdyP, CEA Grenoble).

TEV-his production

BL21 Bacteria cells containing his-TEV plasmid were grown at 37°C in LB liquid medium until stationary phase, then diluted to OD600 ~0.2 and grown again to reach an OD600 ~0.6. TEV production was triggered by 0.1mM IPTG (Fluka) induction 2h at 25°C, and then 4h at 20°C. Cells were centrifuged at 12000g for 10min and pellet resuspended in 10ml cold lysis buffer (PBS 0.1X, 300mM NaCl, 10mM Imidazole). Samples were sonicated for 8 cycles of 30sec (5sec ON/ 5sec OFF) (Sonic Dismembrator, FisherScientific) and, after centrifugation at 12000g for 20min at 4°C, were incubated at 4°C for 1h with prewashed 500µl Ni-NTA Agarose resin (Qiagen). Resin was then washed 8 times in 500µl wash buffer (PBS 0.1X, 300mM NaCl, 20mM Imidazole) and serial elutions (5 fractions in total) were performed in 300µl elution buffer (PBS 0.1X, 300mM NaCl, 250mM Imidazole). Samples were then frozen at -80°C in 20% glycerol. For each his-TEV prurification, all eluted fractions were controlled by coomassie brilliant blue staining.

7. Analysis of interaction between proteins and nucleic acids (ChIP)

Chromatin Immunoprecipitation (ChIP) permits to investigate the binding of a protein to a specific genomic region, or to determine the specific location in the genome of an histone modification. Main experimental steps of this technique are: i) DNA and associated proteins on chromatin in living cells are cross-linked by formaldehyde; ii) The DNA-protein complexes are then fragmented by sonication; iii) cross-linked DNA fragments associated with protein of interest are immunoprecipitated using a specific antibody; iv) DNA fragments are purified and amplified by PCR.

7.1. Samples preparation

50ml cells were grown on YEA at 30°C until OD600 ~1.2 and fixed by 1% formaldehyde (Sigma) in agitation for 15min at room temperature. Glycine was then added to a final concentration of 0.125M to neutralize formaldehyde, and then cells were washed twice in 20ml cold TBS buffer. Pellet was resuspended in 360µl lysis buffer (50mM HEPES pH7.5, 0.1% SDS, 1mM EDTA, 0.1% sodium deoxycholate, 140mM NaCl, 1% Triton X-100, 1mM PMSF, 1mM Benzamidine, 1µg/ml LABP). 1ml of glass beads (0.5mm ø, Retsch) were added to samples and then 3 cycles of 1.30min agitation with a beadbeater (Biospec) were performed (incubation 2min on ice in between cycles). Samples were sonicated at 240W 8 cycles 30sec ON/ 30sec OFF (Bioruptor, de Diagenode), in order to have DNA fragments of around 150-600 nucleotides, and then centrifuged 5min 16000g at 4°C to eliminate cell debris. A volume of 50µl of input was collected to control lysis and sonication homogeneity, diluted in 200µl TE buffer (10mM Tris-Hcl pH8.0, 10mM EDTA, 1% SDS) and stored at 4°C.

7.2. Immunoprecipitation

In order to immunoprecipitate H3K9me2/ H3/ Mmi1/ HA, samples were incubated 2h at 4°C with 2µg of anti-H3K9me2 (Abcam, ab1220)/ or anti-H3 (Millipore, 05928)/ or anti-Mmi1

(Eurogentec 556)/ or anti-HA antibody (Eurogentec, 16B12) (incubation without antibody was performed as control). 20µl of prewashed sepharose beads coupled with protein A (4 Fast Flow, GE Healthcare) were added to each sample. After an incubation of 1h30 at 4°C, beads were washed for 3min at room temperature in: twice 1ml lysis buffer, 1ml wash buffer (10mM Tris-HCl pH8.0, 0.25M LiCl, 0.5% NP-40, 0.5% sodium deoxycholate, 1mM EDTA) and 1ml TE 1X buffer (10mM Tris-Hcl pH8.0, 10mM EDTA). Precipitated proteins were then eluted adding 100µl elution buffer (50mM Tris-Hcl pH8.0, 10mM EDTA, 1% SDS) and incubating 30min at 65°C. After centrifugating 1min at 12000g, eluted material was transferred in another tube. Beads were eluted again with 150µl TES buffer (10mM Tris-Hcl pH8.0, 1mM EDTA, 0.67% SDS), and after vortexing and centrifugating 1min at 12000g, supernatant was added to the tube with previously eluted material (to a total volume of 250µl).

7.3. DNA purification and analysis

To separate immunoprecipitated proteins from bound DNA fragments, eluted samples were reverse cross-linked for ~14h at 65°C. Proteins were then digested in 200µl TE 1X with 100µg Proteinase K (Roche) 30min at 65°C. DNA was purified by phenol-chloroform extraction: one volume of phenol-chloroform isoamyl alcohol (Sigma) with 44µl LiCl 5M was added to samples and, after vortexing and a centrifugation 5min at 16000g, the supernatant was washed with one volume of chloroform. Purified DNAs were then precipitated in 2.5 volume ethanol 100% and 1µl glycogen (20mg/ml) for 20min at -80°C. After washing in 500µl ethanol 70%, DNAs were resuspended in 20µl TE 1X with 0.5µg/ml RNAse (Roche) and incubated 1h at 37°C to digest RNAs. Quantitative PCR (qPCR) was then performed on ¼ dilution purified DNA material (see next section).

7.4. RNA immunoprecipitation (RIP)

RNA Immunoprecipitation (RIP) permits to investigate the binding of a protein to a specific RNA. The main experimental steps of this technique are identical to the ones described for the chromatin-immunoprecipitation. The main difference is a DNAse treatment after the sonication of samples (no RNAse treatment was performed instead), adding 700U DNAse (Sigma) and 50µl DNase Buffer (250mM $MgCl_2$, 50mM $CaCl_2$). Samples were incubated 1h at 30°C

and digestion was stopped adding 20µl EDTA 0.5M. After the immunoprecipitation, the reverse cross-link and the proteinase treatment, RNAs were purified and a Reverse Transcription Quantitative PCR (RTqPCR) was then performed.

8. Analysis of RNA expression

8.1. Extraction and purification of RNA

Cells were grown in 25ml liquid culture until OD600 ~0.5, then collected and centrifuged 5min at 800g. After washing in 1ml dH_20, cells were resuspended in 750µl TES buffer (10mM Tris pH7.5, 10mM EDTA pH8.0, 0.5% SDS) and 750µl acid phenol-chloroform (pH4.5, Ambion). Samples were immediately vortexed, incubated 15min at 65°C (vortexing 10sec every 3min) and then 2min on ice. Lysates were centrifuged 15min 12000g at 4°C and supernatant was extracted again by acid phenol-chloroform and washed in one volume chloroform. RNAs were then precipitated in 2.5 volume of cold ethanol 100% and 1/10 volume of sodium acetate 3M pH5.2 for 20min at -80°C (or 2h at -20°C). Samples were centrifuged 20min at 16000g at 4°C, washed in 500µl ethanol 70% and resuspended in 500µl dH_20. RNA concentration was measured by NanoDrop spectrophotometer and all samples were equilibrated at 1µg/µl. RNA quality was controlled by migrating 1µg of each sample in a 1.5% agarose gel containing ethidium bromide. An additional purification on column was performed for 100µg of each RNA sample using the Qiagen RNEasy kit and following manufacturer's instructions.

8.2. DNAse and RTqPCR

RNA samples were treated with DNAse I (Roche, 2U/µl) for 30min at 37°C in a buffer containing RNAseOut (40U/µl, ThermoFisher), DTT 10mM and DNAse buffer 5X (100mM Tris pH8, 10mM $MgCl_2$). DNAse was then inactivated by adding EDTA 5mM and incubating 10min

at 70°C. Afterwards, samples were incubated 10min at 65°C with primers for cDNA synthesis (either each specific primer 2µM; or 2µl of a mix containing unspecific random hexameric primers 50ng/µl, ThermoFisher) and 1µl $MgCl_2$ (50mM). Tubes were rapidly cooled for 1min at 4°C and mixed with a buffer for reverse transcriptase reaction containing RT buffer 5X (Roche), dNTPs (1mM each) and Transcriptor Reverse Transcriptase (10U/µl, Roche). RNAs were incubated 10min at 25°C, then 30min at 52°C and finally the enzyme was inactivated at 85°C for 5min. In parallel, a control reaction was performed without reverse transcriptase (replaced by dH_2O) to verify that results are not affected by contaminant DNAs. cDNAs were then amplified by quantitative PCR (qPCR). LightCycler 480 (Roche) was used for the qPCR reaction using the SYBR Green, a fluorochrome unspecifically and homogeneously incorporated in all amplified DNAs. qPCR reactions were prepared in a 96-well plate (Roche) in a total volume of 20µl (4µl of ¼ dilution cDNA; 1.2µl of each primer 5µM; 10µl MESA Green Mastermix Plus SYBR, Eurogentec). The program of amplification consists of a first denaturation 10min at 95°C, then 40 cycles of three repeating steps of 15sec each: denaturation at 95°C, hybridization at 60°C and polymerization at 72°C. The program ends with a step required to obtain the dissociation curve (1min at 95°C, 30sec at 60°C and 30sec at 95°C). Analysis of the results was achieved using the LightCycler 480 software (Roche) following manufacturer's instructions. A value was obtained that corresponds to a minimal number of PCR cycles required to amplify a specific genomic region. Each obtained result, corresponding to a genomic region in a specific strain, was normalized both to a control strain (for example *wild type*) and to a control region (for example *act1*).

8.3. Transcriptomic sample preparation and analysis

In order to obtain separated colonies, each strain was plated on YEA solid medium using glass beads and incubated at 30°C for ~6 days. For each genotype, 3 independent 25ml YEA liquid cultures (A, B and C) were inoculated starting from 2-5 separate colonies on plate. For *abo1Δ #1* (SPV3789) just small colonies were selected; on the contrary for *abo1Δ #2* (SPV3790) both small and big colonies were taken (*abo1Δ #2* and *abo1Δ #2 R*). YEA cultures were grown for ~24h at 30°C to reach an OD600 of ~0.2-0.4. At the same time cells were

collected for RNA extraction, they were also tested for reversion of *abo1Δ* phenotype (growth assay on solid medium at 30 and 37°C, analysis of cell morphology and cell mortality). A, B and C cultures for each genotype were pooled only if they share the same phenotype. RNAs were extracted and purified as previously described. 40µg of purified RNAs were then treated using DNAse (Roche), purified again by acid phenol-chloroform and precipitated with ethanol 100% and sodium acetate. RNAs samples were finally eluted in 30µl dH_2O. RNA quality was controlled by migrating 2µg material in a 1.5% agarose gel containing ethidium bromide (2h at 25V), and by RTqPCR. Samples for transcriptomic analysis were obtained by two independent technical replicates.

Transcriptomic analysis was performed by the EMBL Gene Core Facility. Sequencing of RNAs (after elimination of ribosomal RNAs) was performed by Illumina HiSeq2000. Raw data were analyzed in collaboration with Ravi Sachidanandam (MSSM, NY, USA) who annotated each read according to *S. pombe* PomBase database.

Reads were first filtered to remove sequences corresponding to non-annotated regions of the genome, pseudogenes and antisense sequences. All reads corresponding to the same gene ID were then clustered. In order to limit large fold-changes, we implemented total reads of each gene ID to a value of 10. Fold changes (FC) for each gene ID were obtained by a ratio between the total number of reads found in mutants and the one found in *wt* control background (each mutant set of data was compared to both *wt* replicates). Only annotated genomic regions with a FC > 2 or < 0.5 were considered as significantly upregulated or downregulated, respectively.

9. Sporulation assay

This test permits to evaluate the ability of a specific strain to undergo the process of sexual differentiation. *h90* cells were cultivated in YEA liquid medium until OD600 ~0.5. For each genotype, 10^6 cells were washed 3 times in 1ml sterile dH_2O, resuspended in 20µl sterile dH_2O, plated by 3µl drops on SPAS solid medium and incubated for 2 days at 30°C to induce

sporulation. Sporulation efficiency was evaluated by calculating the number of ascospores and zygotes over the total number of counted cells using a phase contrast microscope. For each genotype, ~300 cells were counted and 3 independent experiments were performed.

10. Analysis of mating type identity (PCR)

Cells were grown in liquid YEA culture at 30°C (without reaching saturation). 10^6 cells were washed 3 times in 1ml sterile dH_2O, resuspended in 20µl sterile dH_2O, plated by 3µl drops on SPAS solid medium and incubated for 2 days at 30°C. A patch of cells for each genotype was collected from plates and washed in 1ml dH_2O. DNA was extracted, purified using phenol-chloroform isoamyl alcohol and precipitated by ethanol 100%, as previously explained. DNA samples were then eluted in dH_20. Switching efficiency was studied by PCR analysis of genomic DNA (100µg) to determine the genetic content at the *mat1* locus (Jia, Yamada, and Grewal 2004). Primers used were PV1117 (MT1, common to mat1P and mat1M), PV1118 (MP, mat1P specific) and PV1119 (MM, mat1M specific). PCR reaction was performed using BioMix Red (Bioline) and the program of amplification contains a first denaturation 5min at 95°C, 27 cycles of amplification (30sec denaturation at 95°C, 30sec hybridization at 52°C and polymerization 1min30 at 72°C) and a final step of 10min at 72°C. 0.01µg of ethidium bromide was added directly to PCR amplifications, which were then migrated on a 1.5% agarose gel containing 0.1µg/ml ethidium bromide. Samples were analyzed using UV light after electrophoresis and M/P ratio measured using a software of quantification (ImageJ).

11. Conditional Abo1 knock-down system

A conditional knock-down mutant of Abo1 was obtained using the off-AID strategy (Auxin Inducible Degron) (Nishimura et al. 2009; Kanke et al. 2011). This strategy uses a mechanism of protein degradation present in plants to permit the degradation of a specific protein in

fission yeast. In plants, a vegetal hormone called auxin binds to the TIR1 E3-ubiquitin ligase and this permits recognition, poly-ubiquitination and degradation of members of IAA17 transcription factor family, which all contain a specific IAA17 sequence of 229 amino acids required for protein degradation (Kanke et al. 2011). Therefore, this repressive sequence *IAA17* has been added to *abo1* gene in a fission yeast strain ectopically expressing also the TIR1 protein. The off-AID system was induced adding auxin in the culture medium and the growth of these cells was investigated.

Cells were grown on liquid EMMc medium at 30°C for ~6-8h, diluted at the same concentration and splitted in two culture conditions: either adding 0.5mM auxin NAA (1-naphthaleneacetic acid, Nacalai Tesque), or an equivalent volume of DMSO as control. Cultures were grown then at 26°C for ~14h and diluted again on EMMc medium (containing NAA or DMSO) to a OD600 of 0.05-0.1. This was the first point of the kinetics. Cells concentration was then followed-up in time at least for 24h. Samples were collected also to perform Western blot and RNA extraction.

In the experiment to test the genetic interaction between Abo1 and histone genes, in order to better visualize the contribution of histone deletions to the growth of Abo1 KD strains, we normalized the data (OD600 in presence of NAA) to the one obtained for the control culture condition (in presence of DMSO), and also to the control *wt* strain (not depleted for Abo1).

RESULTS

Overview of my PhD results

As described in the Introduction, in *S. pombe*, heterochromatin formation and gene silencing are mediated by a continuous interplay between transcription and chromatin regulation. Indeed, proteins regulating transcription permits heterochromatin assembly by producing a nascent transcript that may be used as a platform that recruits chromatin modifiers (Holoch and Moazed 2015). On the other hand, chromatin regulators, such as histone chaperones or ATP-dependent chromatin remodelers, precisely modulate transcriptional activity and by this mean can contribute to modulate chromatin compaction (Venkatesh and Workman 2015).

Our team of research focuses on RNA-based chromatin regulations to understand how transcription and RNA can impact on the structure and function of chromatin. The purifications of two proteins known to bind chromatin-associated RNAs led to the identification of new actors possibly involved in the control of gene expression by a RNA-based chromatin regulation in *Schizosaccharomyces pombe.* Among them, we focused our investigation on proteins susceptible to regulate gene expression by acting at the interface between chromatin and transcription.

The first purification concerned Mmi1, an RNA binding protein crucial for facultative heterochromatin formation and gene silencing. This approach led to the finding that Mmi1 interacts with Ccr4-Not, which is a multifunctional complex processing 3'ends of RNAs and regulating gene expression (Collart and Panasenko 2012). The role of the Ccr4-Not complex both at constitutive and facultative heterochromatin regions has been investigated (Chapter 1 Results).

The second purification concerned RITS, an RNAi effector complex involved in heterochromatin formation and gene silencing. Among 9 strong candidates for their physical interaction with RITS, we focused on a known and a putative histone chaperone, both believed to act at the interface between chromatin and transcription regulation: Spt6, an RNA pol II-interacting histone chaperone (Kato et al. 2013); and Abo1, uncharacterized in *S.*

pombe, although its homologues in other organisms were proposed to regulate transcription, likely by acting as histone chaperones (Lombardi, Ellahi, and Rine 2011; Boussouar et al. 2013) (Chapter 2 Results).

ATAD2, the human homologue of Abo1, is a protein ectopically expressed in many tumors (Caron et al. 2010; Morozumi et al. 2015), but little is known about its molecular function. Therefore, we decided to characterize Abo1 in fission yeast, with the aim of providing important insights on the molecular function of this cancer-linked protein ATAD2 (Chapter 3 Results).

1.

Role of Ccr4-Not complex in heterochromatin assembly and gene silencing

1.1. Context and main results

To efficiently repress expression of specific meiotic genes during vegetative growth, *S. pombe* has developed a highly regulated mechanism mainly triggered by Mmi1, which binds to meiotic mRNAs via its YTH RNA-binding domain and induces their degradation by the exosome complex (Harigaya et al. 2006; Yamanaka et al. 2010). In addition, Mmi1 directs methylation of H3K9 to establish facultative heterochromatin at some of its targeted meiotic genes (Zofall et al. 2012; Hiriart et al. 2012) (see Introduction 2.4.2. for more details). To carry out these functions, Mmi1 binds to meiotic mRNAs and use them as 'RNA platforms' to subsequently recruit in *cis* proteins involved in TGS and PTGS.
The mechanisms of Mmi1-mediated gene silencing have just started to be understood and may represent a paradigm of how the binding of proteins to chromatin-associated RNAs could impose a strong gene silencing and modify the structure and function of chromatin.

The identification of new Mmi1 interactors could thus shed light on its mode of action and may also help elucidating other similar mechanisms of silencing in eukaryotes, which are dependent on RNA-binding proteins.

In my hosting team, an interaction between Mmi1 and Ccr4-Not (Carbon Catabolite Repression 4-Negative On TATA) complex was uncovered. Ccr4-Not is a well-known multifunctional complex mainly involved in 3'end RNA maturation. The capacity of Mmi1 to modify the chromatin of meiotic genes prompted us to investigate a possible role of Ccr4-Not in this process. My work has revealed that Ccr4-Not is required for H3K9me deposition at meiotic genes. Surprisingly, I also found that Ccr4-Not is required for proper gene silencing at constitutive heterochromatin. Thus, this work contributed to further define the process of RNA-mediated heterochromatin formation and gene silencing by identifying the RNA-bound Ccr4-Not complex as a potentially important element of this process.

1.2. Purification of Mmi1 revealed its interaction with Ccr4-Not complex

In order to identify new proteins with a function in Mmi1-mediated gene silencing, Mmi1 protein was purified by an immunoaffinity approach. For this purpose, a fission yeast strain containing a *TAP*-tagged *mmi1* gene was generated and used to perform large scale Mmi1-TAP complex purification. Specific bands, which appeared enriched in Mmi1 purification on the acrylamide silver-stained gel, were collected and analyzed by mass spectrometry (MS). Among them, a band corresponding to Not1, a subunit of the Ccr4-Not complex was identified (Figure 21A).

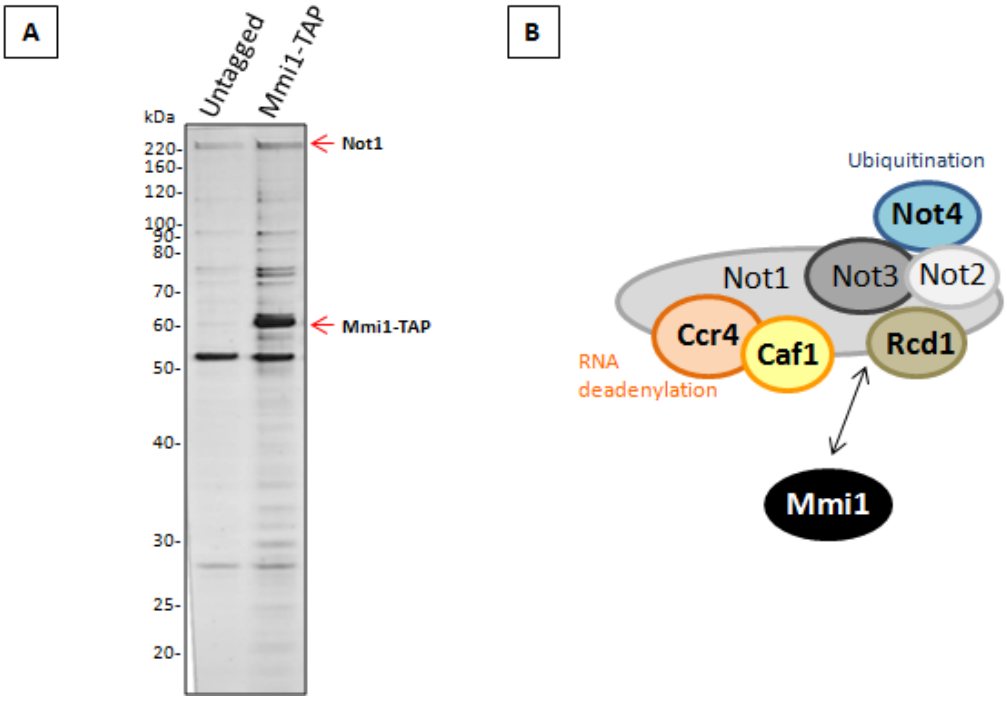


Figure 21. Interaction between Mmi1 and the Ccr4-Not complex. A) Silver staining of an SDS-polyacrylamide gel containing untagged and purified Mmi1-TAP. TAP-purification was performed in triplicates and analyzed by mass spectrometry. Red arrows correspond to specific identified bands: Mmi1-TAP and Not1. **B)** Schematic representation of the Ccr4-Not complex in *S. pombe*, which is composed of two deadenylating enzymes Ccr4 and Caf1; an E3 ubiquitin ligase Not4; Not1, Not2, Not3 and Rcd1, whose functions are still unknown.

The interaction between Mmi1 and Not1 was confirmed by a co-immunoprecipitation (CoIP) and Western blot experiment. Next, quantitative MS analysis on the whole Mmi1-TAP purification revealed that all subunits of Ccr4-Not were highly enriched in Mmi1-TAP purification compared to the untagged one, suggesting that the whole Ccr4-Not complex interacts with Mmi1. Not1-TAP, Not2-TAP and Not3-TAP reciprocal purifications further supported Mmi1's interaction with Ccr4-Not (data not shown).

1.3. Interaction between Mmi1 and Ccr4-Not is mediated by Rcd1

Ccr4-Not (Carbon Catabolite Repression 4-Negative On TATA) is a highly conserved complex in eukaryotes. In *S. pombe,* the Ccr4-Not complex consists of 7 main subunits: (Figure 21B) (Collart and Panasenko 2012). There are three catalytic subunits: two deadenylating enzymes (Ccr4 and Caf1) and an E3 ubiquitin ligase (Not4). The main role of the Ccr4-Not complex is to regulate RNAs turnover by deadenylating poly(A) tails and inducing their degradation (Collart and Panasenko 2012). In addition, Ccr4-Not controls gene expression at multiple levels. In fact, it regulates transcription initiation and elongation, mRNA nuclear export, translation and also protein degradation (mediated by Not4-dependent ubiquitination). Among other subunits without enzymatic activities, Not1 is the biggest of the complex and is believed to function as a scaffold (Bhaskar et al. 2013).

In order to understand which subunit of the Ccr4-Not complex is important to mediate its interaction with Mmi1, we deleted several Ccr4-Not components and we tested if the binding of Mmi1 to the scaffold protein Not1 was still maintained in each mutant. A near complete set of deletion mutants (*not2Δ, not3Δ*, *not4Δ*, *caf1Δ* and *rcd1Δ*) of Ccr4-Not complex were generated in *S. pombe* in which *not1* gene contains a 3HA tag. Mmi1 was immunopurified and co-precipitation of Not1-HA was then tested by anti-HA Western blot. Mmi1-Not1 interaction was maintained after removal of Not2, Not3 and the ubiquitin ligase Not4, thus indicating that these three proteins are not necessary for the interaction (Figure

22). Similarly, no significant change was observed in the absence of the deadenylase subunit Caf1. Since deletion of *caf1* also leads to loss of Ccr4 binding to Ccr4-Not (Mathys et al. 2014), this indicates that Caf1 and Ccr4 deadenylases are not required for Mmi1 binding to Ccr4-Not. In contrast, the removal of Rcd1 strongly reduces the interaction between Mmi1 and Not1 (Not1-3HA is no more visible in Mmi1 purification from 2 different *rcd1Δ* isolates) (Figure 22). Thus, we conclude from these findings that Mmi1 probably interacts with the Ccr4-Not complex by its binding to Rcd1.

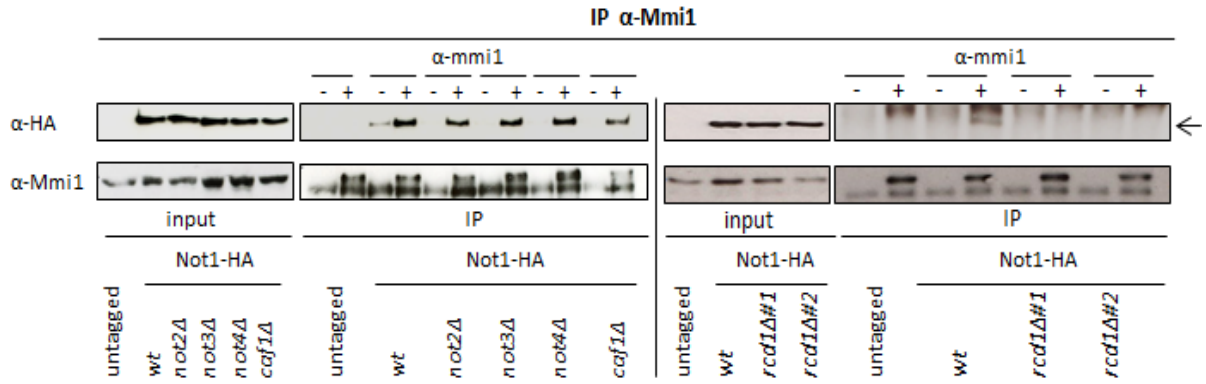


Figure 22. Rcd1 bridges Mmi1 to Ccr4-Not. Mmi1-Not1 interaction analyzed by co-immunoprecipitation in untagged or *not1-HA wt, not2Δ, not3Δ*, *not4Δ*, *caf1Δ* and *rcd1Δ* cells (IP α-Mmi1, WB α-HA or α-Mmi1). -/+ indicate absence/presence of α-Mmi1 antibody for IP; black arrow indicates Not1-HA signal. Co-immunoprecipitation was performed at least in duplicate for each tested strain.

1.4. Ccr4-Not is required for Mmi1-mediated facultative heterochromatin assembly

Since the RNA binding protein Mmi1 has a crucial role in mediating gene silencing and the deposition of repressive histone marks at specific meiotic genes, we next aimed to understand whether Ccr4-Not could also play a role in this process. For this purpose, we used two complementary approaches to investigate the phenotype of mutant strains for Ccr4-Not. First, we analyzed the level of mRNA accumulation of Mmi1 targets. Second, we analyzed the deposition of the heterochromatin repressive mark methyl-H3K9 in the corresponding genes.

We analyzed by RTqPCR the RNA level of two meiotic genes (*mei4*, *ssm4*) in several Ccr4-Not mutant strains (*rcd1Δ, caf1Δ, not4Δ, ccr4Δ and not3Δ*). No relevant increase of *mei4* and *ssm4* mRNA levels (less than 2-fold) was observed in in Ccr4-Not mutants, compared to *wild type* strain (Figure 23A). The only exception is a small but reproducible accumulation of *mei4* mRNA for *caf1Δ* mutants (around 4-fold) (Figure 23A). However, this RNA accumulation does not correspond to an increase in Mei4 protein level. Indeed, Mei4-GFP protein was not visible by Western blot in *wt, caf1Δ* and *not4Δ* cells; whereas, as expected, it is clearly detectable in *mmi1Δ* mutants used as positive control in this experiment (Figure 23B). These results indicate that the Ccr4-Not complex is not required for Mmi1-mediated gene silencing of meiotic genes. Next, we performed a chromatin immunoprecipitation (ChIP) against the histone repressive mark H3K9 dimethylation on several Ccr4-Not mutants (*rcd1Δ, caf1Δ, not4Δ, ccr4Δ and not3Δ*), and copurified DNA fragments were then amplified by qPCR focusing at *mei4* and *ssm4* genes (Figure 23C). Remarkably, a complete or near complete loss of H3K9me2 for both meiotic genes was observed in *caf1Δ, ccr4Δ* or *not4Δ* cells, indicating that the catalytic activities of the complex are necessary for heterochromatin formation or integrity at Mmi1 targets genes.

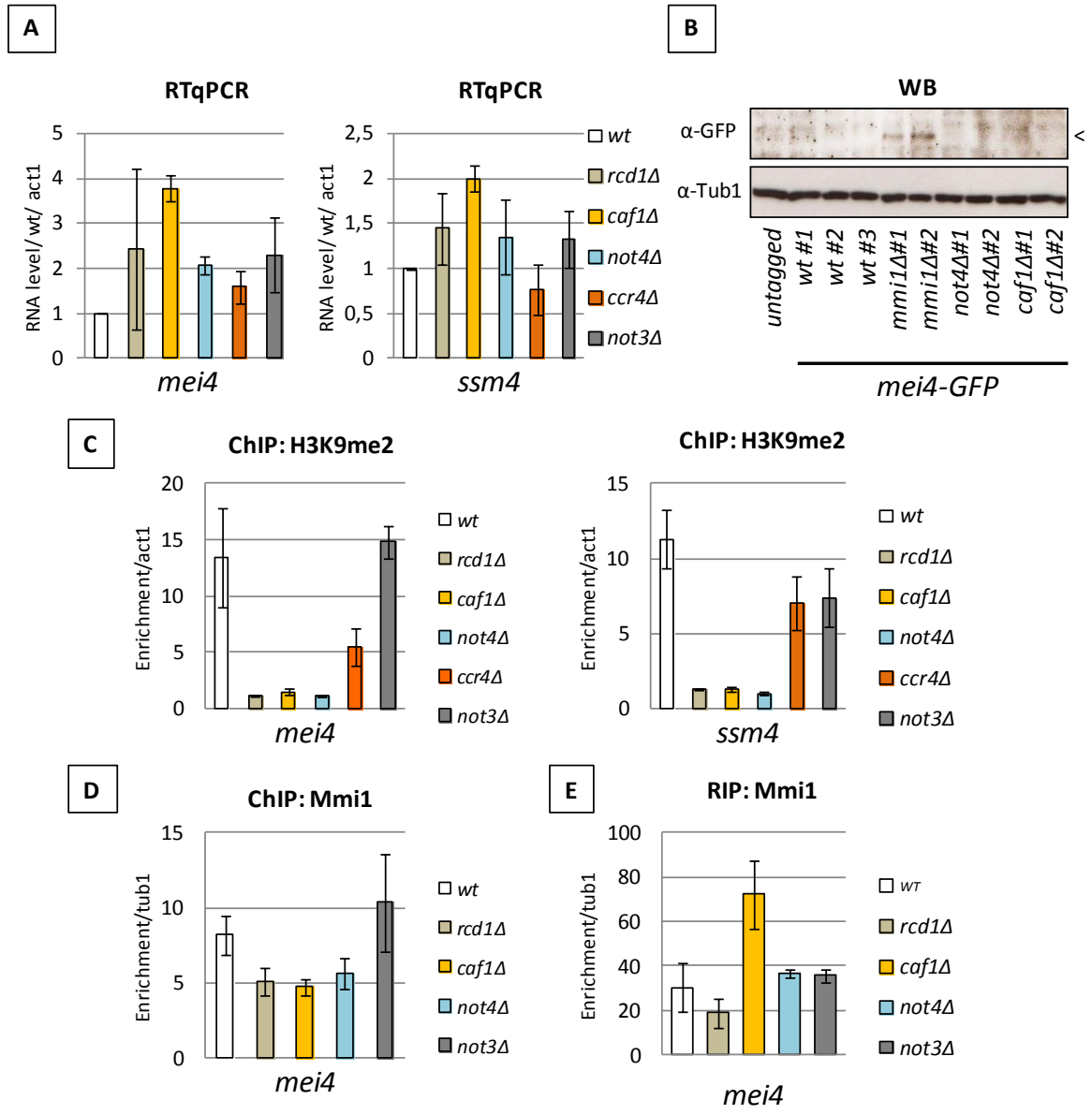


Figure 23. The Ccr4-Not complex is required for facultative heterochromatin integrity. A) RTqPCR analysis of *mei4* and *ssm4* RNA levels in *wt* and Ccr4-Not mutants (*rcd1Δ, caf1Δ, not4Δ, ccr4Δ* and *not3Δ)* relative to *act1* and normalized to the respective RNA levels in *wt* cells. **B)** WB analysis of Mei4-GFP protein levels in untagged and *mei4-gfp wt, mmi1Δ, caf1Δ, not4Δ* strains using Tub1 protein levels as control. Black arrowhead indicates Mei4-GFP signal. **C)** ChIP analysis of H3K9me2 at meiotic genes: qPCR analysis of *mei4* and *ssm4* purified DNA levels in *wt* and Ccr4-Not mutants relative to *act1*. **D)** ChIP **E)** and RIP analysis of Mmi1 binding to *mei4* gene or RNAs: qPCR analysis of *mei4* purified DNA levels in *wt, rcd1Δ, caf1Δ, not4Δ* and *not3Δ* strains relative to *tub1*. All error bars represent s.d. from three independent replicates.

A mild decrease in H3K9me2 was observed in *ccr4Δ* cells, whereas no clear H3K9me2 reduction in *not3Δ* strains (Figure 23C), indicating that not all subunits are important for Mmi1-mediated formation of facultative heterochromatin. Interestingly, H3K9me2 loss is also observed in absence of Rcd1 (Figure 23C), which mediates the interaction between the Ccr4-Not complex and Mmi1. This latter result suggests that the recruitment of Ccr4-Not by Mmi1 to meiotic mRNAs is required for the formation of facultative heterochromatin at meiotic genes.

These results prompted us to investigate whether Mmi1 binding to chromatin depends on Ccr4-Not complex. Interestingly, the ChIP against Mmi1 in *wt* and Ccr4-Not mutants showed a partial decrease in Mmi1 binding to the meiotic gene *mei4* in *rcd1Δ, caf1Δ, not4Δ* cells (in agreement with the importance of these proteins in H3K9me deposition); but not in *not3Δ* cells (Figure 23D).

We next investigated by RNA-immunoprecipitation (RIP) if the Mmi1 binding to the meiotic mRNA *mei4* was also affected in Ccr4-Not mutants. Our RIP experiment showed no significant decrease in the binding of Mmi1 to *mei4* mRNA in Ccr4-Not mutants. In fact, even an increase in Mmi1 binding to this mRNA was observed in *caf1Δ* strains (Figure 23E). Therefore, our ChIP and RIP results showed that Rcd1 and the catalytic subunits of Ccr4-Not participate to Mmi1 recruitment to chromatin of meiotic genes, but not to meiotic RNAs.

Taken together, our data highlight the importance of Rcd1 and the catalytic subunits of Ccr4-Not in the assembly of facultative heterochromatin. According to our findings, this function of Ccr4-Not may rely on its capacity to mediate or facilitate Mmi1's localization to chromatin, which in turns would mediate H3K9 methylation.

1.5. Ccr4-Not promotes gene silencing at constitutive heterochromatin regions

Considering that Ccr4-Not has a role in the formation of facultative heterochromatin, we explored whether it could have a more general function in heterochromatin regions. In support to our hypothesis, a genetic screen conducted in our team showed that Rcd1 is required for proper gene silencing at constitutive heterochromatin regions (unpublished data). This prompted us to further investigate the effects of deleting Ccr4-Not subunits in the context of constitutive heterochromatin.

First, we performed a classical gene silencing assay to test in Ccr4-Not mutants the silencing of an *ura4+* reporter gene inserted into the pericentromeric *imr1R* heterochromatin region. We plated cells on medium containing a specific drug: the 5-FluoroOrotic Acid (5FOA). Mutant strains with defects in centromeric silencing, such as *clr4*Δ, express Ura4 that converts 5FOA in a compound toxic for cells; this results in a loss or reduced growth compared to *wt* cells on medium containing 5FOA. Strains deleted for all Ccr4-Not catalytic subunits (Caf1, Not4 and Ccr4) and Rcd1 are sensitive to 5FOA, reflecting defects in centromeric gene silencing; no effect was observed in *not3Δ* strains (Figure 24A).

In agreement, the level of *ura4* RNA measured by RTqPCR; is highly increased in *rcd1Δ* cells and a modest increase is observed also in *caf1Δ, not4Δ* and *ccr4Δ* mutants, whereas no *ura4* RNA accumulation is observed for *not3Δ* cells (Figure 24B). In order to investigate if the *ura4::imr1R* desilencing in Ccr4-Not mutants was linked to a defect in heterochromatin integrity, we investigated the H3K9 dimethylation level at this reporter gene. A decrease in H3K9me2 was observed in *rcd1Δ* cells, whereas no clear decrease was observed in absence of Caf1, Not4 or Not3, and surprisingly a higher level was observed in *ccr4Δ* cells (Figure 24C).

Finally, in order to better investigate a general impact of Ccr4-Not mutants to constitutive heterochromatin gene silencing, we looked at the level of transcript accumulation and H3K9 methylation profile at other constitutive heterochromatin regions: centromere (*cen dh*) and subtelomere (*tlh1*) (Figure24D and E).

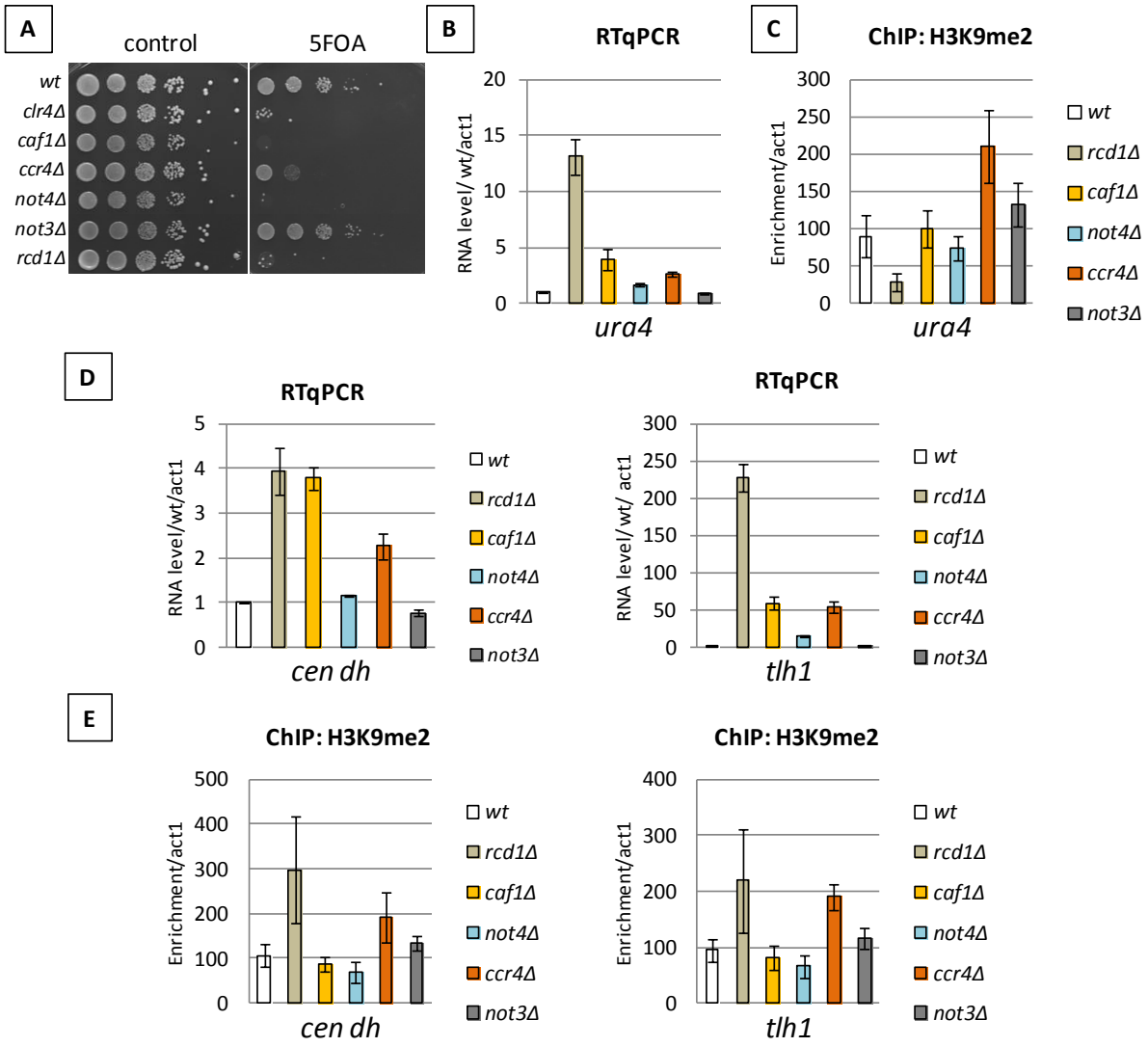


Figure 24. The Ccr4-Not complex is required for constitutive heterochromatin gene silencing. A) Silencing assay of *ura4::imr1R reporter gene;* serial dilution spots of *wt, clr4Δ* and Ccr4-Not mutants (*caf1Δ, ccr4Δ, not4Δ, not3Δ* and *rcd1Δ)* on YEA (control) or medium containing 5FOA. **B)** RTqPCR analysis of *ura4* RNA levels in *wt* and Ccr4-Not mutants relative to *act1* and normalized to the respective RNA levels in *wt* cells. **C)** ChIP analysis of H3K9me2 at *ura4::imr1R*: qPCR analysis of *ura4* purified DNA levels in *wt* and Ccr4-Not mutants relative to *act1*. **D)** RTqPCR analysis of *cen dh* and *tlh1* RNA levels in *wt* and Ccr4-Not mutants relative to *act1* and normalized to the respective RNA levels in *wt* cells. **E)** ChIP analysis of H3K9me2 at constitutive heterochromatin*:* qPCR analysis of *cen dh* and *tlh1* purified DNA levels in *wt* and Ccr4-Not mutants relative to *act1*. All error bars represent s.d. from three independent replicates.

Overall, in agreement with the results obtained using the *ura4+* reporter gene, deletions of Ccr4-Not catalytic subunits and Rcd1 correlate with an increase in transcripts at constitutive heterochromatin regions. The analysis of H3K9me2 mark showed no significant change in its level for *caf1Δ, not4Δ* and *not3Δ* cells, and surprisingly an increased level was observed in *rcd1Δ* and *ccr4Δ*.

Thus, from these last results we conclude that Ccr4-Not has a role in the silencing of a reporter gene placed within pericentromeric heterochromatin. In addition, our data suggest that at constitutive heterochromatin Ccr4-Not may act mostly in regulating RNA expression, rather than mediating H3K9me deposition. Importantly, Mmi1 requirement at constitutive heterochromatin was also investigated in the team. However, in *mmi1Δ* cells, pericentromeric gene silencing and integrity is not impaired, like it is in Ccr4-Not mutants (data not shown). Thus, the function of Ccr4-Not complex at constitutive heterochromatin is independent of Mmi1. Finally, the difference in the action of Ccr4-Not between facultative and constitutive heterochromatin suggests that this complex can mediate heterochromatin formation and gene silencing through multiple mechanisms, which are specific for each heterochromatin region.

1.6. General conclusion

Starting from the purification of Mmi1 complex, which revealed its interaction with the Ccr4-Not complex, we found that Rcd1 subunit is the major actor of this interaction by probably bridging Mmi1 to the Not1 scaffold protein. We next showed a crucial role of Ccr4-Not in heterochromatin assembly and gene silencing in fission yeast. Indeed, Ccr4-Not is required for the integrity of facultative heterochromatin at Mmi1-targeted meiotic genes, permitting the deposition of repressive H3K9 methylation on chromatin. In addition, Ccr4-Not plays also a Mmi1-independent role in gene silencing at constitutive heterochromatin, mostly by PTGS of heterochromatic transcripts.

Therefore, the Ccr4-Not complex plays a role within heterochromatin by at least two different mechanisms: a chromatin-based mechanism at facultative heterochromatin where it mediates heterochromatin formation, and an RNA-based mechanism at constitutive heterochromatin. Further experiments will be required to understand the mechanisms implicated. The work I have conducted and presented in this chapter will constitute part of a manuscript that will describe the function of Mmi1 interaction with the Ccr4-Not complex in a more general manner. Indeed, in addition to the purification of Mmi1, the role of Mmi1/Ccr4-Not interaction in controlling initiation and progression of *S. pombe* sexual differentiation has being addressed.

Finally, as a perspective, it is important to note that even if the molecular function of Ccr4-Not at heterochromatin is still unknown and needs to be further investigated, the complexity of Ccr4-Not and its multifunctional activity might make the understanding of its molecular function challenging. Interestingly, considering the conservation of Ccr4-Not trough the evolution, it would be insightful to explore whether its function in regulating heterochromatin silencing, shown in fission yeast, is also conserved in other eukaryotes. In the discussion section, an overview on the role of Ccr4-Not at heterochromatin will be presented by integrating and commenting our results with data from the literature.

2.

RITS purification reveals a connection with histone chaperones

2.1. Context and main results

RITS is the nuclear RNAi effector complex essential for constitutive heterochromatin formation and gene silencing in *S. pombe* (Verdel et al. 2004). RITS is believed to recognize and bind constitutive heterochromatin regions thanks to a base-pairing mechanism between guide-RNAs, which are loaded into the RITS subunit Ago1, and complementary RNAs that are produced by the RNA polymerase II. Once bound to these nascent transcripts, RITS recruits several protein complexes to induce and reinforce the process of gene silencing (Introduction 2.3.1.). In addition, RITS has been more recently reported to bind to meiotic genes and mRNAs, and to contribute to the silencing of these regions, thereby possibly controlling sexual differentiation (Hiriart et al. 2012).

Nevertheless, the role and mechanisms of action of RITS at constitutive or facultative heterochromatin are still not completely understood and the identification of new actors may help elucidating these fundamental aspects.

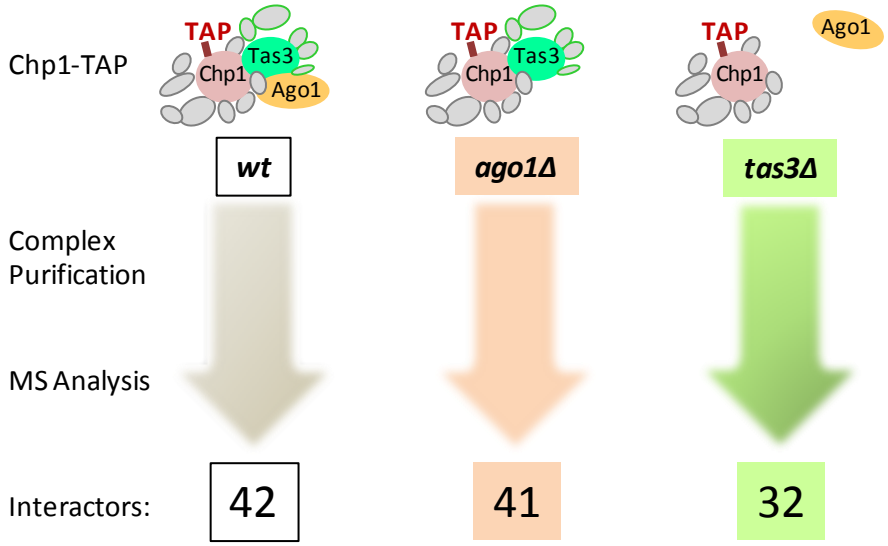
In my team of research, a large-scale purification of RITS complex was conducted to identify new interactors, which could have a role in heterochromatin formation and/or gene silencing. As part of my PhD project, I particularly focused my research on a well-known histone chaperone and a putative one: Spt6 and Abo1, respectively. Here, my contribution has been to validate the interaction between RITS and these two proteins and to investigate their role in heterochromatin integrity and gene silencing in *S. pombe*.

2.2. RITS purification identified new proteins possibly involved in constitutive heterochromatin silencing and/or formation

RITS is composed of three subunits: the Argonaute protein Ago1, the chromodomain protein Chp1 and Tas3, which acts as a bridge between the other two components. In order to identify the network of proteins physically interacting with RITS, its complex was purified from large cultures of a fission yeast strain expressing a *TAP* tagged version of *chp1* gene, used as a bait protein, and its associated proteins were subsequently identified by mass spectrometry. The purification was done under benzonase treatment (which digests both DNA and RNA), in order to get rid of all nucleic acids and obtain direct protein-protein interactions. A high stringency and semi-quantitative analysis was conducted on the identified proteins. Indeed, proteins were considered putative RITS partners only if they showed ≥10 fold enrichment in Chp1 purification over a control one (representing the background of the purification) and if they were identified with at least 10 unique peptides. This approach permitted to identify 42 proteins specifically enriched in Chp1-TAP purification compared to the untagged control (Figure 25). Among them, as expected, the other two RITS subunits Tas3 and Ago1 were the most enriched.

Then, to specifically focus our investigation on proteins that most likely interact with RITS rather than only with the Chp1 subunit, the purification of Chp1-TAP was repeated in *ago1Δ* and *tas3Δ* strains. In *ago1Δ* genetic background, aside Ago1, all other 41 possible interactors still copurified with Chp1-TAP; this indicates that all these proteins do not require Ago1 to interact with Chp1. Tas3 has a structural role to ensure the integrity of the complex, acting as a bridge between Chp1 and Ago1 (Verdel et al. 2004). Accordingly, in *tas3Δ* cells, in which the integrity of the RITS complex is completely lost, Chp1-TAP purification showed a loss of 10 putative interactors (out of 42).

A



B

Name	FC	Unique peptides	Function
Tas3	376	30	RITS (RNAi effector complex)
Ago1	372	44	RITS (RNAi effector complex)
Hrp3	78	20	CHD (chromatin remodeller)
Spt6	61	15	Histone chaperone Transcription regulator
Pla1	42	10	Poly(A) polymerase
Ccq1	41	13	Telomere mantainance SHREC (HDAC)
Pst1	35	12	HDAC complex I
Abo1	35	11	Histone chaperone Transcription regulator*
Cdc20	22	12	DNA repair
Rad52	14	10	DNA repair

Figure 25. Chp1-TAP purification identifies new possible RITS interactors. A) Schematic representation of the proteomic strategy based on RITS mutants: Chp1-TAP purification in *wt, ago1Δ* and *tas3Δ* strains. High stringency quantitative analysis of the MS results permitted the identification of possible RITS interactors. **B)** Sum up the MS results of Chp1-TAP purification in *wt* for 10 proteins not found in Chp1-TAP purification in *tas3Δ* cells. fold enrichment: (∑MS/MS counts Chp1TAP) / (∑MS/MS counts Untagged); Values are average of three TAP purifications. *: putative function.

We focused on these 10 proteins that are more likely to interact with RITS. Among them, aside Ago1 and Tas3, there are 8 proteins not previously described to associate with RITS in fission yeast (Figure 25B). Inside this list of possible RITS interactors, there are 2 proteins involved in DNA repair, replication and cell cycle regulation (Rad52 and Cdc10) (Octobre et al. 2008; Ivanova et al. 2013), the poly(A) polymerase Pla1 and some other proteins that had been already linked to heterochromatin: Ccq1, which is a telomeric protein and a member of the SHREC complex (Sugiyama et al. 2007); the CHD1 protein Hrp3, which is involved in the control of heterochromatin gene silencing (Jae Yoo et al. 2002); Pst1, a component of HDAC complex I (Nicolas et al. 2007). Interestingly there were also two proteins both located at the interplay between transcription and chromatin regulation: the RNA pol II-interacting histone chaperone Spt6 (Kato, Okazaki, and Urano 2013); and Abo1 (ATPase with bromodomain protein), which is uncharacterized in fission yeast, but its homologues in other organisms were also proposed to regulate transcription acting as putative histone chaperones (Lombardi, Ellahi, and Rine 2011; Boussouar et al. 2013).

This new fascinating connection, between RITS and proteins that both work in connection with transcription and chromatin dynamics, could open a new field of investigation to help elucidating mechanisms of RNA-based heterochromatin assembly and gene silencing. For this reason, we decided to focus our investigation on Spt6 and Abo1.

2.3. Spt6 has a role in heterochromatin formation and gene silencing

We first decided to confirm and better characterize Spt6's interaction with RITS. Spt6 is known to interact with RNA pol II and acts, both at heterochromatin and euchromatin regions, as a histone chaperone to maintain histone H3 in their post-translational state (Kato, Okazaki, and Urano 2013; DeGennaro et al. 2013). However, the mechanism of action of Spt6 still needs to be completely understood, especially in the context of heterochromatin.

My contribution to this investigation was limited to confirm the interaction between RITS and Spt6, and to check the implication of Spt6 at heterochromatin regions.

We first confirmed Spt6 interaction with RITS using an Ago1 pull-down approach. Protein extracts from *spt6-TAP* tagged *S. pombe* cells were incubated either with GST or GST-Ago1 resins, after having treated the extract with RNAse and DNAse. GST-Ago1 protein was produced and purified from bacteria. Spt6-TAP appeared to be highly enriched on GST-Ago1 resin, compared to GST resin (Figure 26A), thus confirming the physical interaction between Spt6 and RITS that was initially detected by affinity purification of Chp1 and mass spectrometry analysis.

The identification of a connection between Spt6 and RITS complex suggests that RITS may interact with the RNA polymerase II machinery not only through the RNA platform but also possibly through protein-protein interactions. This possibility prompted us to further investigate the phenotype of cells depleted for Spt6 in heterochromatin assembly and silencing. Considering that *spt6* deletion mutants are viable but too sick to be easily used, we obtained from Fred Winston's laboratory the *spt6-1* strain, in which the sequence encoding for the helix-hairpin-helix motif in *spt6* gene was deleted. This strain has been already used to show a role for Spt6 in regulating constitutive heterochromatin gene silencing, acting both at transcriptional and posttranscriptional levels (Kiely et al. 2011). In addition, these cells present a modest growth defect at 30°C that becomes more severe at 37°C (Kiely et al. 2011).

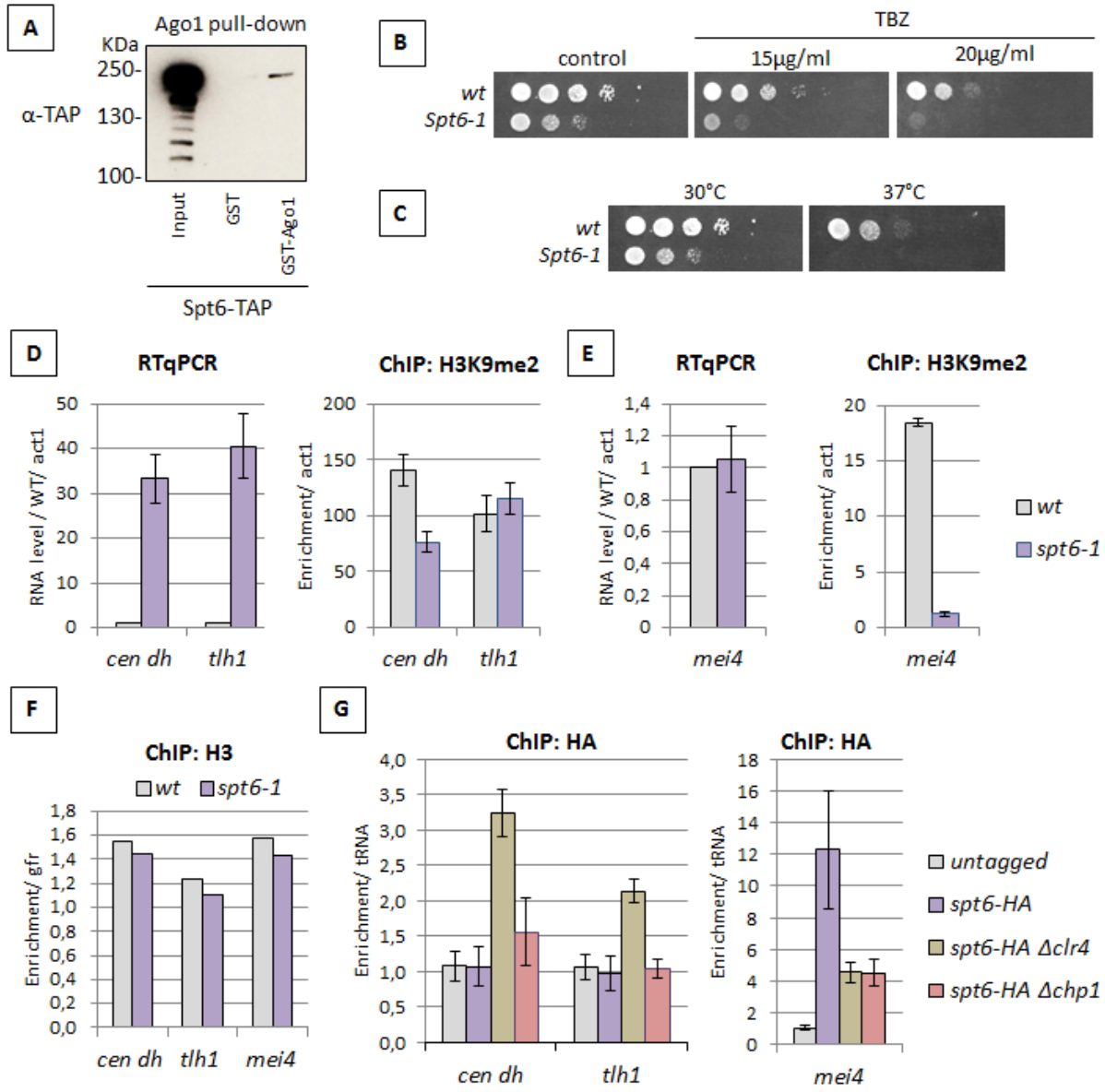


Figure 26. Spt6 is a partner of RITS and regulates heterochromatin assembly. A) Ago1 pull-down: protein extracts from *spt6-TAP* cells used for GST or GST-Ago1 pull-down (done with three independent replicates). WB analysis: α-TAP. **B)** TBZ assay: serial dilution spots of *wt and spt6-1* mutant cells plated on YEA (control) or medium containing TBZ (15 or 20 µg/ml). **C)** Growth assay: serial dilution spots of *wt and spt6-1* mutant cells plated on YEA medium and incubated at 30°C or 37°C. **D)** RTqPCR analysis of *cen dh* and *tlh1* RNA levels in *wt* and *spt6-1* cells relative to *act1* and normalized to the respective RNA levels in *wt* cells. ChIP analysis of H3K9me2: qPCR analysis of *cen dh* and *tlh1* purified DNA levels in *wt* and *spt6-1* relative to *act1*. **E)** RTqPCR analysis of *mei4* RNA levels and ChIP analysis of H3K9me2 at *mei4*. **F)** ChIP analysis of histone H3: qPCR analysis of *cen dh, tlh1* and *mei4* purified DNA levels in *wt* and *spt6-1* relative to *gfr* (gene free region). **G)** ChIP analysis of Spt6-HA binding: qPCR analysis of *cen dh, tlh1* and *mei4* purified DNA levels in *untagged* and *spt6-3HA wt*, *clr4Δ* or *chp1Δ* cells, relative to *tRNA*. All error bars represent s.d. from at least two independent replicates (preliminary results).

We confirmed the published findings of a defect in constitutive heterochromatin formation and gene silencing in *spt6-1* strains. Moreoever, plating cells using serial dilution spots, we confirmed a sensitivity of *spt6-1* strains to thiabendazole (TBZ), a drug that affects microtubule polymerization and centromere integrity and that is often used to suggest a defect in centromeric heterochromatin (Figure 26B). In addition, *spt6-1* mutant presents a strong growth defect at 37°C (Figure 26C). We also confirmed by RTqPCR a desilencing of pericentric and subtelomeric heterochromatin, and we investigated by ChIP if this correlates with a decrease in the repressive histone mark H3K9me2 (Figure 26D). We could confirm a slight decrease in H3K9me2 at centromeric *dh*, whereas no decrease was observed at subtelomeric *tlh1* (Figure 26D). This latter result suggests that the defective gene silencing at constitutive heterochromatin in *spt6-1* cells is not merely caused by a severe reduction of histone repressive marks, but rather by a defective silencing of heterochromatin transcripts that is either due to an increased transcription and/or to defective RNA degradation.

RITS has been also shown to bind and possibly contribute to the efficient silencing of meiotic mRNAs (Hiriart et al. 2012). Therefore, we also decided to investigate whether Spt6 could play a more general role in heterochromatin regulation, by exploring the impact of its deletion on the formation of facultative heterochromatin at meiotic genes. We looked at transcript accumulation and H3K9 methylation profile at the meiotic gene *mei4* in *spt6-1* strain. We could not observe any *mei4* mRNA accumulation in *spt6-1* cells compared to *wt,* indicating that Spt6 may not be involved in Mmi1-mediated gene silencing. In contrast, and quite surprisingly, there is a dramatic loss of H3K9me2 at this gene (Figure 26E). We also confirmed this result at another meiotic gene *ssm4*. In addition, we verified that this lack of H3K9me2 was not due to a loss of histone H3 level at these meiotic genes (preliminary result, Figure 26F), indicating that the drastic decrease in H3K9me2 cannot be explained by a reduced histone occupancy. Therefore, our results revealed a crucial role for Spt6 in facultative heterochromatin integrity.

The loss of H3K9 methylation in *spt6* mutant strains prompted us to test a possible direct role of Spt6 at facultative heterochromatin, by testing whether it localizes to such genomic regions. For this purpose, we performed a ChIP experiment in *wt, chp1Δ* and *clr4Δ* cells, in which *spt6* had been *3HA*-tagged. It has been already shown that Spt6 binding to chromatin correlates with RNA pol II localization. In agreement, Spt6 is hardly detectable at poorly

transcribed constitutive heterochromatin in *wt* cells. However, it is possible to localize Spt6 at constitutive heterochromatin in *clr4Δ* or *chp2Δ* mutants, in which transcription of these regions increases (Kiely et al. 2011) (Figure 26G). In addition, we first showed that in *chp1Δ* strains, despite the increase in transcription at pericentromeric and subtelomeric regions, no detectable Spt6 was observed (Figure 26G), suggesting a possible role of Chp1 in recruiting or stabilizing Spt6 to these regions.

At facultative heterochromatin, meiotic genes are normally transcribed and then degraded by a Mmi1-mediated PTGS mechanism (Yamanaka et al. 2010). As expected, Spt6 binding was already detectable at transcribed *mei4* gene in *wt* cells. However, interestingly, we revealed that Spt6 binding at *mei4* was decreased in both *clr4Δ* and *chp1Δ* mutants (Figure 26F), suggesting a role for both these proteins in Spt6 localization at facultative heterochromatin.

Thus, our Spt6 characterization in the context of heterochromatin revealed an interaction between the RITS complex and the histone chaperone Spt6. My contribution in this study confirmed this interaction and the direct implication of Spt6 at heterochromatin regions. We showed a new function of Spt6 in being essential for facultative heterochromatin formation in fission yeast. Furthermore, while Spt6 localization in the genome has been shown to depend on the presence of the RNA pol II, our findings indicate, interestingly, that the interaction with RITS may also be important for Spt6 localization at heterochromatin regions.

2.4. Abo1 is required for proper constitutive heterochromatin gene silencing

The second RITS interactor that we decided to investigate is Abo1, a completely uncharacterized protein in fission yeast. Its homologues in other organisms regulate both transcription and chromatin structure. Abo1 homologue in human is named ATAD2 and is a cancer-related protein playing a role in cell proliferation and transcription regulation. In addition, Yta7, its homologue in *S. cerevisiae,* has been proposed to regulate gene expression by acting as a chromatin barrier element and a possible histone chaperone (reviewed in Cattaneo et al. 2014; Introduction Chapter3).

In order to characterize Abo1 at heterochromatin regions, my contribution has been first to confirm the interaction between RITS and Abo1, and then to investigate the possible role of Abo1 in constitutive heterochromatin gene silencing. It is of note that, until today, no study has addressed the possibility that an ATAD2-like protein could play a role in heterochromatin gene silencing or in any other epigenetic processes.

We confirmed Abo1-Chp1 interaction by performing a TAP-purification from strains expressing Abo1 TAP-tagged protein and looking at the presence of Chp1 by Western blot. Chp1 was detected specifically in Abo1-TAP purification, while it was not detected in the untagged control one (Figure 27A). This result confirmed thus that Abo1 is likely to be a new interactor of RITS.

We then aimed to investigate whether Abo1 could have a role at heterochromatin regions by looking at heterochromatin gene silencing defects in *abo1Δ* cells. By plating a serial dilution of cells, we could first observe a strong defect in growth for several different *abo1Δ* isolates in control medium (Figure 27B). In addition, similarly to the positive control *chp1Δ, abo1Δ* cells are sensitive to high TBZ doses, therefore suggesting a possible defect of centromeric heterochromatin in *abo1Δ* cells (Figure 27B). In order to test this hypothesis, *abo1* deletion was obtained in cells that contain an *ura4+* reporter gene inserted into centromeric heterochromatin *imr1R* (isolates #2, 4, 5 and 6). Cells were plated in the

presence of 5FOA. Mutant strains with defects in centromeric silencing, such as the *ura4::imr1R chp1*Δ positive control, express Ura4 that converts 5FOA in a compound very toxic for cells. This toxicity results in a reduced growth compared to *wt* cells. Similarly, *ura4::imr1R abo1Δ* cells plated on 5FOA present a higher defect in cells growth, compared to control medium. Of note, the severe growth defect of *abo1Δ* cells, already present in the control medium made the analysis of 5FOA sensitivity difficult. However, no growth defect aggravation was observed in *abo1Δ* strains that lack functional Ura4 (*ura4D18*), indicating that 5FOA sensitivity observed in *ura4::imrR abo1Δ* cells is likely due to a defective centromeric silencing.

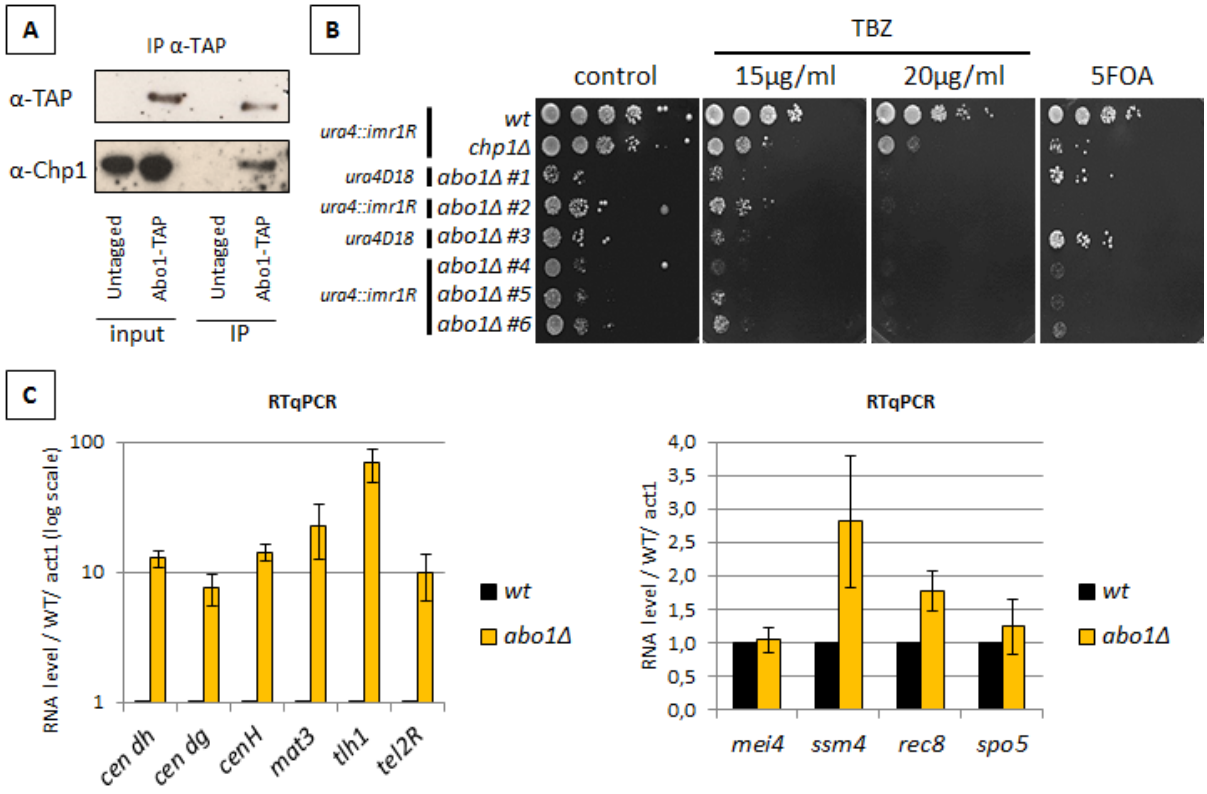


Figure 27. Abo1 is a RITS partner required for constitutive heterochromatin gene silencing. A) Interaction between Abo1-TAP and Chp1 analyzed by Co-immunoprecipitation in untagged and *abo1-tap* cells (IP α-TAP, WB α-Chp1). CoIP was performed from three biological replicates. **B)** TBZ and silencing assay (*ura4::imr1R* reporter gene): serial dilution spots of *wt, chp1Δ* and several *abo1Δ* strains plated on YEA (control) and medium containing TBZ (15 or 20 µg/ml) or 5FOA. **C)** RTqPCR analysis of constitutive heterochromatin (*cen dh, cen dg, cenH, mat3, tlh1* and *tel2R*) and facultative heterochromatin (*mei4, ssm4, rec8* and *spo8*) RNA levels in *wt* and *abo1Δ* cells relative to *act1* and normalized to the respective RNA levels in *wt* cells. All error bars represent s.d. from three independent replicates.

As our results pointed to a possible centromeric heterochromatin defect in *abo1Δ* cells, we further investigated whether Abo1 could have a general role in heterochromatin gene silencing. For this purpose, we analyzed by RTqPCR the level of RNA accumulation at both constitutive and facultative heterochromatin regions. Interestingly, we observed an increase in heterochromatic transcripts level for all three tested constitutive heterochromatin regions: centromeres (*cen dh* and *cen dg*), mating type (*cenH* and *mat3*) and subtelomeres (*tlh1 and tel2R*) regions. Conversely, no significant RNA accumulation was observed at facultative heterochromatin, looking at 4 different meiotic mRNAs (*mei4, ssm4, rec8* and *spo5*) (Figure 27C).

Thus, taken together, our study of Abo1 identified this protein as a probable new interactor of RITS complex. In addition, it revealed a clear requirement of Abo1 in gene silencing at all constitutive heterochromatin regions in fission yeast.

2.5. General conclusion

RITS purification permitted the identification of several new possible RITS interactors, potentially involved in heterochromatin assembly and gene silencing. Among them, we further investigated two proteins, Spt6 and Abo1, which are potentially involved in transcription regulation by acting as histone chaperones.

Our investigations showed that Spt6 plays a role both at constitutive and facultative heterochromatin, and that the interaction with RITS may be important for its localization. Importantly, so far, no protein-protein interaction has been found to physically link RITS to transcription and to directly contribute to RNAi-mediated heterochromatin formation and/or gene silencing. Our results suggest that Spt6 binding to RITS may represent such physical link with transcription that could be crucial for RNAi to mediate heterochromatin formation. Testing this possibility will be of great interest in order to understand the contribution of transcription and of this histone chaperone to the well-studied RNA-dependent chromatin regulation mediated by RITS.

In addition, in the case of Abo1, our investigations demonstrated that the ATAD2-like protein in *S. pombe* interacts with the RNAi effector complex RITS and is required for proper heterochromatin gene silencing. These findings may open a new research area aimed at understanding the role of Abo1, and possibly other members of the ATAD2 protein family, in regulating gene silencing in the context of heterochromatin or other related silent chromatin.

3.

Characterization of *S. pombe* Abo1, a model to dissect the function of ATAD2 in cancer

3.1. Context and main results

As described in the previous chapter, we found an interaction between RITS and Abo1. In addition, we uncovered that Abo1 is required for proper constitutive heterochromatin gene silencing, although its mechanism of action is not determined yet.

In this chapter, our more extensive characterization of Abo1 in fission yeast is presented. Our results showed that Abo1, like its human homologue ATAD2, regulates cell growth in *S. pombe*. In order to investigate a possible function of Abo1 in transcription regulation in a genome-wide manner, we performed a transcriptomic analysis on *abo1Δ* cells and we discovered that Abo1 is a regulator of gene expression for many protein-coding but also a large proportion of non-coding genes. In parallel, we purified the Abo1-TAP complex and we discovered that Abo1 interacts with several chromatin-related proteins, including histones. Altogether, the work presented in this chapter suggests that Abo1 may have a role as a histone chaperone in *S. pombe*. Of note, this study was the main topic of investigation of my PhD project and will be described in a manuscript under preparation.

Studies in other organisms showed that Abo1 homologues play a general role in transcription regulation. Indeed, Yta7 in *S. cerevisiae* has been shown to regulate the transcription of histones and inducible genes, possibly acting as histone chaperone at promoter boundaries sites (Lombardi, Ellahi, and Rine 2011). In addition, human ATAD2 activates transcription of estrogen and androgen responsive genes (Zou et al. 2007; Zou et al. 2009) and acts as coactivator of E2F and Myc to induce transcription of genes involved in cell proliferation and survival (Revenko et al. 2010). Interestingly, ATAD2 is a male germ factor ectopically expressed in many cancer types (Caron et al. 2010), however its contribution in the processes of carcinogenesis is not completely understood yet. ATAD2 has been shown to regulate cell proliferation, indeed a knock-down of ATAD2 in cancerous cells lines, which normally overexpress ATAD2, is known to inhibit cell growth, increases apoptosis and leads to G1 phase cell cycle arrest (Zheng et al. 2015; Caron et al. 2010).

Therefore, the characterization of Abo1 presented in this chapter brings more information on its cellular and molecular function, and mode of action, which is susceptible to be true for other ATAD2-like proteins, including the cancer-linked human.

3.2. Abo1, but not Abo2, is critical for proper cell growth in fission yeast

In fission yeast there are two uncharacterized ATAD2-like proteins, Abo1 and Abo2, which share a similar domain organization. Conversely to Abo1, Abo2 was not found in RITS purification. This suggested that these two ATAD2 paralogues might have different functions in *S. pombe*. The percentage of sequence identity between human ATAD2 and either Abo1 or Abo2 is quite comparable and is of 25.8 and 24.3%, respectively (Figure 1A). The highest sequence identity corresponds to the known functional domains: the two AAA+ ATPase and the bromodomain. In addition, there is a fourth conserved region located at the C-terminal part of Abo1 and Abo2, which is found only in ATAD2-like proteins and does not correspond to any annotated domain (Cattaneo et al. 2014). Based on sequence alignment, it is thus difficult to state whether one of the two Abo proteins is more functionally related to human ATAD2. Therefore, we started by characterizing both Abo1 and Abo2 proteins.

We first generated strains deleted either for *abo1* or *abo2* genes and we studied their growth abilities. By plating cells to obtain isolated colonies, we observed that both w*t* and *abo2Δ* colonies appear quite similar and homogeneous on plate. Conversely, a clear and remarkable difference in the size of colonies and in their heterogeneity of size was observed for *abo1Δ s*trains (Figure 1B). The majority of the colonies were smaller than the *wt* ones, indicating a defect in growth. Interestingly, the fact that *abo1Δ* colonies with a size comparable to *wt* appeared spontaneously on plate suggested that these are probably revertant (*abo1Δ R*; Figure 1B), which have recovered their ability to grow as *wt* cells. The appearance of this revertant phenotype was observed in all tested *abo1Δ* clonal populations and the frequency of reversion was higher as the number of cell divisions increased.

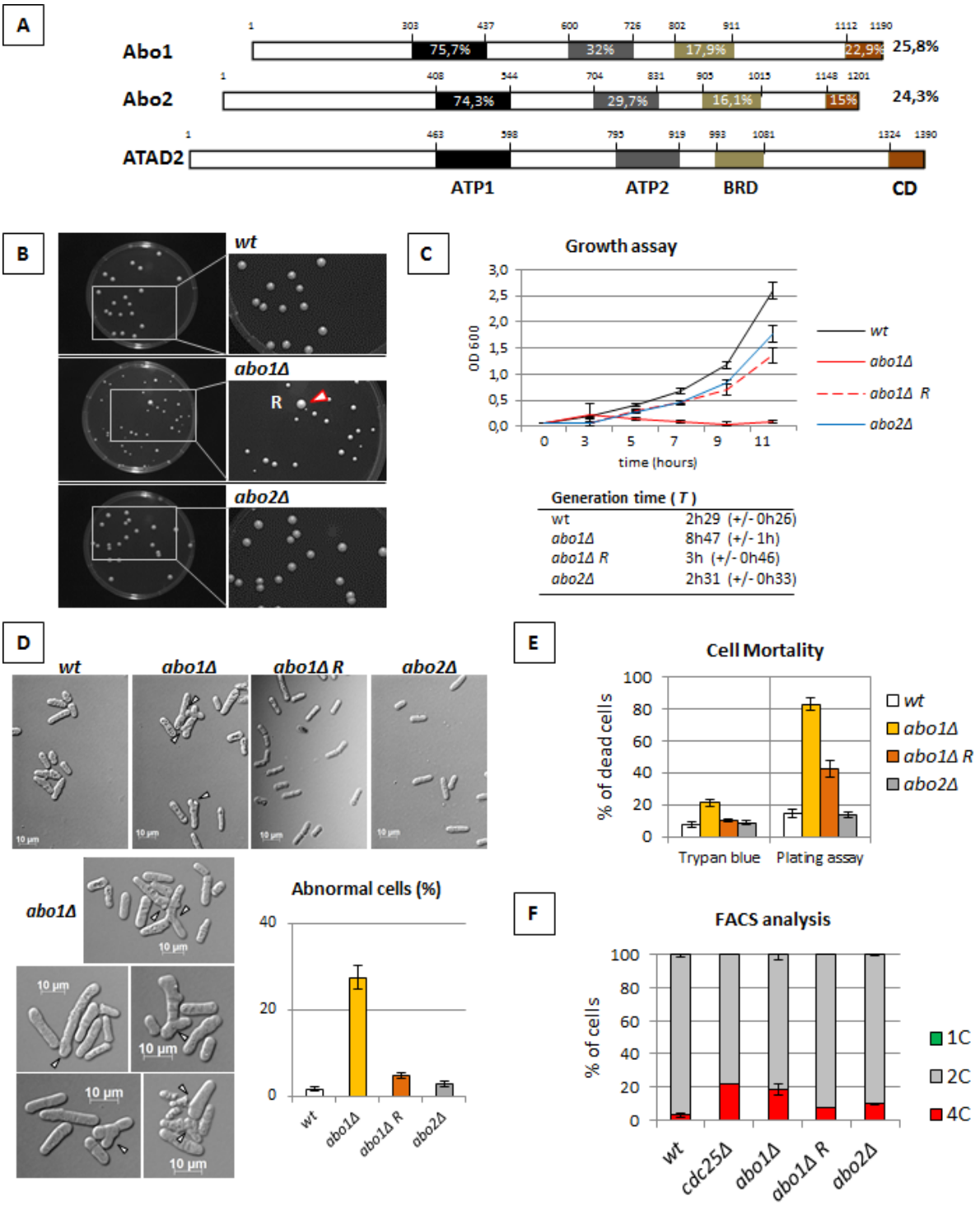


Figure 1. Severe growth defect of *abo1Δ* cells associated to abnormal morphology, cell mortality and delayed cell cycle. A) Schematic representation of human ATAD2 and S. *pombe* Abo1 and Abo2. The percentages of identity between ATAD2 and Abo1/Abo2 are indicated for the overall proteins and for each conserved domain (alignment done with CLUSTALO): ATP1 and ATP2, AAA+ ATPase domain 1 and 2; BRD, BRomoDomain; CD, C-terminal Domain. **B)** Plate with isolated *wt, abo1Δ* and *abo2Δ* colonies. Magnifications show colonies size. 'R' indicates *abo1Δ* revertant colony (red arrowhead). **C)** Graph showing kinetics of cell growth in liquid medium (OD600nm) of *wt, abo1Δ*, *abo1Δ R* and *abo2Δ* cells. Table indicates generation time (*T*) for these strains. **D)** Microscopy images of *wt, abo1Δ*, *abo1Δ R* and *abo2Δ* cells. Magnification of *abo1Δ* cells, white arrows indicate cells with abnormal morphology. Histogram showing % of abnormal cells in *wt, abo1Δ*, *abo1Δ R* and *abo2Δ*

strains (n = 1000). **E)** Histogram showing percentage of dead cells in *wt, abo1Δ*, *abo1Δ R* and *abo2Δ* strains, obtained by trypan blue staining (n = 1000) or by plating assay (3 plates for each tested genotype). **F)** FACS analysis of *wt, cdc25 ts, abo1Δ*, *abo1Δ R* and *abo2Δ* cells. Histogram presents the % of cells with 1C, 2C and 4C DNA content for each genotype. All error bars represent s.d. from three independent replicates.

The growth defect of *abo1Δ* was also confirmed by plating cells in serial dilution spots on solid medium (Figure S2A), whereas *abo2Δ* cells have no major growth defect.

The cell growth of *abo1Δ* and *abo2Δ* cells was monitored more accurately in liquid cultures (Figure 1C). The generation time (*T*) of *wt* cells, grown in rich medium in optimal conditions, was, as expected, of around 2h30. The severe growth defect of *abo1Δ* strains was confirmed with a generation time of ~9h, while it was mainly rescued in revertant *abo1Δ R* cells (*T* of ~3h). The generation time was equal to *wt* for *abo2Δ* cells (*T*~2h30).

In order to confirm that the growth defect observed in *abo1Δ* strains is indeed due to the absence of Abo1 in cells, we transformed both *wt* and *abo1Δ* cells with a plasmid expressing the coding sequence of *abo1* fused to the tag *3HA.* In parallel, cells were transformed with a control plasmid lacking *abo1* coding sequence (empty vector, ø). The expression of Abo1 in cells was checked first at the transcript level by RTqPCR (for endogenous copy and plasmids), and second at the protein level by Western blot anti-HA (Figure S1A). Growth assay in liquid culture showed as expected that *abo1Δ* cells transformed with the empty vector present a severe growth defect compared to *wt* strains. Conversely, the expression of Abo1 in *abo1Δ* cells completely rescued their growth defect (Figure S1B), confirming that it is indeed the absence of Abo1 that is responsible for the severe growth defect observed in *abo1Δ S. pombe* cells. Of note, this experiment also showed that increasing the expression of Abo1 protein in *S. pombe wt* strains does not impact negatively on cell growth.

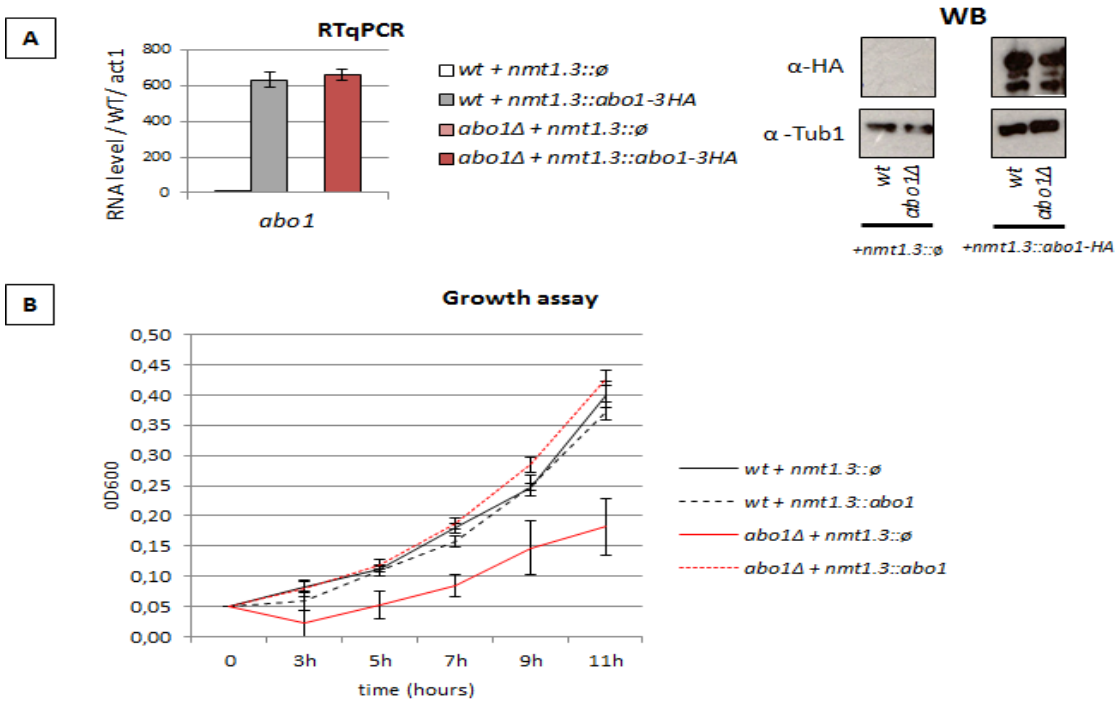


Figure S1. Abo1 complements growth defect of *abo1Δ* cells. A) RTqPCR analysis in *wt* and *abo1Δ* cells transformed with control vector *nmt1.3::ø* or *nmt1.3::abo1-3HA.* RNA level of total *abo1* is relative to *act1* and normalized to the respective RNA levels in wt cells transformed with control vector. WB analysis of Abo1-3HA protein levels in same strains using Tub1 protein levels as control. **B)** Graph showing kinetics of growth in liquid medium (OD600nm) of *wt* and *abo1Δ* cells transformed with control vector *nmt1.3::ø* or *nmt1.3::abo1-3HA.*

In addition, we tested the sensitivity of *abo1Δ* strains to grow at higher temperature (37°C). We found that the growth defect of *abo1Δ* cells is even more dramatic compared to standard temperature (30°C) (Figure S2A). Interestingly, we observed that the growth of *abo2Δ* (Figure S2A) and *abo1Δ R* (Figure S5A) cells was not impaired at 37ºC.

To investigate if the strong growth defect of *abo1Δ* cells at 37°C could also be observed under other stress conditions, we tested their growth ability in the presence of several genotoxic agents (HU, MMS and CPT). We observed that *abo1Δ* cells were sensitive to all tested genotoxic drugs, whereas no particular sensitivity was detected in *abo2Δ* cells (Figure S2B). Altogether, our results showed that the removal of Abo1, but not Abo2, leads to an increased sensitivity to stress conditions.

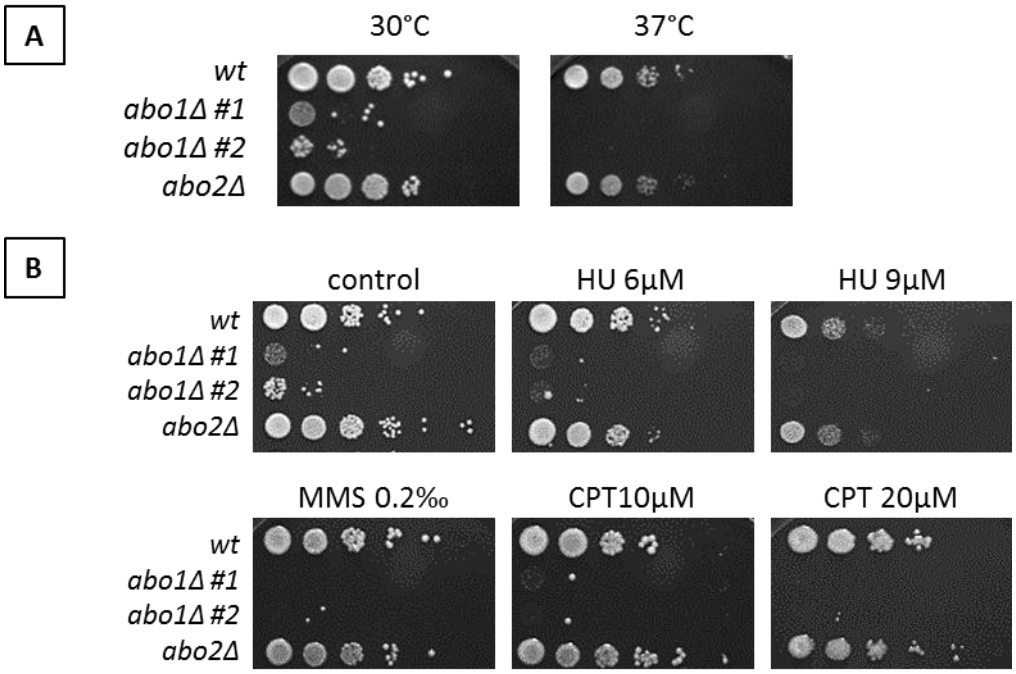


Figure S2. Increased growth defect of *abo1Δ* cells under stress condition. A) Growth assay on solid medium: serial dilution spots of *wt, abo1Δ* (2 isolates) and *abo2Δ* strains plated on YEA medium and incubated at 30°C or 37°C. B) Genotoxic agents sensitivity: serial dilution spots of *wt, abo1Δ* (2 isolates) and *abo2Δ* strains plated on YEA control medium or medium containing HU (6 and 9µM), MMS (0.2‰) or CPT (10 and 20µM).

The severe growth defect of *abo1Δ* strains prompted us to further investigate this phenotype, first by analyzing the morphology of cells. While no noticeable abnormalities were observed in *abo2Δ* and *abo1Δ R* cells, ~30% of *abo1Δ* cells present an evident altered morphology with a variety of shapes: elongated, rounded, curved cells or containing additional abnormal knobs (Figure 1D).

In parallel, we also determined if the severe growth defect of *abo1Δ* cells could be due to increased cell mortality. We used two complementary approaches: the trypan blue staining, which specifically stains dead cells; and a plating assay, in which we plated a known number of cells and counted the number of colonies that eventually appeared after 6 days of growth at 30°C. The trypan blue assay showed that ~20% of the population of *abo1Δ* cells corresponds to dead cells, at the time of the experiment, whereas ~8% of *wt* cells and ~11% of *abo1Δ R* cells (Figure 1E). Similarly, the cell mortality for *abo2Δ* cells is compable to the one of *wt*. Of note, looking individually at the shape of cells after trypan blue staining

showed no positive correlation between cells with an abnormal shape and dead cells. The plating assay showed a similar tendency in cell mortality (Figure 1E). Interestingly, the mortality was significantly higher for *abo1Δ* and *abo1Δ* R cells, whereas it only modestly increased for *abo2Δ* and *wt* cells (Figure 1E). This latter result indicated that the proportion of dead cells probably increases over-time *in abo1*Δ cells, and also in *abo1*Δ R cells although to a lesser extent.

In *S.* pombe, an altered morphology is often associated to defects in the cell cycle (Hayles et al. 2013), thus we also examined by FACS analysis whether *abo1Δ* cells have a problem in the cell cycle progression. In *wt* exponentially growing *S. pombe* cells, the majority of cells are in G2, which is the longest phase of their cell cycle, and the G1 phase is extremely short. Our FACS results showed that, as expected, the vast majority of *wt* cells were in G2 phase (2C DNA content), while G1 cells (1C DNA content) were hardly detectable (Figure 1F). In addition, few 4C cells were observed corresponding to binucleated cells arrested in G2, in which two DNA duplication events occurred before the process of cytokinesis had finished. In this experiment we also included, as a positive control of cells with a G2 arrest (Oliva et al. 2005), a *cdc25* thermosensitive mutant (*ts*) in which ~20% of cells were arrested in G2 (4C DNA content). The population of *abo1Δ* cells*,* similarly to *cdc25 ts* mutants, presented an increased number of arrested cells in the late G2 phase, showing that *abo1Δ* cells are also defective in the cell cycle progression (Figure 1F). Conversely, results that need to be reproduced showed that the cell cycle is not or less affected in *abo2Δ* and *abo1Δ R* strains.

None of the tested phenotypes, which we have shown for *abo1Δ*, were found altered in *abo2Δ* cells, strongly suggesting that Abo1 and Abo2 are not, or poorly, functionally redundant. To further test a possible genetic interaction between Abo1 and Abo2 we constructed the double mutant *abo1Δ abo2Δ* strains. The phenotype of *abo1Δ abo2Δ* cells was overall comparable to the one of *abo1Δ* cells when looking at growth, temperature sensitivity, cell mortality and heterochromatin defects (Figure S3). Thus, altogether these data indicate no important functional redundancy between the *S. pombe* Abo1 and Abo2.

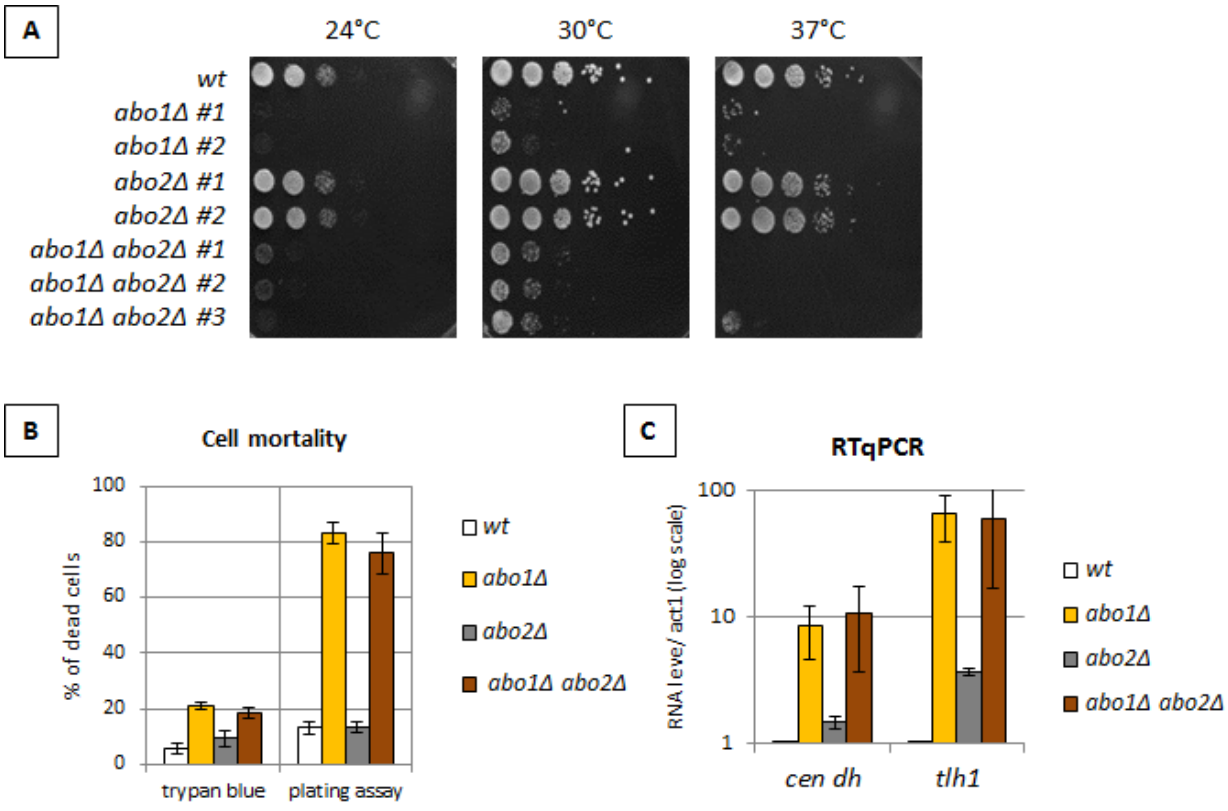


Figure S3. No apparent functional redundancy between Abo1 and Abo2. A) Growth assay on solid medium: serial dilution spots of *wt, abo1Δ* (2 isolates), *abo2Δ* (2 isolates) and double mutants *abo1Δ abo2Δ* (3 isolates) strains plated on YEA medium and incubated at 24°C, 30°C or 37°C. **B)** Histogram showing percentage of dead cells in *wt, abo1Δ*, *abo2Δ* and *abo1Δ abo2Δ* strains. Results obtained by trypan blue staining (n = 1000) or by plating assay (3 plates for each tested genotype). **C)** RTqPCR analysis of constitutive heterochromatin (*cen dh* and *tlh1*) RNA levels in *wt* and *abo1Δ* cells relative to *act1* and normalized to the respective RNA levels in *wt* cells. All error bars represent s.d. from three independent replicates.

Taken together, and in agreement with the function of human ATAD2 in cell proliferation, our results showed a severe growth defect for *abo1Δ* cells, which correlates with an increase in abnormal morphology, cell mortality and a defect in cell cycle progression. Interestingly, *abo1Δ* can spontaneously rescue these defects, although the mechanism of reversion is so far unknown. Finally, none of the cellular defects observed in *abo1Δ* was found in *abo2Δ* cells.

3.3. Abo1 and human ATAD2 share functional redundancy

The role of the fission yeast Abo1 and human ATAD2 proteins in regulating cell growth and their high sequence similarity suggested that these two proteins could share similar functions. To directly test this possibility, we investigated whether the expression of human ATAD2 in *abo1Δ* fission yeast cells could rescue their growth defect. We cloned the DNA sequence of human *ATAD2* on three plasmids that allow its expression in *S. pombe*, and which differ only for the strength of the promoter (strong *nmt1.3*, medium *nmt1.41* or weak *nmt1.81* expression). These plasmids were then used to transform both *wt* and *abo1Δ* cells. In parallel, *wt* and *abo1Δ* cells were transformed with control plasmids lacking the *ATAD2* coding sequence (control vector, ø). The expression of human ATAD2 in yeast cells was verified, both at the RNA and protein levels. Noticeably, ATAD2 protein level appeared higher in *wt* than in *abo1*Δ cells, whereas *ATAD2* RNA levels were comparable between these two strains (Figure S4). This suggests that the presence of Abo1 in cells may increase ATAD2 protein stability.

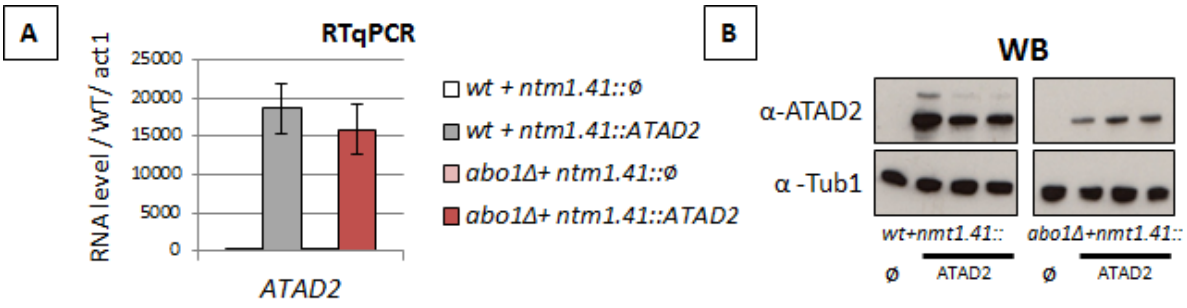


Figure S4. Complementation of *abo1Δ* growth defect with human ATAD2, control of expression of human ATAD2. A) RTqPCR analysis in *wt* and *abo1Δ* cells transformed with control vector *nmt1.41::ø* or *nmt1.41::ATAD2.* RNA level of *ATAD2* is relative to *act1* and normalized to the respective RNA level in wt cells transformed with control vector. **B)** WB analysis of ATAD2 protein levels in same strains as A) using tubulin1 (Tub1) protein levels as control. All error bars represent s.d. from three biological replicates.

We then performed a growth assay on liquid culture and followed over-time the increase in the cell population. Remarkably, the expression of human ATAD2 in *abo1Δ* cells, under the stronger promoter (*nmt1.3*), completely rescued their growth defect; indeed we could observe a growth for these cells comparable to *wt* (Figure 2). A partial rescue of the growth defect was also obtained in *abo1*Δ cells expressing human ATAD2 under medium (*nmt1.48*) and weak (*nmt1.81*) promoters. This latter result further suggested that the rescue of the growth defect of *abo1*Δ cells depends on the level of ATAD2 present in these cells. In addition, we noticed that the ectopic expression of human ATAD2 in *wt* cells has no effect on their growth, as already observed for the overexpression of Abo1. Taken together, these results showed that the expression of human ATAD2 protein rescues the severe growth defect of *abo1*Δ cells, suggesting that, despite their relatively long distance in evolution, the function of human ATAD2 in regulating cell proliferation is conserved in Abo1.

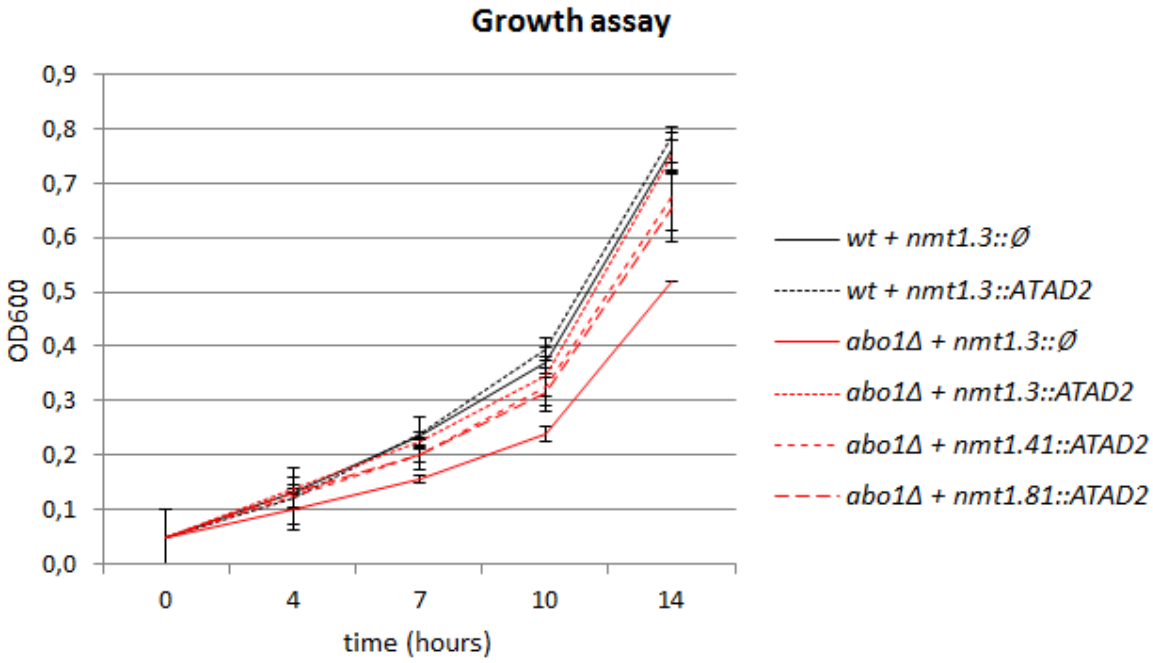


Figure 2. Human ATAD2 complements growth defect of *abo1Δ* cells. Graph showing kinetics of growth in liquid medium (OD600nm) of *wt* and *abo1Δ* cells transformed with control vector *nmt1.41::ø*, *nmt1.3::ATAD2, nmt1.41::ATAD2* or *nmt1.81::ATAD2.* All error bars represent s.d. from three independent replicates.

3.4. Transcriptomic analysis of *abo1Δ* cells revealed a role for Abo1 in transcription regulation and gene silencing

Our initial results on the characterization of Abo1 showed that it is required for the silencing of gene expression at constitutive heterochromatin regions in fission yeast. In order to determine whether Abo1 plays a role in regulating gene expression at other places in the genome, we performed a transcriptomic analysis of *abo1Δ* cells. For this investigation, we decided to perform a transcriptomic analysis on two different *abo1*Δ strains. Additionally, we included in our study an *abo1*Δ revertant strain issued from one of the two *abo1*Δ strains used for the transcriptomic analysis. Analysis of the transcriptome of this *abo1*Δ *R* strain has been conducted with the aim of obtaining some indications on the molecular mechanism of *abo1Δ* reversion.

The transcriptomic analysis was performed by using massive RNA-sequencing on total RNA. Total RNA samples from each *wt, abo1Δ* (*abo1Δ* #1 and *abo1Δ* #2) and *abo1Δ #2R* strains (Figure 3A) were obtained in two independent technical replicates for each genotype. For each replicate, three independent cultures (named A, B, C) were made. Several controls, such as the existence of a growth defect at 30°C and 37°C, cell morphology defects and higher mortality, were conducted to verify the absence of reversion in *abo1Δ* mutants in the cultures made for RNA preparations (Figure S5A and B). Indeed, as previously explained, *abo1Δ* (non revertant) strains present a growth defect at 30°C, which is even more drastic at 37°C, an increased number of cells with altered morphology, an elevated cell mortality and an accumulation of constitutive heterochromatic RNAs compared to *wt* cells (Figure 1, Results 2.4.). Conversely, we observed in *abo1Δ* revertant cells a rescue of these phenotypes, except for the desilencing of constitutive heterochromatin (Figure S5C). This latter finding suggested that the function of Abo1 in cell proliferation may be separated from its function in heterochromatin gene silencing.

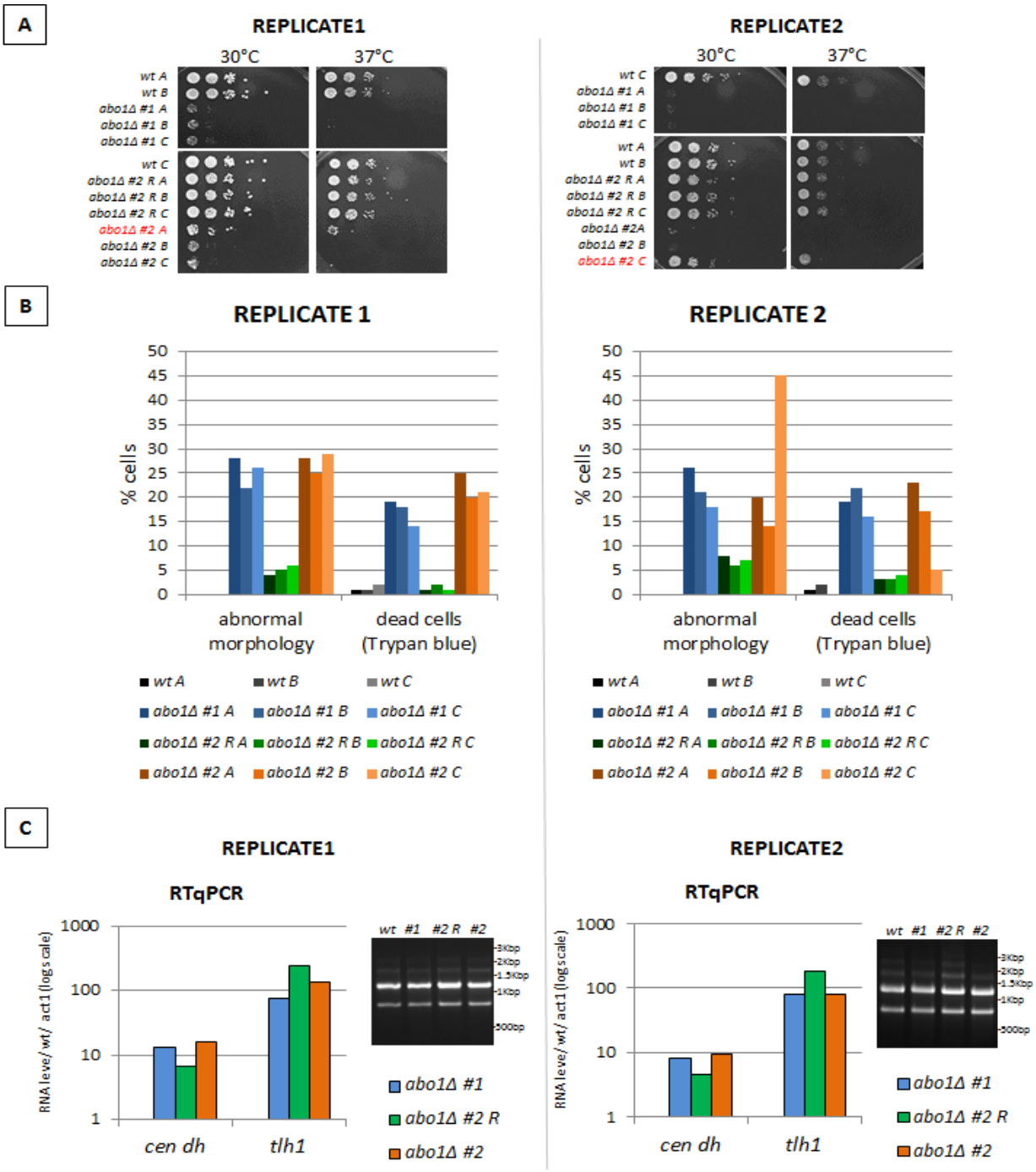


Figure S5. Samples for *abo1Δ* transcriptomic analysis, control of reversion. A) Growth assay on solid medium: serial dilution spots of *wt, abo1Δ* #1, *abo1Δ* #2 and *abo1Δ #2 R* strains (A, B and C cultures; replicates 1 and 2) plated on YEA medium and incubated at 30°C or 37°C. Labelled in red, cultures removed from the analysis. **B)** Histogram showing percentage of abnormal and dead cells (trypan blue staining) for *wt, abo1Δ* #1, *abo1Δ* #2 and *abo1Δ #2 R* strains (A, B and C cultures, replicates 1 and 2) (n = 200). **C)** Verification of RNA preparations migrating on agarose gel and by RTqPCR analysis of heterochromatin *cen dh and tlh1* RNAs (relative to *act1* and normalized to the respective RNA levels in *wt* cells).

We thus removed from our analysis every *abo1Δ* cultures with a clear or suspected reverted phenotype. This was the case for the *abo1Δ* #2 A replicate 1 and the *abo1Δ* #2 B replicate 2 (Figure S5A). The quality and purity of total RNA preparations were also checked. RNA was migrated on agarose gel, analyzed with a spectrophotometer at different wavelengths to check for the absence of proteins and verified by qPCR for the previously shown desilencing at constitutive heterochromatin regions (Figure S5C, Results 2.4).

The analysis of our transcriptomics data showed that the levels of 910 RNAs were significantly altered in *abo1Δ* cells (Figure 3B and C). Interestingly, 95% of genes are up-regulated (868/910, with a fold change FC ≥ 2), and only 5% are downregulated (42/910, FC ≤ 0,5), compared to *wt*. This first result indicated that Abo1 act as a general negative regulator of transcription, potentially inducing gene silencing rather than mediating transcription activation.

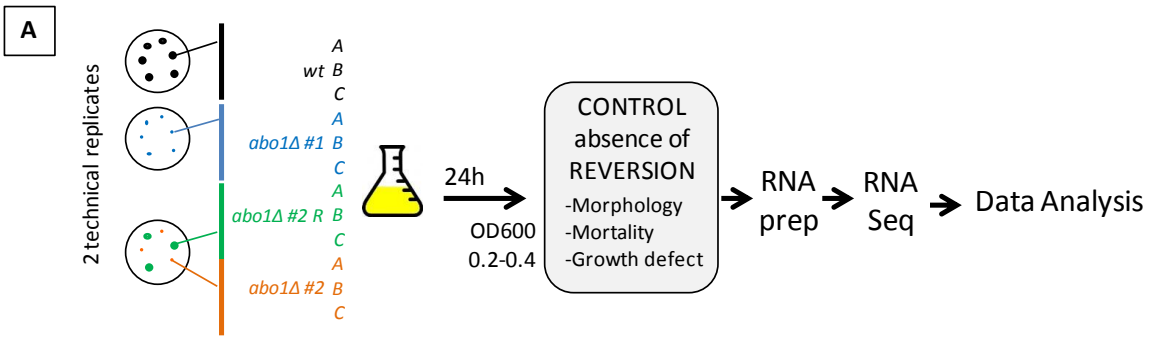
In addition, among upregulated genes, half of them are protein-coding genes (PCGs), whereas the other half are non-coding RNAs (ncRNAs) (Figure 3B). Importantly, the ncRNAs upregulated in *abo1Δ* cells represent approximately 30% of all *S. pombe* annotated ncRNAs, thus indicating a rather general role for Abo1 in mediating the silencing of ncRNAs.

The strain *abo1Δ R*, despite its reversion of the growth defect, also showed a global deregulation in gene expression, although less drastic than *abo1Δ* cells (Figure 3B and C). Indeed, 363 genes are upregulated in revertant cells, while 2 genes are downregulated (*abo1* being one of them). As in the case of *abo1Δ* cells, about 50% of upregulated genes correspond to PCGs and 50% to ncRNAs (Figure 3B). Interestingly, a majority (274 out of 363) of upregulated genes in *abo1Δ R* are also upregulated in *abo1Δ* cells (Figure 3C), while none of the downregulated genes in *abo1Δ* is upregulated in revertant strain (Figure 3D).

Next, we investigated if the deregulated genes in *abo1Δ* and *abo1ΔR* cells were dispersed or clustered in specific regions of the genome. Noticeably, we found a high density of misregulated genes at subtelomeric regions of chromosomes 1 and 2 with, respectively, up to 65% and 44% of genes contained within these regions that are upregulated (Figure 3E). This is not the case for both subtelomeric regions from chromosome 3, which host the highly repeated rDNA units. In the case of *abo1Δ R* cells, up to 67% and 54 % of genes contained at

the subtelomeres of chromosomes 1 and 2, respectively, are upregulated. This indicates a crucial role of Abo1 in silencing gene expression at subtelomeric regions.

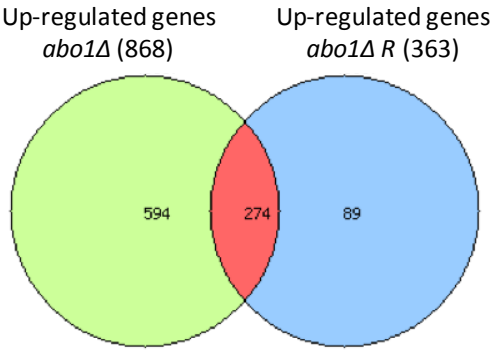
Finally, we investigated whether Abo1 could regulate the expression of several genes that belong to the same pathway. Gene ontology analysis was carried out using the *Gene Ontology Enrichment Analysis Software Toolkit* (http://omicslab.genetics.ac.cn/GOEAST/php/batch_genes.php). Due to the large set of upregulated genes in *abo1Δ* or *abo1Δ R* cells, no clear biological, cellular or molecular affected process was identified. We next focused our analysis on upregulated genes with a higher misregulation in the revertant strain compared to *abo1Δ* (FC *abo1ΔR* / FC *abo1Δ* ≥ 2). Among the 53 genes more deregulated in *abo1Δ R* than in *abo1Δ*, 6 are grouped in the RNA-directed DNA polymerase activity molecular function (GO:0003964, p-value $5{,}72.10^{-8}$). Remarkably, these genes belong to the *Tf2* transposon family (*Tf2-1, Tf2-2, Tf2-3, Tf2-6, Tf2-7 and Tf2-11*) and are more upregulated in *abo1ΔR* cells (from 14 to 52 times compare to *wt*) than in *abo1Δ* (from 4 to 13 times). Transposon RNAs accumulation was verified by RTqPCR measuring the levels of transcripts of the *TE reverse transcriptase* gene and of the LTR regions (Long Terminal repeats) (Figure 3F). Thus, the RTqPCR analysis confirmed a higher level of transposable transcripts in *abo1Δ R* cells compared to *abo1Δ*. Finally, we checked if the misregulation of Tf2 transposable elements could also affect the expression of neighbouring genes. None of the genes in proximity of the 7 upregulated *Tf-2* showed an altered expression (data not shown), indicating that Abo1's action at *Tf-2* is specific for transposable sequences, conversely to its regional role in gene silencing at subtelomeric regions.



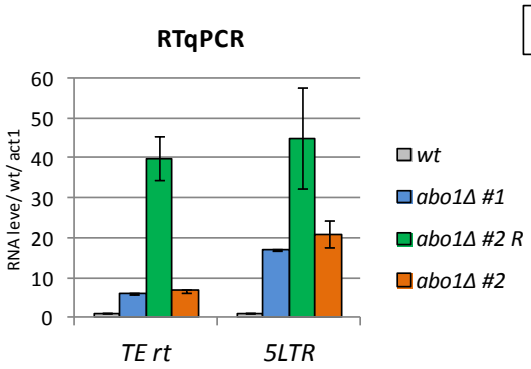
B

	Up-regulated				Down-regulated			
	PCG	%	ncRNA	%	PCG	%	ncRNA	%
abo1Δ	423	8,5	445	29,7	41	0,8	1	0,07
abo1Δ R	180	3,6	183	12,2	2	0,4	0	0

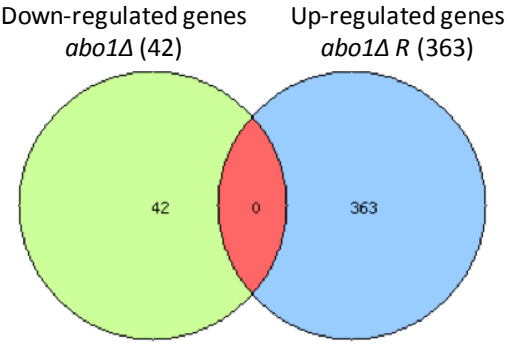
C



F



D



E

	Chr. 1		Chr. 2		Chr. 3	
	Tel. left	Tel. right	Tel. left	Tel. right	Tel. left	Tel. right
abo1Δ	65 % (33/51)	57 % (21/37)	44 % (21/48)	38 % (17/45)	4 % (2/50)	0 % (0/49)
abo1Δ R	66 % (34/51)	67 % (25/37)	54 % (26/48)	46 % (21/45)	4 % (2/50)	0 % (0/49)

Figure 3. Transcriptomic analysis of *abo1Δ* cells reveals a wide deregulation of both protein-coding and non-coding genes. A) Scheme representing the protocol used to obtain RNA samples for transcriptomic analysis (see text). **B)** Table showing numbers and percentages (%) of protein-coding genes (PCGs) and ncRNAs (ncRNAs) significantly up- and down-regulated in *abo1Δ* and *abo1Δ R*, compared to *wild* type. **C)** Venn diagram showing common genes upregulated in *abo1Δ* and *abo1Δ R* cells. **D)** Venn diagram showing no common genes down-regulated in *abo1Δ* and upregulated in *abo1Δ R* cells. **E)** Table showing numbers and percentages of misregulated genes within 100kb of subtelomeric sequences (left and right telomeres) on the three chromosomes in *abo1Δ* and *abo1Δ R*

cells. **F)** RTqPCR analysis of transposable transcripts (*TE rt* and *5LTR*) in *wt, abo1Δ* #1, *abo1Δ* #2 and *abo1Δ #2 R* cells. RNA levels are relative to *act1* and normalized to the respective RNA levels in *wt* cells. All error bars represent s.d. from three independent replicates.

Altogether, this first analysis of our transcriptomic data showed an important role of Abo1 in regulating gene expression of both protein-coding and non-coding genes. In addition, we discovered that Abo1 is important for the silencing of subtelomeric regions and of transposable elements. Revertant *abo1Δ* cells also present a general misregulation of gene expression and, interestingly, an even higher level of RNA accumulation for transponsable elements. It has not been addressed yet whether this increased expression of transposable elements observed in *abo1Δ R* cells is key for rescuing the *abo1Δ* growth defect. Of note, at the moment, the transcriptomic data analysis is still ongoing. Further findings may come from this analysis and help to better define the function of Abo1 as well as the mechanism by which *abo1Δ* cells revert.

3.5. Abo1 is connected to the chromatin- and transcription-linked protein Tfg3

To address the molecular function of Abo1, we also purified Abo1 and identified its potential partners by mass spectrometry analysis. We made a strain in which a *TAP*-tagged sequence was added at the C-terminal end of *abo1* gene. We then conducted large-scale purifications of Abo1-TAP. Since, Abo1 homologues were shown to localize mostly within the insoluble fraction during the early step of protein purification, we decided to perform the Abo1-TAP purification using a strategy that permits to fractionate DNA in order to release at least part of Abo1 proteins from chromatin. The protocol we used consisted of a combination of gentle sonication and benzonase treatment to obtain DNA fragments with a peak of around 200bp (Figure S6A). Similar approaches have been already described in the literature to purify chromatin-associated protein networks and also for the purification of Yta7, the Abo1 homologue in *S. cerevisiae* (Lambert et al. 2009).

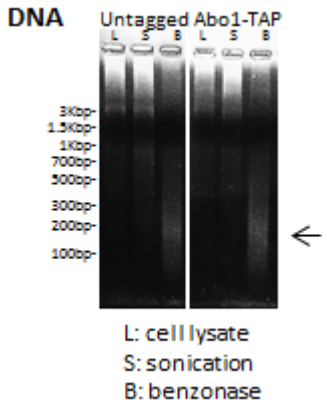


Figure S6. Abo1-TAP purification: control of DNA digestion. Agarose gel to check DNA digestion on extracted DNAs from untagged and Abo1-TAP strains after cell lysis (L), after sonication (S) and after benzonase treatment (B). Arrow shows obtained DNA fragments (less than 200 bp).

The Abo1-TAP purification was performed five times. For each purification, a whole cell extract from an untagged strain, used as negative control, was submitted to the exact same conditions as the Abo1-TAP purification. Eluates from Abo1-TAP and untagged purifications were loaded on an acrylamide gel, which was later silver-stained (Figure 4A). Two specific bands appeared highly enriched in Abo1-TAP purification compared to the untagged one. Mass spectrometry (MS) analysis was performed on these two bands and demonstrated that

the higher band corresponded to Abo1-TAP, as expected, whereas the second band corresponded to Tfg3.

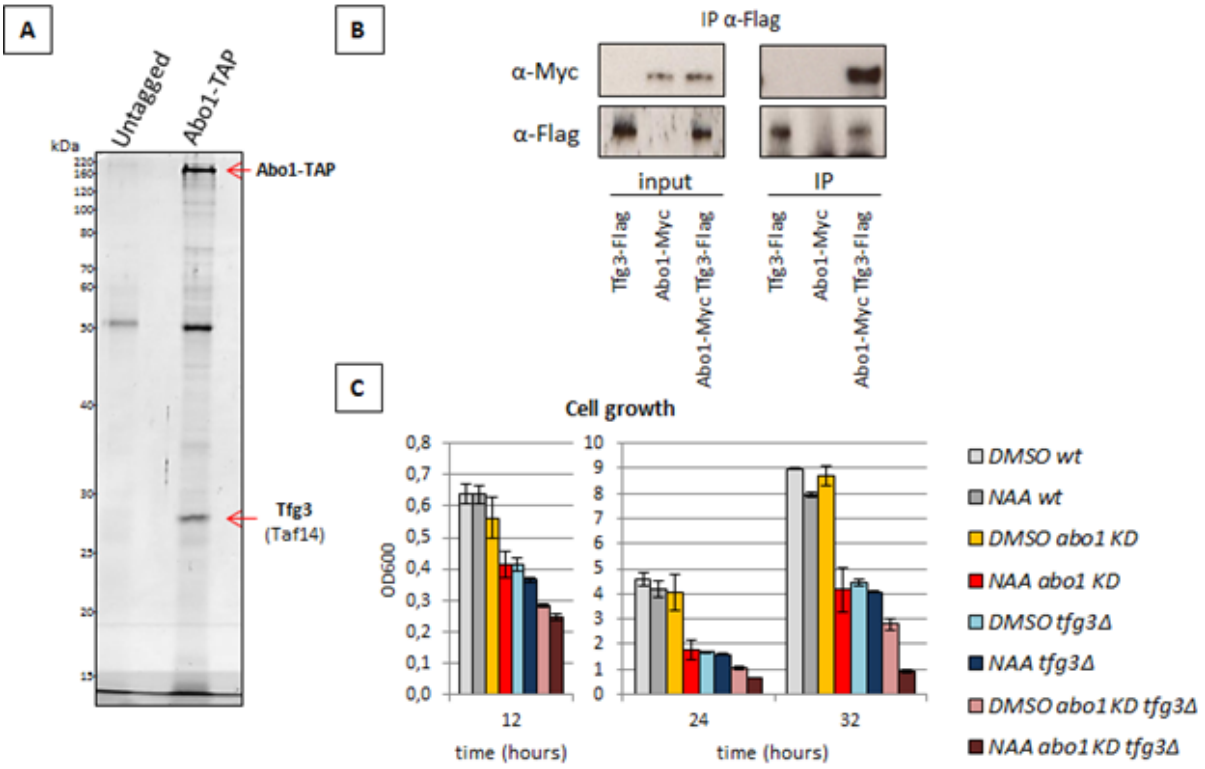


Figure 4. Abo1 is connected to the chromatin and transcription-linked protein Tfg3. A) Silver staining of an SDS-polyacrylamide gel containing untagged and purified Abo1-TAP. TAP-purification was performed in 5 replicates and analyzed by mass spectrometry. Red arrows correspond to specific identified bands: Abo1-TAP and Tfg3. **B)** Abo1-Tfg3 interaction analyzed by co-immunoprecipitation (CoIP in strains *tfg3-flag*, *abo1-13myc* and *tfg3-flag abo1-13myc* (IP α-Flag, WB α-Myc or α-Flag). CoIP was performed twice with two biological replicates. **C)** Histogram showing cell growth (OD600nm) over-time for *wt, abo1* KD, *tfg3*Δ and *abo1* KD *tfg3*Δ strains in presence of NAA (auxin) or DMSO (control condition). All error bars represent s.d. from three biological replicates.

Interestingly, the Tfg3 homologue in *S. cerevisiae*, called Taf14 (TATA binding protein-Associated Factor 4), has been shown to have a crucial role in RNA polymerase II transcription initiation and in chromatin modification (Schulze, Wang, and Kobor 2009), and, its homologue in human, called MLLT3 (Mixed-Lineage Leukemia Translocated To Chromosome 3 Protein), has been strongly associated to cancer (Soler et al. 2008). In *S. pombe*, Tfg3 has been shown to interact with the transcription factor TFIIF, which is important for general RNA pol II binding to promoter of genes. Moreover, at elevated

temperature, Tfg3 has been also reported to interact with TFIID, another transcription factor that constitutes the RNA pol II pre-initiation complex (Kimura and Ishihama 2004). Tfg3 is connected to chromatin also in fission yeast, indeed it was shown to be a member of the HAT Mst2 complex (Wang et al. 2012) and to interact with several chromatin remodeler complexes, as INO80 or SWI/SNF (Hogan et al. 2010; Monahan et al. 2008).

To confirm Tfg3-Abo1 interaction, we generated strains expressing Flag-tagged Tfg3 and Myc-tagged Abo1. Tfg3-Flag was immunoprecipitated and the presence of Abo1-Myc was checked by Western blot (Figure 4B). This experiment showed that Abo1-Myc copurifies with Tfg3-Flag, whereas no Abo1-Myc was detected in Flag purified materials from control strains. Thus, confirming that Tfg3 is indeed a partner of Abo1.

To assess whether Abo1-Tfg3 physical interaction plays a role in the function of Abo1 we explored the genetic interaction between these two proteins, focusing on cell growth. To avoid any misinterpretation that could be caused by *abo1Δ* cells spontaneous and frequent reversion of the growth defect, we generated a conditional Abo1 knock-down strain (off-AID, Auxin Inducible Degron) (Kanke et al. 2011). The advantage of using an inducible Abo1 knock-down system for our epistasis analysis, compared to stable *abo1* deleted strains, is that the possible appearance of cells that reverted Abo1 mutants phenotype is eliminated.

The off-AID knock-down strategy exploits a mechanism of protein degradation present in plants to specifically degrade a fission yeast protein. A repressive sequence *IAA17* (fused to an *HA-tag)* was added to *abo1* gene in fission yeast strains that ectopically express a plant E3-ubiquitin ligase named TIR1. The off-AID system is induced adding a vegetal hormone called auxin to culture medium; this compound activates TIR1 that after recognizing the IAA17 sequence linked to Abo1 induces Abo1 specific degradation (Material and methods 11.).

In agreement with the growth defect of *abo1Δ* cells, the induction of Abo1-IAA17 protein degradation generated a rapid appearance of a defect in cell growth compared to control cells (clearly visible after ~8-12h) (Figure S7A). The growth defect was correlated to the dramatic loss of Abo1 protein level (Figure S7B). In addition, Abo1 knock-down (KD) also led to an increase in transposable elements transcripts, as observed previously for *abo1Δ* cells (Figure S7C).

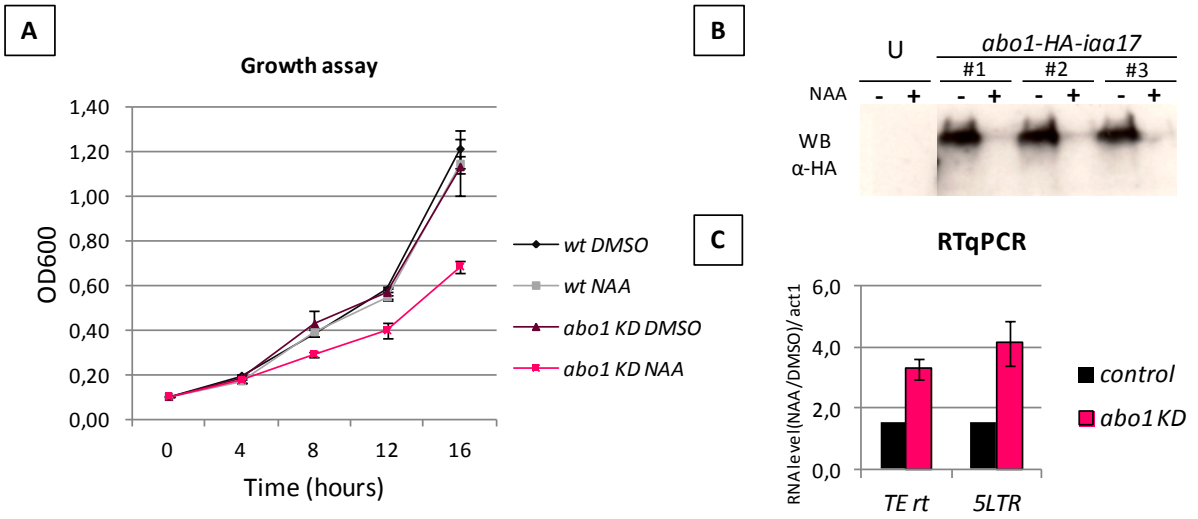


Figure S7. Characterization of Off-AID Abo1 knock-down system. A) Graph showing cell growth (OD600nm) over-time for *wt* and *abo1* KD cells in presence of NAA (auxin) or DMSO (control condition). **B)** WB analysis of Abo1-HA protein levels in *wt* (U, in which Abo1 is untagged) and *abo1-HA-iaa7* strains (3 isolates). +: cultures in presence of NAA; -: culture in presence of DMSO. **C)** RTqPCR analysis of transposable transcripts (*TE rt* and *5LTR*) in *wt* and *abo1KD* strains. RNA levels are relative to *act1* and expressed as a ratio between RNAs expression in cells grown under NAA and DMSO. All error bars represent s.d. from three biological replicates.

We then deleted *tfg3* gene in the conditional *abo1 KD* cells and examined their cell growth, before and after the knock-down of Abo1 (Figure 5C). The depletion of Abo1 was obtained in cells growing at a logarithmic rate in presence of auxin (NAA). In parallel, cells were also grown without auxin, as a control. Cells depleted for Abo1, as expected, present a growth defect compared to control conditions (Figure 4C). In addition, *tfg3Δ* strains also present a growth defect in our culture conditions (EMMc medium, 26°C). Importantly, depletion of Abo1 in *tfg3Δ* cells led to a more severe phenotype than each of the single mutants (Figure 4C).

Here, the negative genetic interaction that we found between Abo1 and Tfg3 is in agreement with the idea that they may act together in contributing proper cell proliferation in *S. pombe*. Indeed, our genetic analysis showed that the function of these two proteins is not completely redundant; in fact both single mutants are already defective in growth. However, the double mutant presents a more severe phenotype, indicating that cells tolerate more a single mutation than the double one. Knowing that Abo1 and Tfg3 physically

interact, this negative genetic interaction most likely reflects proteins that belong to the same complex or act in the same pathway (Forsburg 2001).

3.6. Analysis of Abo1 purification by quantitative proteomics uncovered multiple physical links to chromatin

In parallel to the classical mass spectrometry analysis conducted by sequencing proteins found in the two bands that were cut on the silver stained gel, we also performed a quantitative mass spectrometry analysis from total mixtures of Abo1-TAP or untagged purifications. Such analysis permitted not only to identify new proteins enriched in Abo1-TAP purification compared to the untagged one, but also to quantify their differences in enrichment, providing some information on the "proximity" of each of these proteins to Abo1.

Proteins found in Abo1-TAP purification are displayed in a volcano plot, which separate proteins according to their average enrichment, compared to the untagged strain, and the statistical significance of their enrichment (p-value) within the three purifications conducted for this analysis (Figure 5A). The names of some specific proteins, which we will later analyze in this study, are indicated in the volcano plot.

In addition, the most enriched proteins in Abo1-TAP compared to the untagged purification are represented in a scheme that combines together all subunits that belong to same complexes (Figure 5B).

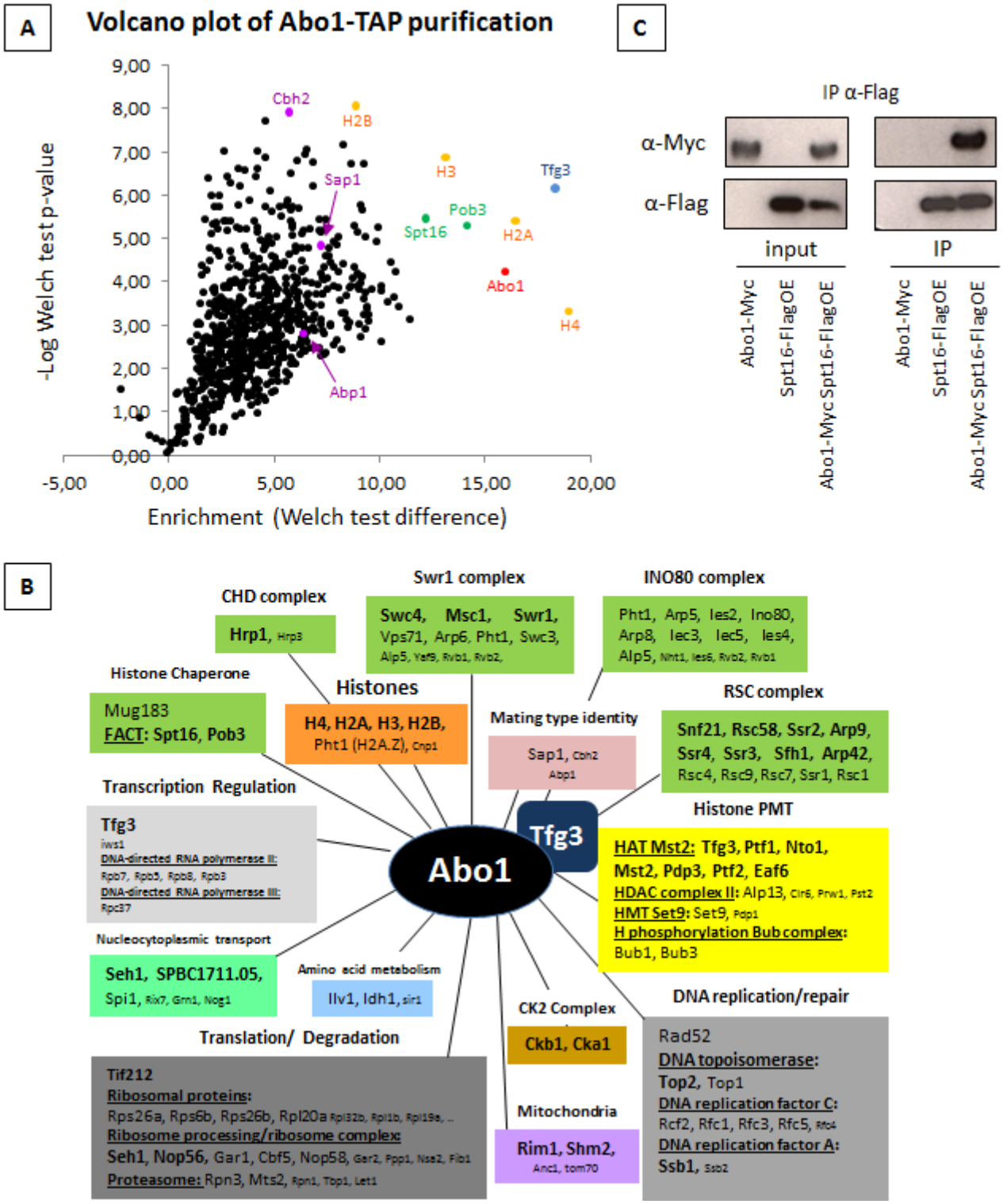


Figure 5. Analysis of Abo1-TAP purification. A) Statistical analysis of Abo1-TAP purification using a volcano plot. Each protein found in the purification is represented as a dot to which corresponds an average enrichment (welch test difference), compared to the untagged strain, and a p-value (-Log welch test p-value) within three purifications conducted for this analysis. Names of proteins of interest for this study are indicated: Abo1 (red), Tfg3 (blue), Histones (H3, H4, H2A and H2B; orange), FACT subunits (Spt16 and Pob3; green), Sap1 and CENPB proteins (Abp1, Cbh2) (violet). **B)** Schematic

view of Abo1's network shows its strong association to histones and protein regulating chromatin and transcription. All these proteins were found enriched in Abo1-TAP purification compared to untagged (fold change > 2). **C)** Abo1-Spt16 interaction analyzed by co-immunoprecipitation in strains *spt16-flagOE*, *abo1-13myc* and *spt16-flagOE abo1-13myc* (IP α-Flag, WB α-Myc or α-Flag). CoIP was performed three times with two biological replicates. *OE:* overexpression.

Interestingly, among the list of proteins identified in Abo1-TAP purification, we found Tfg3 but not the other subunits of the transcription factor TFIIF, Tfg1 and Tfg2 (Kimura and Ishihama 2004). Moreover, several other known Tfg3's partners, such as the HAT Mst2 complex or the chromatin remodeler complex INO80 were found in this list (Figure 5B). This suggests that Tfg3 may have different functions depending on whether it associates with Abo1 or Tfg1/2.

Remarkably, the set of proteins enriched in Abo1-TAP purification shows a strong enrichment for proteins linked to chromatin and DNA-based processes, such as transcription, DNA replication and repair. Among the major putative partners, we found all histones (both canonical and histone variants), histone chaperones (FACT and Mug183) and many chromatin remodelers expressed in *S. pombe* (CHD, Swr1, INO80 and RSC). Interestingly, most subunits of these complexes were actually identified in Abo1 purification, supporting the idea of a tight link between Abo1 and these chromatin-related complexes. Several complexes containing histone modifiers were also found enriched in Abo1-TAP purification, such as the HAT complex Mst2, the HDAC complex II, the HMT H4K20 Set9 and the histone phosphorylation Bub complex.

Therefore, the analysis of Abo1-TAP purification uncovered multiple links between Abo1 and chromatin, suggesting that this protein may be a hub on which a large number of complexes acting on chromatin would connect to.

Interestingly, similarly to Abo1, the FACT complex (composed of Spt16 and Pob3) is known to act at the interplay between chromatin and transcription regulation. Because FACT acts as a histone chaperone and is required for efficient transcription elongation (Lejeune et al. 2007), we decided to further test the interaction between Abo1 and FACT. For that, we generated strains expressing Flag-tagged Spt16 and Myc-tagged Abo1 that were used to perform a co-immunoprecipitation, in which we purified Spt16-Flag and analyzed the

presence of Abo1-Myc by Western blot (Figure 5C). Abo1 clearly copurified with Spt16, whereas no Abo1-Myc was detected in Flag purified materials from control strains expressing either Abo1-Myc or Spt16-Flag alone.

In addition, Abo1 purification revealed a possible interaction between Abo1 and proteins regulating mating type switching, protein translation and degradation, as well as mitochondrial factors, proteins involved in nucleocytoplasmic transport and amino acid metabolism. Interestingly, a possible interaction with Abo1 was found also for Sap1 and 2 homologues to human CENPB (Abp1 and Cbh2), whose depletion generates a phenotype that has many similarities with *abo1*Δ cells (Cam et al. 2008; Zaratiegui, Vaughn, et al. 2011; Noguchi and Noguchi 2007). The confirmation of the interaction between Abo1 and these proteins and a first investigation of its possible functional relevance is presented at the end of this chapter (Additional Results section 2).

Purification of *S. cerevisiae* Yta7 has already been done (Kurat et al. 2011; Tackett et al. 2005; Lambert et al. 2009). This permitted us to compare the list of interactors obtained in Yta7 purification with the one we conducted on Abo1. Several proteins are shared between Abo1 and Yta7 protein purifications: histones, histone chaperones (FACT, Rtt106), chromatin remodelers (CHD, RSC), the HAT Mst2 complex and proteins involved in transcription and DNA replication (Figure S8A). This is in agreement with the idea that the molecular function of these homologues could be conserved. In addition, the kinase complex CK2, which was previously shown to interact with Yta7 and to induce its S-phase specific phosphorylation and subsequent detachment from chromatin (Kurat et al. 2011), is also found in Abo1 purification, indicating that CK2 may also regulate Abo1 in fission yeast.

Recently, the TAP-purification of human ATAD2 was published (Morozumi et al. 2015). We also compared Abo1 purification with the one of ATAD2. This showed, not surprisingly, that they both interact with histones and proteins linked to chromatin, such as FACT or CHD complexes, or to DNA replication (Figure S8B). The similarities among the protein network of Abo1, Yta7 and ATAD2 further support the idea of common molecular function(s) for these ATAD2-like proteins.

A

S. cerevisiae Yta7	*S. pombe* Abo1	
Yta7	Abo1	
Histones		
H4	H4	
H2A	H2A	
H3	H3	
H2B	H2B	
Htz1	Pht1	
Histone Chaperones		
Spt16	Spt16	FACT
Pob3	Pob3	
Rtt106	Mug183	
Chromatin remodelers		
Rsc58	Rsc58	RSC
Rsc6	Ssr3	
Rsc8	Ssr1	
Rsc4	Rsc4	
Arp9	Ssr2	
Chd1	Hrp1	CHD
Post-translational modifier		
Sas3	Mst2	Mst2 HAT
Pdp3	Pdp3	
CK2 Kinase Complex		
Ckb2/Ckb1	Ckb1	
Cka1/ Cka2	Cka1	
DNA topoisomerase		
Top1	Top1	
Top2	Top2	
DNA-directed RNA polymerase II		
Rpb2	Rpb2	
Rpb5	Rpb5	

B

Human ATAD2	S. pombe Abo1
Atad2	Abo1
Histones	
H4	H4
H2A	H2A
H3	H3
H2B	H2B
H2A.Z	Pht1
Histone Chaperone (FACT)	
Supt16	Spt16
Ssrp1	Pob3
Chromatin remodeler (CHD)	
Chd1/Chd2	Hrp1
Chd1/Chd3	Hrp3
Post-translation modifiers	
Kat7/ Kat6b	Mst2
Kdm5b	Msc1
DNA replication factor C	
Rfc1	Rfc1

Figure S8. Similarities between the purifications of Abo1, Yta7 and ATAD2. A) Table showing common proteins found in the purifications of Abo1 and Yta7 (Kurat et al. 2011; Tackett et al. 2005; Lambert et al. 2009). **B)** Table showing common proteins found in the purifications of Abo1 and ATAD2 (Morozumi et al. 2015).

3.7. The critical function of Abo1 in cell growth is linked to histones

Our MS analysis on Abo1-TAP purification indicated that Abo1 binds to histones, in agreement with its suspected histone chaperone activity. We thus decided to investigate if there was also a genetic interaction between Abo1 and histones genes in relation to cell growth. To do so, we analyzed the growth of Abo1 knock-down strains when genes encoding for histone H3 and H4 were deleted.

In *S. pombe*, histone genes are found in pairs (either coding for H3-H4 or H2A-H2B) that are transcribed in a divergent way from a common regulation region (histones H3-H4 genes represented in Figure 6A). We deleted either *hht1-hhf1* or *hht2-hhf2* (two different copies of genes encoding for histones H3-H4) in off-AID Abo1 knock-down cells and we investigated their growth over-time. Cells *hht1-hhf1Δ* and *hht2-hhf2Δ* grew similarly to *wt* strains, while Abo1 KD cells, as expected, presented a growth defect compared to *wt* (2-fold decrease after 32h of culture) (Figure 6B). However, and importantly, cells that were both depleted for Abo1 and deleted for either *hht1-hhf1* or *hht2-hhf2* histones pair showed a significant rescue of the growth defect (Figure 6B). Of note, this latter result is from an experiment that has been conducted with three different biological replicates, but done only once so far. The experiment will be redone before making a solid conclusion of this result.

Therefore, our preliminary results showed that a decrease in the amount of histones partially rescues the growth defect of Abo1 depleted strains, thus supporting the hypothesis that Abo1 could act as histone chaperone. This suggests that the growth defect of Abo1 KD or *abo1Δ* cells may be caused by a deregulation of histone protein levels.

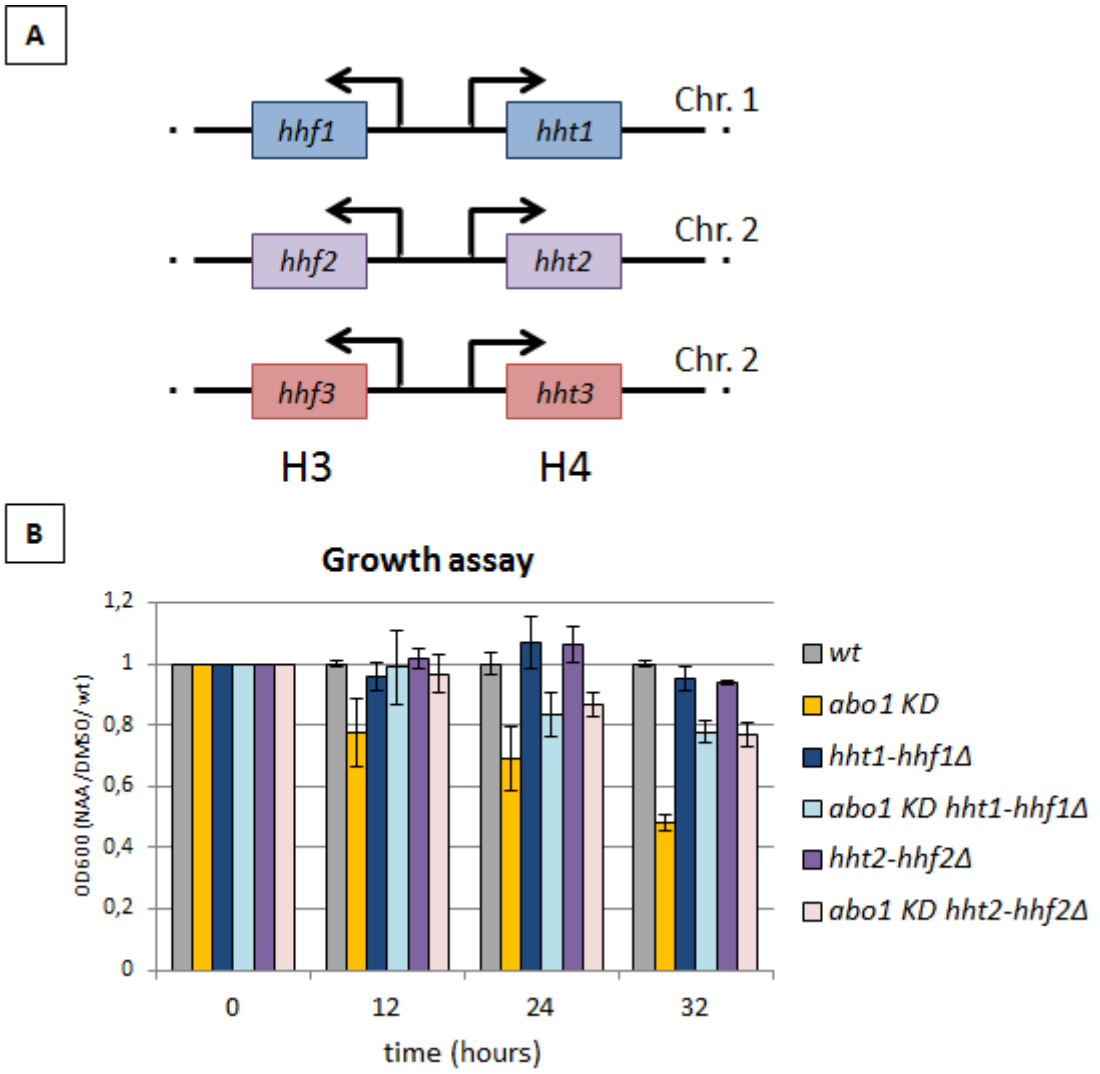


Figure 6. Abo1 could act as a possible histone chaperone. A) Scheme representing the organization of the 3 copies of histones genes H3 and H4 in *S. pombe* (*hht1-hhf1, hht2-hhf2, hht3-hhf3*). Arrows indicate transcription orientation. **B)** Histogram showing cell growth (OD600nm) over-time for *wt, abo1* KD, *hht1-hhf1*Δ, *hht2-hhf2*Δ and double mutants *abo1* KD *hht1-hhf1*Δ and *abo1* KD *hht2-hhf2*Δ. Data are expressed as a ratio of OD600 nm in presence of NAA/ OD in presence of DMSO (control condition), and normalized to *wt*. All error bars represent s.d. from three biological replicates (preliminary result).

3.8. General conclusion

In this chapter, we performed an in depth characterization of Abo1 in *S. pombe.* We found that Abo1 is critical for proper cell growth and this function seems to be conserved between Abo1 and its human homologue ATAD2. Therefore, this study supports the idea that a better understanding of Abo1 may also contribute toward elucidating the role of ATAD2 in cancer.

We initially found that Abo1 interacts with the RITS complex and is required for proper constitutive heterochromatin gene silencing. Our transcriptomic analysis of *abo1Δ* cells further showed that Abo1 is a general regulator of gene expression, controlling the expression of approximately 10 and 30% of the protein-coding and non-coding genes in fission yeast, respectively. Intriguingly, a large fraction of genes requiring Abo1 for their silencing are localized at subtelomeric regions. In addition, we also showed that Abo1 is required to silence transposable elements.

The purification of Abo1 complex uncovered a clear interaction with Tfg3, which also links Abo1 to chromatin and transcription regulation, and our genetic analysis further suggests that these two proteins together are critical for proper cell growth in *S. pombe.* Abo1 purification also discovered multiple other physical links between Abo1, transcription and chromatin. Importantly, we showed that deletions of histone genes H3-H4 partially rescues the growth defect of Abo1 deficient cells, indicating that the requirement of Abo1 for cell growth and gene silencing may be linked to histones and Abo1's putative histone chaperone activity.

In conclusion, Abo1 is likely to play an important function at the interplay between chromatin and transcription, which is key for an efficient gene silencing. Proteins with such a function are crucial in eukaryotes and they are deregulated in several pathologies, including cancer. Our results permitted to uncover new possible molecular functions of Abo1 in fission yeast that could be conserved throughout the evolution in other ATAD2-like proteins.

Additional results:

1. The bromodomain of Abo1 binds to histone H4 independently of its acetylation status

Histones were among the most enriched proteins in Abo1-TAP purification, and the interaction with histones had been also shown for Abo1 homologues in human and *S. cerevisiae*. Interestingly however, while ATAD2 bromodomain is known to bind preferentially acetylated histones H3 and H4 (Caron et al. 2010; Revenko et al. 2010), Yta7 binds histones but in an acetylation-independent manner (Gradolatto et al. 2009; Jambunathan et al. 2005). The bromodomain of Abo1 is unconventional and, similarly to the one of Yta7, lacks some residues critical for binding acetylated histones (Gradolatto et al. 2009; Cattaneo et al. 2014).

We decided to experimentally investigate whether the bromodomain of Abo1 could preferentially bind to acetylated histones. For this purpose, we cloned a truncated sequence of *abo1* gene, corresponding to its annotated putative bromodomain (BD), fused to the *GST*-tag sequence. This GST-Abo1BD protein was expressed in bacterial cells and protein extract was used to perform a histone H4/ H4ac (tetra-acetylated) peptide pull-down experiment, in collaboration with S. Khochbin's team. The presence of GST-Abo1BD, copurifying with beads coupled to H4/H4ac peptides, was observed by Western blot. The bromodomain of ATAD2 was used as positive control and, as expected, preferentially binds acetylated histones H4 (Caron et al., 2010). The results showed that the bromodomain of Abo1 binds to histone H4, but independently of its acetylation status (Figure 28).

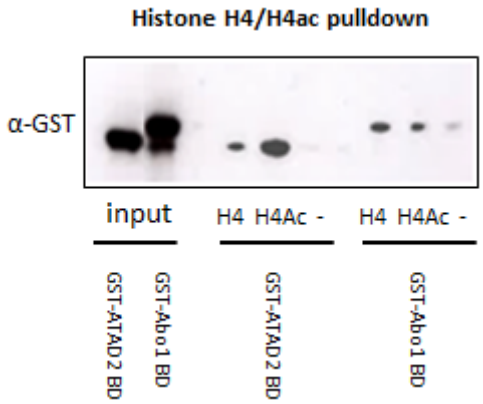


Figure 28. The bromodomain of Abo1 binds to histone H4 independently of its acetylation. Histone H4/H4ac/- pull-down of GST-Abo1BD and GST-ATAD2BD (BD: bromodomain; '-'is a control, beads without histone peptides). WB α-GST. Experiment performed once.

2. Functional similarities between Abo1 and Sap1/ CENPB proteins

Among Abo1 partners, we became interested in the possible interaction with Sap1 and proteins homologues to CENPB in humans (Abp1, Cbh1 and Cbh2). As mutant strains for these proteins have many phenotypic similarities with *abo1* deleted cells, we further investigated the physical and functional link between Abo1 and Sap1 and CENPB homologues. Indeed, CENPB proteins have been reported to be involved in the silencing of centromeric heterochromatin and of transposable elements (Cam et al. 2008), whereas Sap1 has been recently shown to be required for transposon integration in the genome (Jacobs et al. 2015; Hickey et al. 2015). In addition, a deregulation of both Sap1 and CENPB proteins is associated with cellular morphological defects, increase in cell mortality and abnormal mitosis (de Lahondes, Ribes, and Arcangioli 2003; Zaratiegui, Vaughn, et al. 2011).

Therefore, first we decided to confirm the interaction between Abo1 and Sap1 or Abp1 by co-immunoprecipitation. We constructed strains expressing Myc-tagged Abo1 and either Sap1 or Abp1 tagged by Flag. Then, we purified Flag-tagged proteins and looked at Abo1-

Myc presence by Western blot. The interaction between Abo1 and Sap1 was clearly confirmed compared to control purifications (Abo1-Myc or Sap1-Flag strains alone) in which Abo1-Myc was not detected (Figure 29A). Our results also indicated that the interaction between Abo1 and Abp1 is probably true, although the Western blot signals were much weaker (Figure 29B).

Interestingly, similarly to *abo1*Δ, it was shown that CENPB deletion mutants have a severe growth defect (in double mutants *abp1Δ cbh1Δ* or *abp1Δ cbh2Δ*, triple deletion mutants are inviable) that can quickly revert due to a mutation in Sap1 (Zaratiegui, Vaughn, et al. 2011). Therefore, we investigated whether Sap1 depletion (strains deleted for *sap1* are indeed not viable) could also rescue *abo1Δ* growth defect. We obtained two different temperature-sensitive (ts) mutants of Sap1 (*sap1-1* and *sap1-48*), which at 24°C sensitive temperature grow comparably to *wt* cells, but present a severe growth defect at the restrictive temperature of 30°C (Noguchi and Noguchi 2007) (Figure 29C). Then, we deleted *abo1* in these two *sap1 ts* strains and we analyzed the growth of these double mutants on solid medium, incubating plates either at 24°C or 30°C. Cells deleted for *abo1,* present a growth defect compared to *wt*, which is even more severe at 24°C. Double mutant *abo1Δ sap1 ts* strains also present a growth defect at the permissive temperature of 24°C, but with certain heterogeneity among isolates. Strikingly, at the restrictive temperature of 30°C, none of these double mutants was viable.

This result demonstrates that, differently to CENPB mutants, no rescue of *abo1Δ* growth defect occurred upon Sap1 depletion. On the contrary, the additive phenotype observed in double mutants suggests that Abo1 and Sap1 are both required for normal cell growth and their function is not completely redundant in cell.

.

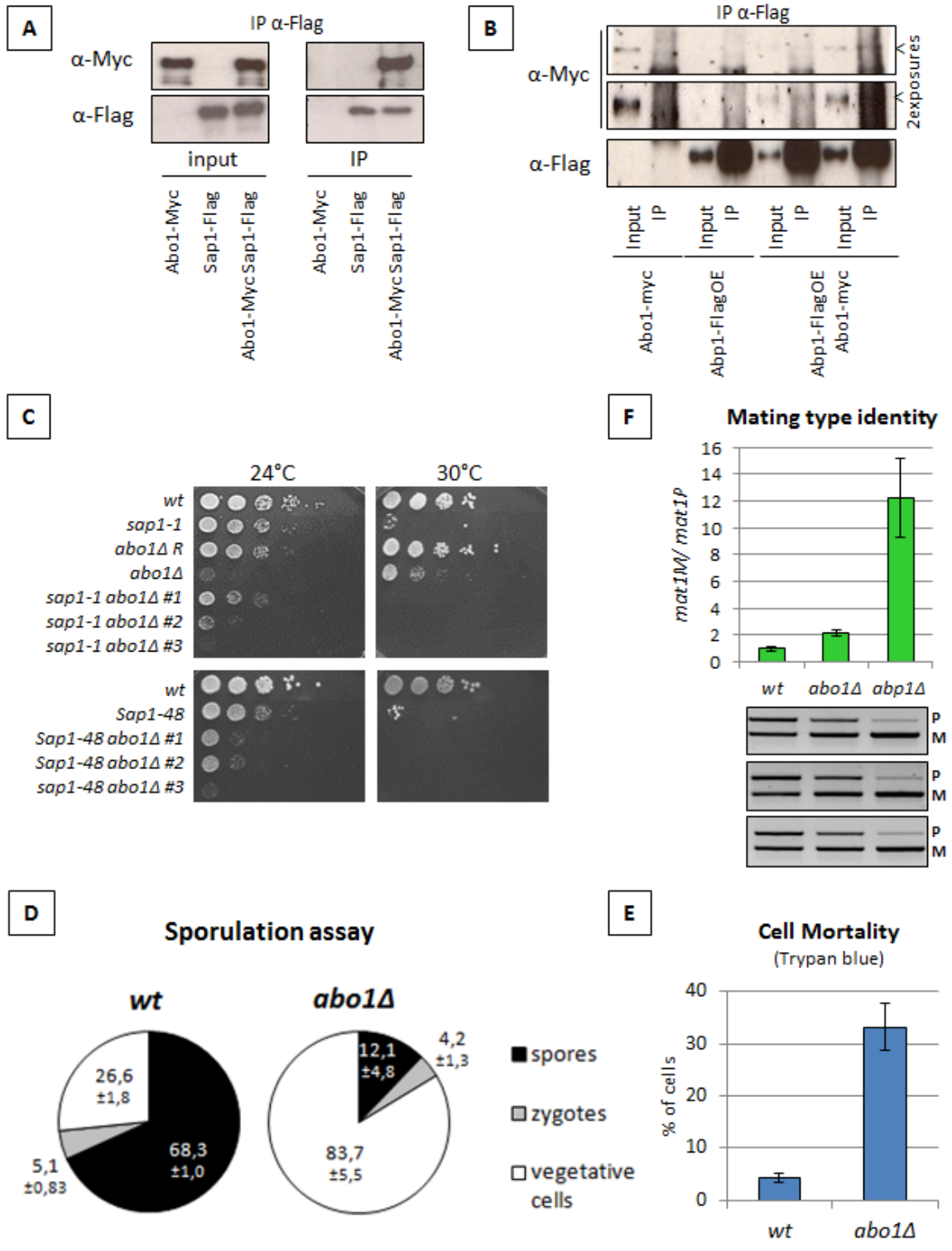


Figure 29. Functional link between Abo1 and Sap1/ Abp1. A) Abo1/Sap1 interaction analyzed by CoIP in strains *sap1-flag*, *abo1-13myc* and *sap1-flag abo1-13myc* (IP α-Flag, WB α-Myc or α-Flag). CoIP was performed twice with two biological replicates. **B)** Abo1/Abp1 interaction analyzed by CoIP in strains *abp1-flagOE*, *abo1-13myc* and *abp1-flagOE abo1-13myc* (IP α-Flag, WB α-Myc or α-Flag). CoIP was performed once with two biological replicates. **C)** Growth assay on solid medium: serial dilution spots of *wt, sap-1, sap1-48*, *abo1Δ, abo1Δ R* and double mutants *sap1-1 abo1Δ* (3 isolates)

and *sap1-48 abo1Δ* (2 isolates) strains plated on YEA medium and incubated at 24°C or 30°C. **D)** Pie charts showing percentage of vegetative cells, zygotic cells and spores in *wt* and *abo1Δ h90* cells plated on SPAS medium (2days). **E)** Histogram showing percentage of dead vegetative cells (trypan blue staining) for the same *wt* and *abo1Δ* strains. (n = 300) **F)** Mating type identity analysis of *wt, abo1Δ a*nd *abp1Δ* cells grown on SPAS medium. Agarose gels showing specific bands for *mat1P* and *mat1M*. Histogram showing quantified signals expressed as a ratio *mat1M/mat1P*. All error bars represent s.d. from three independent replicates.

CENPB proteins and Sap1 were also shown to play a crucial role in the process of mating type switching (Introduction 2.2.3.3.). Sap1 is required to generate a double-strand break in the mating type region, which is then healed by a translocation event that permits cells to switch their mating type information (*mat1M* or *mat1P*) (Arcangioli, Copeland, and Klar 1994). CENPB regulates the directionality of switching, for instance removal of Abp1 correlates with preferential *mat1M* (rather than *mat1P*) (Aguilar-Arnal, Marsellach, and Azorin 2008). Therefore, we decided to explore whether Abo1 could also share this function with CENPB and Sap1. First, we tested the ability of *h90 abo1Δ* cells to undergo the process of sexual differentiation. We plated cells on nitrogen-free sporulation-induced medium (SPAS), and then counted the percentage of spores and zygotes after 2 days. Similarly to what was reported for *abp1Δ* cells (Aguilar-Arnal, Marsellach, and Azorin 2008), we observed a severe defect in sporulation for *abo1Δ* (12% of spores observed) compared to *wt* cells (68% of spores) (Figure 29D). This result could only be partially explained by an increase in cell mortality, as only ~30% of dead *abo1Δ* cells were observed on SPAS medium (Figure 29E). This major problem in sexual differentiation prompted us to investigate a possible defect in mating type identity of *h90 abo1Δ* cells by PCR, using specific primers to amplify *mat1M* and *mat1P*. Purified PCR products were then migrated on agarose gel, ethidium bromide signal of the two bands (corresponding to *mat1M* and *mat1P*) was quantified using an image quantification software (ImageJ) and expressed as *mat1M / mat1P* ratio. Our analysis showed a weak but consistent defect in mating type identity for *abo1Δ* cells. Indeed, we could observe an increase in the ratio *mat1M / mat1P* for *abo1Δ* (2.13 ± 0.22) compared to *wt* cells (0.95 ± 0.14), although modest compared to *abp1Δ* (12.26 ± 2.95) (Figure 29F).

Altogether, we confirmed the interaction between Abo1 and Sap1 or the CENPB protein Abp1. In addition, we could observe that these three proteins share many functional similarities, including a common role in regulating the process of mating type identity.

DISCUSSION AND PERSPECTIVES

In this section, the main results obtained during my PhD are first resumed and then discussed in the light of other studies published in this field of research. In addition, our study has opened the way for new possible future investigations that will be also discussed.

In this study, we exploited the purification of two proteins known to bind chromatin-associated RNAs in order to characterize new actors in chromatin-based gene silencing in *Schizosaccharomyces pombe.*

We found that Mmi1, an RNA binding protein required for facultative heterochromatin formation, interacts with Ccr4-Not, which is a complex mainly known to process 3'ends of RNAs (Collart and Panasenko 2012). Interestingly, we uncovered that Ccr4-Not mediates both chromatin- and RNA-based silencing at heterochromatin regions.

In parallel, we studied two new partners of RITS (RNA-Induced Transcriptional Silencing), an RNAi effector complex required for heterochromatin formation and gene silencing. Both partners, Spt6 and Abo1, are believed to be potentially involved in transcription regulation by acting as histone chaperones.

Our initial functional characterization of Spt6 showed its role in gene silencing at constitutive and facultative heterochromatin. Interestingly, the interaction between RITS and Spt6 may be important for its localization and function at heterochromatin.

The homologue of Abo1 in human is the cancer-linked protein ATAD2, whose function has not been yet understood. Therefore, we decided to characterize Abo1 in fission yeast with the aim of better understanding also the role of ATAD2 in cancer. Our study on Abo1 uncovered a crucial role for this protein in regulating gene silencing at constitutive heterochromatin regions and also of hundreds of protein-coding and non-coding genes at euchromatin. In addition, the identification of Abo1's protein network revealed several links with chromatin- and transcription-linked proteins and our latter results suggest a possibly function of Abo1 as histone chaperone in fission yeast. Interestingly, we showed that the severe growth defect observed in *abo1Δ* cells is completely rescued by the expression of human ATAD2, thus suggesting that the function of these proteins in regulating cell growth seems conserved between yeast and humans.

Mmi1 interacts with Ccr4-Not, a new regulator of heterochromatin formation and gene silencing

The RNA-binding protein Mmi1 is a key protein for the silencing of meiotic genes in *S. pombe*, regulating both meiotic RNA levels and the establishment of facultative heterochromatin in these regions. Indeed, the YTH domain of Mmi1 permits its binding to meiotic RNAs, where it then mediates their degradation by recruiting the exosome machinery. In addition, Mmi1 has been shown to be required for the deposition of the methyl-H3K9 repressive mark at meiotic genes (Zofall et al. 2012; Hiriart et al. 2012).

In our study, we revealed a new interaction between Mmi1 and Ccr4-Not, a multi-subunit complex, highly conserved in eukaryotes, that regulates gene expression at multiple levels. In particular, Ccr4-Not contains three catalytic subunits: two deadenylating enzymes (Ccr4 and Caf1), and an E3 ubiquitin ligase (Not4) (Collart and Panasenko 2012). The role of the Ccr4-Not subunit Rcd1 is mostly unknown in eukaryotes, aside from a study in mammals that suggests a function for this protein in mediating miRNA silencing (Chen et al. 2014). Interestingly, we showed that Rcd1 is required for the interaction between Mmi1 and Ccr4-Not, suggesting thus that the deletion of this subunit may also affect a possible function of Ccr4-Not mediated by Mmi1.

In this study, we discovered a previously unknown role of the Ccr4-Not complex in regulating heterochromatin formation and/or gene silencing at all heterochromatin regions (both facultative and constitutive) in *S. pombe*. Cells deleted for the catalytic subunits of this complex showed more severe phenotypes. Therefore, although the mechanism of action of Ccr4-Not at heterochromatin is not yet fully understood, our results suggest that both deadenylation and ubiquitination are required for its function. We can speculate that these catalytic subunits regulate, either by RNA deadenylation or protein ubiquitination, the expression of proteins that control heterochromatin spreading, chromatin dynamics or transcriptional activation. Interestingly, Not4 has been shown to ubiquitinate and induce the proteasome-mediated degradation of Jdh2, a demethylase that regulates the methylation level of H3K4 (a mark of active transcription); this role of Not4 is conserved between yeast and humans (Mersman et al. 2009).

Aside from the catalytic subunits, Rcd1 also has a crucial role in heterochromatin assembly and gene silencing, and this is also the case at constitutive heterochromatin, where Mmi1 is not known to have a function. Our results indicate that Rcd1, apart from mediating the interaction with Mmi1, seems to have a general key contribution to the function of Ccr4-Not.

The high conservation of the Ccr4-Not complex in eukaryotes suggests that its function in regulating heterochromatin assembly and gene silencing may also be conserved throughout evolution. Interestingly, in support of this hypothesis, a recent publication has shown a role of Ccr4-Not in controlling telomeric repeat silencing in germ cells during *D. melanogaster* early development (Morgunova et al. 2015).

In the next two sections of this discussion, the role of Ccr4-Not at facultative and constitutive heterochromatin will be analyzed in more detail in order to provide a more complete overview of the function of this complex at heterochromatin regions.

Ccr4-Not is required for facultative heterochromatin integrity

A recent publication showed that Mmi1 permits the recruitment of the Ccr4-Not complex to meiotic RNAs (Cotobal et al. 2015). Despite the known role of Ccr4-Not in regulating RNA and protein stability in cells, we did not observe any contribution of this complex in regulating the expression of meiotic genes, either at the RNA or the protein level.

Surprisingly, we found that Ccr4-Not is required for facultative heterochromatin integrity, regulating the deposition of the methyl-H3K9 repressive mark at meiotic genes. In addition, we showed that Ccr4-Not catalytic subunits and Rcd1 also mediate the binding of Mmi1 to chromatin at meiotic genes. Interestingly, the function of Ccr4-Not in recruiting Mmi1 in these regions may be specific for chromatin; indeed, the same Ccr4-Not subunits do not seem to mediate Mmi1 binding to meiotic RNAs.

The molecular mechanism by which Ccr4-Not mediates methyl-H3K9 deposition at meiotic genes is yet to be understood. We can speculate that Crr4-Not recruits other chromatin-linked proteins that, in addition to Mmi1, could contribute to the assembly of facultative heterochromatin in these regions. For instance, Ccr4-Not was shown to interact with the RITS complex and to mediate its recruitment to meiotic genes (Cotobal et al. 2015). In order to mechanistically explain our results, it would be interesting to better characterize the functional link between Ccr4-Not and RITS and, furthermore to explore whether Ccr4-Not could mediate the recruitment of other heterochromatin proteins to meiotic genes, such as the H3K9 HMT Clr4 or the HP1 protein Swi6.

Ccr4-Not promotes constitutive heterochromatin gene silencing

Our results showed that Rcd1 and catalytic subunits of the Ccr4-Not complex are required for proper gene silencing at constitutive heterochromatin. The role of Ccr4-Not in these regions, different from that at facultative heterochromatin, is mainly based on regulating the level of heterochromatin transcripts, rather than acting on the chromatin state. Indeed, in mutant cells for Ccr4-Not, we observed an increased level of heterochromatin transcripts, with no major change in the H3K9me repressive mark at centromeric *dh* and subtelomeric *tlh1* regions. We can speculate that a possible role of Ccr4-Not in regulating constitutive heterochromatin integrity could be masked by the predominant role of the RNAi-pathway in these regions. In support of this hypothesis, a clear decrease in H3K9me was indeed observed in Ccr4-Not mutant cells at subtelomeres, but in regions outside the RNAi-targeted sequences *tlh1* and *tlh2* (Cotobal et al. 2015). Therefore, this result suggests that Ccr4-Not may contribute more to constitutive heterochromatin spreading, rather than to its assembly.

The mechanism of action of Ccr4-Not in the silencing of constitutive heterochromatin is still unclear and, in addition, the localization of this complex in these regions still needs to be proven. The RITS complex is a crucial regulator of constitutive heterochromatin formation and gene silencing, so a functional characterization of the interaction between RITS and

Ccr4-Not could help elucidate the mechanism of recruitment and function of Ccr4-Not at constitutive heterochromatin.

Analysis of the RITS purification to identify new proteins involved in heterochromatin silencing and/or formation

The RITS complex is the nuclear RNAi effector in fission yeast and it was shown to be required for heterochromatin assembly and gene silencing. RITS is a trimeric complex: Ago1 permits RITS recruitment to newly transcribed heterochromatin RNAs and Chp1 permits RITS anchoring to H3K9 methylation by its chromodomain, whereas the third subunit, Tas3, mainly acts as a bridge between the other two components (Verdel et al. 2004).

In our study, we purified RITS with the aim of better understanding its mechanism of action at heterochromatin and, in addition, to reveal new possible functions associated with this complex. Purification of Chp1-TAP coupled to high stringency semi-quantitative analysis of the mass spectrometry (MS) results permitted identification of 42 proteins. Interestingly, aside from proteins already known to be involved in heterochromatin silencing and chromatin regulation, we were also able to identify proteins connected to other biological functions such as transcription and DNA replication and repair. The possible involvement of RITS in those processes is currently investigated by our team.

In order to specifically identify proteins interacting with the RITS complex, and not just with the Chp1 subunit, we repeated the Chp1-TAP purification in cells deleted for *ago1* or *tas3*. Interestingly, we showed that 9 proteins (including Ago1) no longer interact with Chp1 in the absence of Tas3, when the integrity of the RITS complex is lost.

However, there is evidence in the literature of a possible role of Chp1-Tas3 dimer in heterochromatin silencing without Ago1, therefore independently of RITS (Schalch et al. 2011). Our proteomic approach cannot actually distinguish whether these 9 proteins interact with Chp1-Tas3 dimer or with the entire RITS complex. In order to discriminate between these two possibilities, we could generate *tas3-TAP* strains *in wt* and *ago1Δ background* to be used for a TAP purification. Among the 9 identified proteins, those interacting with the entire RITS complex would be expected to copurify with Tas3-TAP in *wt* but not in *ago1Δ* strains.

Chp1 is known to tightly link the RITS complex to chromatin via its chromodomain. In order to uncover new functions of RITS outside of the chromatin context, we thus aimed to repeat the Chp1-TAP purification in strains in which RITS was no longer able to bind to heterochromatin. The most straightforward strategy consists in using strains in which the *clr4* gene, which codes for the HMT responsible for H3K9me, has been deleted. However, the severe phenotype resulting from the deletion of this gene could influence the stability of RITS and therefore our proteomic analysis. We also undertook a second strategy to loosen RITS binding to heterochromatin which consists of using a mutant of Chp1 (Chp1V24R) that has been shown *in vitro* to reduce 500-fold its binding to H3K9me (Schalch et al. 2009). Fission yeast cells expressing this Chp1V24R mutant present a defect in heterochromatin comparable to *chp1Δ* strains (Schalch et al. 2009). In addition, we were also able to confirm *in vivo* by ChIP that the binding of Chp1V24R at centromeres and meiotic genes was strongly decreased compared to wild type Chp1; interestingly, Chp1V24R binding to Ago1 was conserved (data not shown). My contribution to this ongoing study has been to generate *chp1v24r-TAP* strains that were used to repeat the TAP purification in triplicate, also including the *untagged*, *chp1-TAP* and *chp1-TAP clr4Δ* strains in the analysis. The MS raw results of these purifications are now available in our team and their analysis will integrate our results on the RITS interactome, in particular providing new insights into unknown possible functions of the RITS complex outside the chromatin context.

RITS interacts with the transcription-coupled histone chaperone Spt6

Our results revealed an interaction between the RITS complex and Spt6, which is a conserved protein in eukaryotes acting at the interplay between transcription and chromatin regulation. Spt6 contains an N-terminal acid domain known to interact with nucleosomes (McDonald et al. 2010) and a tandem SH2 domain at the C-terminus known to bind to phosphorylated RNA pol II (Johnson et al. 2008). Transcription causes a transient dissociation of histones from chromatin, requiring the presence of specific proteins ensuring the integrity of the chromatin state during transcription. Spt6 has been suggested to interact with elongating RNA pol II and preserve post-translationally modified histone H3 during transcription, so that the epigenetic state of chromatin is maintained (Kato et al. 2013). Consistently with this idea, the epigenetic state of chromatin is generally impaired in cells depleted for Spt6, displaying both a decrease of H3K9me at heterochromatin and of H3K4me/H3K36me at euchromatin regions (Kato et al. 2013; DeGennaro et al. 2013).

Although the mechanism of action of Spt6 is not yet completely understood, it has been proposed that it could both regulate nucleosome positioning and also recruit histone modifiers to chromatin. For instance, it has been shown that Spt6 recruits the H3K4 HMT Set1 and the H3K36 HMT Set2 to euchromatin (DeGennaro et al. 2013). In addition, Spt6 seems to have a general role in the regulation of gene expression. Indeed, mutant cells for *spt6* present genome-wide transcriptional deregulation, with an increase of antisense transcription in more than 70% of genes (DeGennaro et al. 2013).

Role of Spt6 in heterochromatin formation and gene silencing

Our study was mostly focused on investigating the role of Spt6 at heterochromatin regions. Mutant cells for s*pt6* (*spt6-1*, deletion of the helix-hairpin-helix domain) present a severe defect in constitutive heterochromatin silencing. Interestingly, Spt6 seems to act in regulating constitutive heterochromatin silencing both at the transcriptional and post-transcriptional level. Indeed, *spt6-1* cells showed an increase in RNA pol II binding to pericentromeric sequences and also a loss of centromeric siRNA production (Kiely et al. 2011). Surprisingly, this constitutive heterochromatin desilencing does not seem to correlate with a clear decrease in H3K9me in *spt6-1* mutant cells. However, a clear decrease in H3K9me was reported in other *spt6* mutants, *spt6-K20* (deleted for the YqgFc RNase H-like domain) and *spt6Δ*, suggesting thus that Spt6 is also involved in preserving the heterochromatin integrity of these regions (Kato et al. 2013). Spt6 localization depends on RNA pol II, indeed its binding to constitutive heterochromatin is detected only in *clr4Δ* and *chp2Δ* strains, in which transcription is increased (Kiely et al. 2011). Interestingly, Spt6 was not detected at constitutive heterochromatin in *chp1Δ* strains, despite the increase in transcription. This result suggests that RITS could help the recruitment of Spt6 to constitutive heterochromatin. Therefore, investigating the function of the interaction between Spt6 and the RITS complex may also help to elucidate the role of Spt6 in constitutive heterochromatin silencing.

Surprisingly, we also discovered an unknown function of Spt6 at facultative heterochromatin regions. Indeed, as for Ccr4-Not mutants, we showed that *spt6-1* mutant cells loosen the methyl-H3K9 repressive mark at meiotic genes; however, this does not correlate with an increase in meiotic transcripts accumulation. We still need to understand how Spt6 regulates H3K9me deposition at these genes, but our results indicate that it does not seem to regulate nucleosome occupancy in these regions. Therefore, we can speculate that Spt6 could help the recruitment of other chromatin factors to meiotic genes, such as the H3K9 HMT Clr4 or the HP1 protein Swi6, as has already been shown for Set1 and Set2 at euchromatin (DeGennaro et al. 2013). The role of Spt6 at facultative heterochromatin seems direct as we

showed that Spt6 binds to transcribed meiotic genes in *wt* cells. However, Spt6 localization to meiotic genes is reduced both in *chp1Δ* and *clr4Δ* cells, suggesting that these two proteins could have a role in recruiting Spt6 to these regions, collaborating probably with RNA pol II. In order to test this hypothesis, we would need to investigate the ability of RITS (or Clr4) to recruit Spt6 in the absence of RNA pol II. For this aim, we could investigate the binding to meiotic genes of a mutant Spt6 protein with a deletion of its SH2 domain, which is known to mediate its interaction with RNA pol II.

Finally, to better understand the role of Spt6, we decided to identify its protein interactors. For this purpose, we obtained a *TAP*-tagged *spt6* strain that we used for protein complex purification. The analysis of Spt6-TAP purification by MS will provide additional clues on Spt6 function and its mechanism of action at heterochromatin regions.

Abo1 interacts with RITS and is a crucial regulator of gene expression in fission yeast

In this study, we revealed an unknown interaction between the RITS complex and an uncharacterized protein in fission yeast named Abo1. RITS is known to be crucial for constitutive heterochromatin assembly and interestingly, we showed that Abo1 is also required for gene silencing of these regions. However, the mechanism of action of Abo1 at constitutive heterochromatin is not known.

Abo1 homologues in other organisms have been shown to play a role in transcription regulation. Indeed, Yta7 in *S. cerevisiae* has been shown to regulate transcription of histones and inducible genes (early meiotic and galactose) (Lombardi, Ellahi, and Rine 2011), whereas human ATAD2 activates for example the transcription of estrogen and androgen responsive genes (Zou et al. 2009; Zou et al. 2007). Additionally, our investigation of Abo1 purification revealed its interaction with many proteins known to regulate transcription, such as Tfg3, the FACT complex and the DNA-directed RNA pol II and III.

Moreover, our transcriptomic analysis in *abo1Δ* cells indicated that Abo1 seems to act as a general regulator of transcription and gene silencing, controlling the level of expression of hundreds of both protein-coding and non-coding genes. The deeper analysis (in progress) of these data may indicate a class of genes specifically deregulated in *abo1Δ* strains that could be responsible for the defective phenotype of these cells. Alternatively, these defects could be the consequence of a massive gene desilencing observed mainly at subtelomeric regions.

Surprisingly, our *abo1Δ* transcriptomic data analysis revealed a strong accumulation of transcripts derived from transposable elements. It is necessary to investigate whether the accumulation of these transcripts correlates with increased integrations of transposable elements into the genome. For this purpose, we have planned to use a transposon mobility assay in *abo1Δ* cells, as already described in the literature (Levin 1995; Hoff, Levin, and Boeke 1998). A possible increase in transposon integration could generate a drastic genomic instability and be responsible for the fast appearance of mutations in these cells. This could also explain the quick phenotypic reversion of *abo1Δ* cells; in fact, the appearance of new additional mutations could have permitted cells to rescue their severe growth defect. It would be interesting to test whether a depletion of ATAD2 in cancer cells could also generate a desilencing of transposable elements. If this is the case, a specific drug to inhibit ATAD2 could generate a dangerous additional increase in genomic stability in treated cells.

We have also demonstrated that Abo1 interacts with Tfg3 and we showed that these two proteins could act together in regulating cell growth. Tfg3 has been previously suggested to be involved in transcriptional regulation under stress conditions (Kimura and Ishihama 2004). Therefore, it could be interesting to also investigate the transcriptomic profile of *abo1Δ* cells under stress conditions and compare it to the transcriptomic profile of *tfg3Δ* cells. Interestingly, to support our hypothesis, our results showed that the growth defect of *abo1Δ* strains becomes even more severe in cells growing at higher temperatures or in presence of genotoxic agents. However, a general sensitivity of *abo1Δ* cells to stress still need to be tested under other conditions, as for example under osmotic or oxidative stress, or in presence of heavy metals (Kimura and Ishihama 2004).

ATAD2 is a male germ specific protein and Yta7 has been shown to regulate the transcription of sporulation-induced genes (Lombardi, Ellahi, and Rine 2011). Therefore, we can

hypothesize that Abo1 could also regulate the expression of meiotic genes during sexual differentiation in fission yeast. In addition, we showed that *h90 abo1Δ* cells present a severe defect in sporulation and that Abo1 has a role in the process of mating type switching, supporting our hypothesis of involvement of this protein in sexual differentiation.

Abo1 is a chromatin-linked protein, possibly acting as histone chaperone

Abo1-TAP purification revealed its probable tight connection with several proteins and complexes involved in different chromatin-related processes. Indeed, Abo1 may interact with all histones, some histone chaperons (FACT, Mug183), many chromatin remodelers and several histone modifiers. Interestingly, Abo1 seems to act at the interplay between transcription regulation and chromatin, although its mechanism of action is still not completely understood. Further functional characterization of the Abo1's protein network could allow us to better understand the biological relevance of these protein interactions and could also help elucidate the mechanism of action of Abo1 in cells.

Surprisingly, our MS analysis did not show any enrichment for RITS subunits in Abo1-TAP purification. However, by Western blot we were able to observe that Chp1 copurified specifically with Abo1-TAP after the incubation with IgG beads, thus confirming the interaction between these two proteins.

We confirmed that Abo1 interacts with histones but, unlike ATAD2, the bromodomain of Abo1 seems to bind to histone H4 in an acetylation-independent manner, as it has been already shown for Yta7. Interestingly, studies in Yta7 have revealed a region in the N-terminal region of this protein that contains a stretch of acidic residues responsible for interaction with histones (Gradolatto et al. 2009). Remarkably, Abo1 also seems to contain a negatively charged region in its N-terminal portion that could likewise contribute to its binding to histones.

Yta7 has been proposed to regulate transcription of inducible genes by acting as a histone chaperone that evicts and degrades histones H3 and H4 in budding yeast (Lombardi, Ellahi, and Rine 2011). Therefore, we investigated whether Abo1 could also act as a histone chaperone in fission yeast. Our hypothesis was that the severe growth defect observed in *abo1Δ* strains could be due to a toxic accumulation of free histones in cells. Interestingly, we observed that the growth defect of Abo1 depleted cells was clearly reduced by deleting two different copies of histone *H3-H4* genes. Therefore, our results suggest that a possible decrease in the level of histones in cells could rescue the growth defect caused by the absence of Abo1, thus supporting our hypothesis that Abo1 could act as histone chaperone in fission yeast. To confirm our results, we could investigate a general role of Abo1 in regulating histone turnover and nucleosome occupancy. For instance, we could perform a MNase-seq (MNase digestion coupled to sequencing) or a ChIP-seq anti histone H3 (ChIP coupled to sequencing) in *abo1Δ* cells. Overlapping these data with our transcriptomic analysis could eventually permit us to demonstrate that the histone chaperone activity of Abo1 correlates with its role in regulating transcription of specific genes. Then, in order to show the direct function of Abo1, we could also test its localization in these regions. However, performing a ChIP-seq to investigate the genome-wide localization of Abo1 could be a challenge as we were not able to detect Abo1 localization at heterochromatin regions by ChIP experiments. In addition, it would be interesting to understand which domains of Abo1 are important for its function. For this aim, we could generate strains expressing several truncated mutants of Abo1 and investigate their phenotypes.

Abo1, possible orthologue of human ATAD2, is required for cell growth in *S. pombe*

ATAD2 is ectopically expressed in many cancer types and this correlates with poor prognosis (Caron et al. 2010), although its molecular function still remains to be understood. The ectopic expression of ATAD2 in cancerous cells is required for their proliferation. Indeed, a knock-down of ATAD2 in these cells is known to inhibit cell growth and invasion, to increase

apoptosis and to lead to G1 phase cell cycle arrest (Zheng et al. 2015; Caron et al. 2010). Therefore, inactivation of ATAD2 could be a promising anti-cancer therapy, although further studies are necessary to elucidate the role of this protein in the process of cancerogenesis and to develop specific and effective ATAD2 inhibitors. Fission yeast is a good model for investigating the function of human ATAD2 since Abo1, the ATAD2 homologue in this organism, is normally expressed in vegetative cells.

In our study, we investigated the phenotype of *S. pombe* cells deleted for *abo1* and we were able to observe that the deletion of this gene is not completely lethal for cells, although it causes a severe growth defect in standard culture conditions.

In addition, we showed that the growth defect of *abo1Δ* cells correlates with an increase of cell mortality in these strains. Moreover, many *abo1Δ* cells that succeed in escaping cell death present an evident altered morphology with variegated types of abnormal shapes: elongated, rounded, curved cells or containing additional abnormal knobs. An altered morphology has been often associated to a defect in mitosis, for instance elongated cells grow but fail to divide since they are often blocked or delayed in the cell cycle (Hayles et al. 2013). Indeed, we could observe that *abo1Δ* cells present a defect in the cell cycle progression compared to *wt* strains, with an increase in the number of binucleate cells stopped in G2. It would be interesting to pursue our study looking more carefully at possible mitotic defects in *abo1Δ* strains, for example investigating by DAPI staining the presence of possible lagging chromosomes in these cells.

Fission yeast Abo1 and human ATAD2 seem to have both a role in regulating cell growth. In addition, these two proteins share ~26% of sequence identity, which is even higher considering only their functional domains. Therefore, we investigated their possible functional complementation and we showed that the growth defect of *abo1Δ S. pombe* cells was completely rescued by expressing human ATAD2. This demonstrates that, despite their distance in evolution, the function of these two homologues in regulating cell growth could be conserved. Interestingly, no function in cell growth was reported for Yta7, ATAD2 homologue in *S. cerevisiae*. Yta7 shares with Abo1 ~32% of sequence identity, which is even higher than the identity between Abo1 and human ATAD2. Therefore, it would be interesting to test whether expressing Yta7 in *abo1Δ S. pombe* cells could also rescue their

growth defect. If not, this would highlight a significant difference between Yta7 and Abo1/ATAD2, and its understanding could be of great interest in cancer research.

In conclusion, our results further emphasize the importance of our study. Indeed, due to their functional conservation, a better understanding of the molecular function of Abo1 could help elucidate the role of ATAD2 in cancer.

No functional redundancy between Abo1 and Abo2 in fission yeast

In *S. pombe* there are actually two ATAD2-like paralogues, Abo1 and Abo2, both uncharacterized. The percentage of identity between human ATAD2 and either Abo1 or Abo2 is quite comparable and corresponds to 25.8% and 24.3%, respectively; Abo1 and Abo2 are identical for the 28.8% of their sequences.

In our study, we showed that Abo1, and not Abo2, is probably the functional homologue in fission yeast of human ATAD2. In *abo2Δ* cells, we did not observe any of the phenotypes shown for *abo1Δ* strains: no defect in cell growth, no increased cell mortality, no altered morphology or delay in the cell cycle progression. In addition, Abo2 was not found in the RITS interactome and the level of constitutive heterochromatin RNAs was not significantly increased in *abo2Δ* cells. We also investigated whether the function of Abo2 in cells could be masked by the presence of Abo1, but the analysis of the *abo1Δ abo2Δ* double mutant cells do not suggest any functional redundancy between these two proteins.

A phylogenetic analysis of ATAD2-like proteins in eukaryotes reveals that several organisms actually contain two paralogs of this protein (Cattaneo et al. 2014). However, fission yeast Abo1 and Abo2, despite their sequence similarity, seem to be functionally divergent. Interestingly, a similar case is found in mammals that contain a paralogue of ATAD2 called ATAD2B, which does not seem to be associated with cancer (Caron et al. 2010).

A way to escape *abo1Δ* phenotype: mechanism of reversion

In our study, we were able to observe that some *abo1Δ* cells spontaneously and quickly find a way to survive and to rescue their growth defect, thus coming back to a phenotype more similar to *wt* strains. For this reason, we called these cells 'revertants' (*abo1Δ R*).

Interestingly, we noticed that the reversion occurs in all tested *abo1Δ* isolates, that the frequency of reversion seems to increase after several cell divisions and that, once *abo1Δ* cells have reverted, the process seems irreversible. However, we showed that the rescue of the phenotype is not complete in *abo1Δ R* cells. Indeed, the desilencing of heterochromatin regions is not rescued in these cells; in contrast, the accumulation of subtelomeric and transposable transcripts seems even higher. This suggests that the function of Abo1 in cell proliferation may be independent from its function in heterochromatin gene silencing.

We showed that Abo1 interacts with Sap1 and protein homologues to human CENPB, whose depletion generates a similar phenotype to that of *abo1*Δ cells. Interestingly, similarly to *abo1*Δ, CENPB deletion mutants have a severe growth defect that can quickly revert due to a mutation in the *sap1* gene (Zaratiegui, Vaughn, et al. 2011). Therefore, we tested whether depletion of Sap1 could also explain the reversion of *abo1Δ* cells, but we found no rescue of *abo1Δ* growth defect upon Sap1 depletion, suggesting different modes of action between Abo1 and CENPB proteins.

Alternatively, as the appearance of reversion in *abo1Δ* cells seems to increase after several cell divisions, we could speculate that Abo1 may regulate the length of specific genomic sequences, such as telomeric or rDNA repeats. In *abo1Δ* cells the length of these repeats could therfore become shorter after each division leading to genome instability and cell death. This could correlate with a severe phenotype that *abo1Δ* cells could eventually overcome, finding a way to survive. For instance, it has been shown that mutant cells for the telomerase catalytic subunit Trt1 present severe cell mortality due to decreased telomeric length. However, a subpopulation of these cells succeed in surviving, either by circularizing

their chromosomes or through the activation of specific telomeric recombination events (Nakamura, Cooper, and Cech 1998).

In order to understand whether *abo1Δ* reversion depends on mutations in specific genes, we could backcross *abo1Δ R* with *wt* strains and test if the obtained *abo1Δ* cell progeny re-acquires a severe growth defect. In addition, sequencing the genomic DNA of several *abo1Δ R* strains could permit identification of the possible mutated gene or genes responsible for the reversion.

Alternatively, we can speculate that the fast reversion of *abo1Δ* cells may be caused by an epigenetic mechanism. For instance, a combinatory loss of the HAT Mst2 and the boundary protein Epe1 has been shown to be associated with a severe growth defect due to ectopic heterochromatin spreading that silences essential genes. Interestingly, these cells can rapidly rescue this growth defect by an adaptation mechanism in which heterochromatin spreading also silences genes that are essential for heterochromatin assembly, such as Clr4 or Rik1, thus leading to restoration of a normal heterochromatin landscape (Wang, Reddy, and Jia 2015). We tested whether a similar mechanism could explain the reversion in *abo1Δ* strains, but no decrease of Clr4 or Rik1 RNA levels was observed in *abo1Δ R* cells.

The mechanism of reversion in *abo1Δ* cells is still unknown and a deep analysis of the transcriptomic profile of *abo1Δ R* cells will help to molecularly elucidate this process. A better understanding of the reversion is even more important when one speculates that a similar mechanism could also be conserved in humans. Indeed, this could mean that cancerous cells treated with inhibitors of ATAD2 may also find a way to escape the cell death caused by a depletion of ATAD2, thus becoming resistant to treatment.

Conclusive remarks

Gene expression is controlled in cells by interplay between transcription and chromatin regulation. Therefore, proteins controlling these processes are conserved in eukaryotes and deregulated in many pathologies.

Our results have permitted a better understanding of three actors involved in RNA- and histone chaperone-based silencing in fission yeast: the Ccr4-Not complex, Spt6 and Abo1.

In addition, our characterization of Abo1 suggests a possible function of this protein in regulating transcription by acting as histone chaperone. Interestingly, our study on Abo1 may also help to elucidate the function of ATAD2 and its role in cancer.

REFERENCES

Aguilar-Arnal, L., F. X. Marsellach, and F. Azorin. 2008. 'The fission yeast homologue of CENP-B, Abp1, regulates directionality of mating-type switching', *EMBO J*, 27: 1029-38.

Ahmad, K., and S. Henikoff. 2002. 'The histone variant H3.3 marks active chromatin by replication-independent nucleosome assembly', *Mol Cell*, 9: 1191-200.

Alabert, C., and A. Groth. 2012. 'Chromatin replication and epigenome maintenance', *Nat Rev Mol Cell Biol*, 13: 153-67.

Alfredsson-Timmins, J., F. Henningson, and P. Bjerling. 2007. 'The Clr4 methyltransferase determines the subnuclear localization of the mating-type region in fission yeast', *J Cell Sci*, 120: 1935-43.

Alfredsson-Timmins, J., C. Kristell, F. Henningson, S. Lyckman, and P. Bjerling. 2009. 'Reorganization of chromatin is an early response to nitrogen starvation in Schizosaccharomyces pombe', *Chromosoma*, 118: 99-112.

Alizadeh, A. A., M. B. Eisen, R. E. Davis, C. Ma, I. S. Lossos, A. Rosenwald, J. C. Boldrick, H. Sabet, T. Tran, X. Yu, J. I. Powell, L. Yang, G. E. Marti, T. Moore, J. Hudson, Jr., L. Lu, D. B. Lewis, R. Tibshirani, G. Sherlock, W. C. Chan, T. C. Greiner, D. D. Weisenburger, J. O. Armitage, R. Warnke, R. Levy, W. Wilson, M. R. Grever, J. C. Byrd, D. Botstein, P. O. Brown, and L. M. Staudt. 2000. 'Distinct types of diffuse large B-cell lymphoma identified by gene expression profiling', *Nature*, 403: 503-11.

Allshire, R. C., and K. Ekwall. 2015. 'Epigenetic Regulation of Chromatin States in Schizosaccharomyces pombe', *Cold Spring Harb Perspect Biol*, 7: a018770.

Allshire, R. C., E. R. Nimmo, K. Ekwall, J. P. Javerzat, and G. Cranston. 1995. 'Mutations derepressing silent centromeric domains in fission yeast disrupt chromosome segregation', *Genes Dev*, 9: 218-33.

Alper, B. J., G. Job, R. K. Yadav, S. Shanker, B. R. Lowe, and J. F. Partridge. 2013. 'Sir2 is required for Clr4 to initiate centromeric heterochromatin assembly in fission yeast', *EMBO J*, 32: 2321-35.

Aravin, A. A., R. Sachidanandam, D. Bourc'his, C. Schaefer, D. Pezic, K. F. Toth, T. Bestor, and G. J. Hannon. 2008. 'A piRNA pathway primed by individual transposons is linked to de novo DNA methylation in mice', *Mol Cell*, 31: 785-99.

Aravind, L., H. Watanabe, D. J. Lipman, and E. V. Koonin. 2000. 'Lineage-specific loss and divergence of functionally linked genes in eukaryotes', *Proc Natl Acad Sci U S A*, 97: 11319-24.

Arcangioli, B., T. D. Copeland, and A. J. Klar. 1994. 'Sap1, a protein that binds to sequences required for mating-type switching, is essential for viability in Schizosaccharomyces pombe', *Mol Cell Biol*, 14: 2058-65.

Arnoult, N., A. Van Beneden, and A. Decottignies. 2012. 'Telomere length regulates TERRA levels through increased trimethylation of telomeric H3K9 and HP1alpha', *Nat Struct Mol Biol*, 19: 948-56.

Ayoub, N., K. Noma, S. Isaac, T. Kahan, S. I. Grewal, and A. Cohen. 2003. 'A novel jmjC domain protein modulates heterochromatization in fission yeast', *Mol Cell Biol*, 23: 4356-70.

Bahler, J., J. Q. Wu, M. S. Longtine, N. G. Shah, A. McKenzie, 3rd, A. B. Steever, A. Wach, P. Philippsen, and J. R. Pringle. 1998. 'Heterologous modules for efficient and versatile PCR-based gene targeting in Schizosaccharomyces pombe', *Yeast*, 14: 943-51.

Banerjee, T., and D. Chakravarti. 2011. 'A peek into the complex realm of histone phosphorylation', *Mol Cell Biol*, 31: 4858-73.

Bannister, A. J., and T. Kouzarides. 2011. 'Regulation of chromatin by histone modifications', *Cell Res*, 21: 381-95.

Baum, M., and L. Clarke. 2000. 'Fission yeast homologs of human CENP-B have redundant functions affecting cell growth and chromosome segregation', *Mol Cell Biol*, 20: 2852-64.

Bayne, E. H., S. A. White, A. Kagansky, D. A. Bijos, L. Sanchez-Pulido, K. L. Hoe, D. U. Kim, H. O. Park, C. P. Ponting, J. Rappsilber, and R. C. Allshire. 2010. 'Stc1: a critical link between RNAi and chromatin modification required for heterochromatin integrity', *Cell*, 140: 666-77.

Bedford, M. T., and S. G. Clarke. 2009. 'Protein arginine methylation in mammals: who, what, and why', *Mol Cell*, 33: 1-13.

Belancio, V. P., P. L. Deininger, and A. M. Roy-Engel. 2009. 'LINE dancing in the human genome: transposable elements and disease', *Genome Med*, 1: 97.

Bell, D. W., N. Sikdar, K. Y. Lee, J. C. Price, R. Chatterjee, H. D. Park, J. Fox, M. Ishiai, M. L. Rudd, L. M. Pollock, S. K. Fogoros, H. Mohamed, C. L. Hanigan, Nisc Comparative Sequencing Program, S. Zhang, P. Cruz, G. Renaud, N. F. Hansen, P. F. Cherukuri, B. Borate, K. J. McManus, J. Stoepel, P. Sipahimalani, A. K. Godwin, D. C. Sgroi, M. J. Merino, G. Elliot, A. Elkahloun, C. Vinson, M. Takata, J. C. Mullikin, T. G. Wolfsberg, P. Hieter, D. S. Lim, and K. Myung. 2011. 'Predisposition to cancer caused by genetic and functional defects of mammalian Atad5', *PLoS Genet*, 7: e1002245.

Belotserkovskaya, R., S. Oh, V. A. Bondarenko, G. Orphanides, V. M. Studitsky, and D. Reinberg. 2003. 'FACT facilitates transcription-dependent nucleosome alteration', *Science*, 301: 1090-3.

Bhaskar, V., V. Roudko, J. Basquin, K. Sharma, H. Urlaub, B. Seraphin, and E. Conti. 2013. 'Structure and RNA-binding properties of the Not1-Not2-Not5 module of the yeast Ccr4-Not complex', *Nat Struct Mol Biol*, 20: 1281-8.

Bourc'his, D., and T. H. Bestor. 2004. 'Meiotic catastrophe and retrotransposon reactivation in male germ cells lacking Dnmt3L', *Nature*, 431: 96-9.

Boussouar, F., M. Jamshidikia, Y. Morozumi, S. Rousseaux, and S. Khochbin. 2013. 'Malignant genome reprogramming by ATAD2', *Biochim Biophys Acta*, 1829: 1010-4.

Bowen, N. J., I. K. Jordan, J. A. Epstein, V. Wood, and H. L. Levin. 2003. 'Retrotransposons and their recognition of pol II promoters: a comprehensive survey of the transposable elements from the complete genome sequence of Schizosaccharomyces pombe', *Genome Res*, 13: 1984-97.

Braun, S., J. F. Garcia, M. Rowley, M. Rougemaille, S. Shankar, and H. D. Madhani. 2011. 'The Cul4-Ddb1(Cdt)(2) ubiquitin ligase inhibits invasion of a boundary-associated antisilencing factor into heterochromatin', *Cell*, 144: 41-54.

Brennecke, J., C. D. Malone, A. A. Aravin, R. Sachidanandam, A. Stark, and G. J. Hannon. 2008. 'An epigenetic role for maternally inherited piRNAs in transposon silencing', *Science*, 322: 1387-92.

Briggs, M. W., K. T. Burkard, and J. S. Butler. 1998. 'Rrp6p, the yeast homologue of the human PM-Scl 100-kDa autoantigen, is essential for efficient 5.8 S rRNA 3' end formation', *J Biol Chem*, 273: 13255-63.

Brodersen, P., and O. Voinnet. 2006. 'The diversity of RNA silencing pathways in plants', *Trends Genet*, 22: 268-80.

Buhler, M., W. Haas, S. P. Gygi, and D. Moazed. 2007. 'RNAi-dependent and -independent RNA turnover mechanisms contribute to heterochromatic gene silencing', *Cell*, 129: 707-21.

Buhler, M., A. Verdel, and D. Moazed. 2006. 'Tethering RITS to a nascent transcript initiates RNAi- and heterochromatin-dependent gene silencing', *Cell*, 125: 873-86.

Buker, S. M., T. Iida, M. Buhler, J. Villen, S. P. Gygi, J. Nakayama, and D. Moazed. 2007. 'Two different Argonaute complexes are required for siRNA generation and heterochromatin assembly in fission yeast', *Nat Struct Mol Biol*, 14: 200-7.

Burgess, R. J., and Z. Zhang. 2013. 'Histone chaperones in nucleosome assembly and human disease', *Nat Struct Mol Biol*, 20: 14-22.

Buscaino, A., R. Allshire, and A. Pidoux. 2010. 'Building centromeres: home sweet home or a nomadic existence?', *Curr Opin Genet Dev*, 20: 118-26.

Buscaino, A., E. Lejeune, P. Audergon, G. Hamilton, A. Pidoux, and R. C. Allshire. 2013. 'Distinct roles for Sir2 and RNAi in centromeric heterochromatin nucleation, spreading and maintenance', *EMBO J*, 32: 1250-64.

Cam, H. P., K. Noma, H. Ebina, H. L. Levin, and S. I. Grewal. 2008. 'Host genome surveillance for retrotransposons by transposon-derived proteins', *Nature*, 451: 431-6.

Cam, H. P., T. Sugiyama, E. S. Chen, X. Chen, P. C. FitzGerald, and S. I. Grewal. 2005. 'Comprehensive analysis of heterochromatin- and RNAi-mediated epigenetic control of the fission yeast genome', *Nat Genet*, 37: 809-19.

Canzio, D., E. Y. Chang, S. Shankar, K. M. Kuchenbecker, M. D. Simon, H. D. Madhani, G. J. Narlikar, and B. Al-Sady. 2011. 'Chromodomain-mediated oligomerization of HP1 suggests a nucleosome-bridging mechanism for heterochromatin assembly', *Mol Cell*, 41: 67-81.

Caron, C., C. Lestrat, S. Marsal, E. Escoffier, S. Curtet, V. Virolle, P. Barbry, A. Debernardi, C. Brambilla, E. Brambilla, S. Rousseaux, and S. Khochbin. 2010. 'Functional characterization of ATAD2 as a new cancer/testis factor and a predictor of poor prognosis in breast and lung cancers', *Oncogene*, 29: 5171-81.

Castel, S. E., J. Ren, S. Bhattacharjee, A. Y. Chang, M. Sanchez, A. Valbuena, F. Antequera, and R. A. Martienssen. 2014. 'Dicer promotes transcription termination at sites of replication stress to maintain genome stability', *Cell*, 159: 572-83.

Cattaneo, M., Y. Morozumi, D. Perazza, F. Boussouar, M. Jamshidikia, S. Rousseaux, A. Verdel, and S. Khochbin. 2014. 'Lessons from yeast on emerging roles of the ATAD2 protein family in gene regulation and genome organization', *Mol Cells*, 37: 851-6.

Chen, E. S., K. Zhang, E. Nicolas, H. P. Cam, M. Zofall, and S. I. Grewal. 2008. 'Cell cycle control of centromeric repeat transcription and heterochromatin assembly', *Nature*, 451: 734-7.

Chen, H. M., B. Futcher, and J. Leatherwood. 2011. 'The fission yeast RNA binding protein Mmi1 regulates meiotic genes by controlling intron specific splicing and polyadenylation coupled RNA turnover', *PLoS One*, 6: e26804.

Chen, Y., A. Boland, D. Kuzuoglu-Ozturk, P. Bawankar, B. Loh, C. T. Chang, O. Weichenrieder, and E. Izaurralde. 2014. 'A DDX6-CNOT1 complex and W-binding pockets in CNOT9 reveal direct links between miRNA target recognition and silencing', *Mol Cell*, 54: 737-50.

Ciro, M., E. Prosperini, M. Quarto, U. Grazini, J. Walfridsson, F. McBlane, P. Nucifero, G. Pacchiana, M. Capra, J. Christensen, and K. Helin. 2009. 'ATAD2 is a novel cofactor for MYC, overexpressed and amplified in aggressive tumors', *Cancer Res*, 69: 8491-8.

Clapier, C. R., and B. R. Cairns. 2009. 'The biology of chromatin remodeling complexes', *Annu Rev Biochem*, 78: 273-304.

Collart, M. A., and O. O. Panasenko. 2012. 'The Ccr4--not complex', *Gene*, 492: 42-53.

Colmenares, S. U., S. M. Buker, M. Buhler, M. Dlakic, and D. Moazed. 2007. 'Coupling of double-stranded RNA synthesis and siRNA generation in fission yeast RNAi', *Mol Cell*, 27: 449-61.

Cook, A. J., Z. A. Gurard-Levin, I. Vassias, and G. Almouzni. 2011. 'A specific function for the histone chaperone NASP to fine-tune a reservoir of soluble H3-H4 in the histone supply chain', *Mol Cell*, 44: 918-27.

Cooper, J. P., E. R. Nimmo, R. C. Allshire, and T. R. Cech. 1997. 'Regulation of telomere length and function by a Myb-domain protein in fission yeast', *Nature*, 385: 744-7.

Corpet, A., L. De Koning, J. Toedling, A. Savignoni, F. Berger, C. Lemaitre, R. J. O'Sullivan, J. Karlseder, E. Barillot, B. Asselain, X. Sastre-Garau, and G. Almouzni. 2011. 'Asf1b, the necessary Asf1 isoform for proliferation, is predictive of outcome in breast cancer', *EMBO J*, 30: 480-93.

Cotobal, C., M. Rodriguez-Lopez, C. Duncan, A. Hasan, A. Yamashita, M. Yamamoto, J. Bahler, and J. Mata. 2015. 'Role of Ccr4-Not complex in heterochromatin formation at meiotic genes and subtelomeres in fission yeast', *Epigenetics Chromatin*, 8: 28.

Cowieson, N. P., J. F. Partridge, R. C. Allshire, and P. J. McLaughlin. 2000. 'Dimerisation of a chromo shadow domain and distinctions from the chromodomain as revealed by structural analysis', *Curr Biol*, 10: 517-25.

Crabbe, L., A. J. Cesare, J. M. Kasuboski, J. A. Fitzpatrick, and J. Karlseder. 2012. 'Human telomeres are tethered to the nuclear envelope during postmitotic nuclear assembly', *Cell Rep*, 2: 1521-9.

de Lahondes, R., V. Ribes, and B. Arcangioli. 2003. 'Fission yeast Sap1 protein is essential for chromosome stability', *Eukaryot Cell*, 2: 910-21.

DeGennaro, C. M., B. H. Alver, S. Marguerat, E. Stepanova, C. P. Davis, J. Bahler, P. J. Park, and F. Winston. 2013. 'Spt6 regulates intragenic and antisense transcription, nucleosome positioning, and histone modifications genome-wide in fission yeast', *Mol Cell Biol*, 33: 4779-92.

Dehe, P. M., and J. P. Cooper. 2010. 'Fission yeast telomeres forecast the end of the crisis', *FEBS Lett*, 584: 3725-33.

Demont, E. H., C. W. Chung, R. C. Furze, P. Grandi, A. M. Michon, C. Wellaway, N. Barrett, A. M. Bridges, P. D. Craggs, H. Diallo, D. P. Dixon, C. Douault, A. J. Emmons, E. J. Jones, B. V. Karamshi, K. Locke, D. J. Mitchell, B. H. Mouzon, R. K. Prinjha, A. D. Roberts, R. J. Sheppard, R. J. Watson, and P. Bamborough. 2015. 'Fragment-Based Discovery of Low-Micromolar ATAD2 Bromodomain Inhibitors', *J Med Chem*, 58: 5649-73.

Dereeper, A., S. Audic, J. M. Claverie, and G. Blanc. 2010. 'BLAST-EXPLORER helps you building datasets for phylogenetic analysis', *BMC Evol Biol*, 10: 8.

Dereeper, A., V. Guignon, G. Blanc, S. Audic, S. Buffet, F. Chevenet, J. F. Dufayard, S. Guindon, V. Lefort, M. Lescot, J. M. Claverie, and O. Gascuel. 2008. 'Phylogeny.fr: robust phylogenetic analysis for the non-specialist', *Nucleic Acids Res*, 36: W465-9.

Di Tommaso, P., S. Moretti, I. Xenarios, M. Orobitg, A. Montanyola, J. M. Chang, J. F. Taly, and C. Notredame. 2011. 'T-Coffee: a web server for the multiple sequence alignment of protein and RNA sequences using structural information and homology extension', *Nucleic Acids Res*, 39: W13-7.

Djupedal, I., I. C. Kos-Braun, R. A. Mosher, N. Soderholm, F. Simmer, T. J. Hardcastle, A. Fender, N. Heidrich, A. Kagansky, E. Bayne, E. G. Wagner, D. C. Baulcombe, R. C. Allshire, and K. Ekwall. 2009. 'Analysis of small RNA in fission yeast; centromeric siRNAs are potentially generated through a structured RNA', *EMBO J*, 28: 3832-44.

Drazkowska, K., R. Tomecki, K. Stodus, K. Kowalska, M. Czarnocki-Cieciura, and A. Dziembowski. 2013. 'The RNA exosome complex central channel controls both exonuclease and endonuclease Dis3 activities in vivo and in vitro', *Nucleic Acids Res*, 41: 3845-58.

Du, H. N., and S. D. Briggs. 2010. 'A nucleosome surface formed by histone H4, H2A, and H3 residues is needed for proper histone H3 Lys36 methylation, histone acetylation, and repression of cryptic transcription', *J Biol Chem*, 285: 11704-13.

Dunleavy EM1, Roche D, Tagami H, Lacoste N, Ray-Gallet D, Nakamura Y, Daigo Y, Nakatani Y, Almouzni-Pettinotti G. HJURP is a cell-cycle-dependent maintenance and deposition factor of CENP-A at centromeres. Cell. 2009 May 1;137(3):485-97. doi: 10.1016/j.cell.2009.02.040.

Durand-Dubief, M., and K. Ekwall. 2008. 'Heterochromatin tells CENP-A where to go', *Bioessays*, 30: 526-9.

Egan, E. D., C. R. Braun, S. P. Gygi, and D. Moazed. 2014. 'Post-transcriptional regulation of meiotic genes by a nuclear RNA silencing complex', *RNA*, 20: 867-81.

Egel, R. 1973. 'Genes involved in mating type expression of fission yeast', *Mol Gen Genet*, 122: 339-43.

Egel, R. 2004. 'DNA replication: stalling a fork for imprinting and switching', *Curr Biol*, 14: R915-7.

Enukashvily, N. I., R. Donev, I. S. Waisertreiger, and O. I. Podgornaya. 2007. 'Human chromosome 1 satellite 3 DNA is decondensed, demethylated and transcribed in senescent cells and in A431 epithelial carcinoma cells', *Cytogenet Genome Res*, 118: 42-54.

Eymery, A., B. Horard, M. El Atifi-Borel, G. Fourel, F. Berger, A. L. Vitte, A. Van den Broeck, E. Brambilla, A. Fournier, M. Callanan, S. Gazzeri, S. Khochbin, S. Rousseaux, E. Gilson, and C. Vourc'h. 2009. 'A transcriptomic analysis of human centromeric and pericentric sequences in normal and tumor cells', *Nucleic Acids Res*, 37: 6340-54.

Fagegaltier, D., A. L. Bouge, B. Berry, E. Poisot, O. Sismeiro, J. Y. Coppee, L. Theodore, O. Voinnet, and C. Antoniewski. 2009. 'The endogenous siRNA pathway is involved in heterochromatin formation in Drosophila', *Proc Natl Acad Sci U S A*, 106: 21258-63.

Fazly, A., Q. Li, Q. Hu, G. Mer, B. Horazdovsky, and Z. Zhang. 2012. 'Histone chaperone Rtt106 promotes nucleosome formation using (H3-H4)2 tetramers', *J Biol Chem*, 287: 10753-60.

Ferreira, M. E., K. Flaherty, and P. Prochasson. 2011. 'The Saccharomyces cerevisiae histone chaperone Rtt106 mediates the cell cycle recruitment of SWI/SNF and RSC to the HIR-dependent histone genes', *PLoS One*, 6: e21113.

Filippakopoulos, P., and S. Knapp. 2012. 'The bromodomain interaction module', *FEBS Lett*, 586: 2692-704.

Fillingham, J., P. Kainth, J. P. Lambert, H. van Bakel, K. Tsui, L. Pena-Castillo, C. Nislow, D. Figeys, T. R. Hughes, J. Greenblatt, and B. J. Andrews. 2009. 'Two-color cell array screen reveals interdependent roles for histone chaperones and a chromatin boundary regulator in histone gene repression', *Mol Cell*, 35: 340-51.

Fire, A., S. Xu, M. K. Montgomery, S. A. Kostas, S. E. Driver, and C. C. Mello. 1998. 'Potent and specific genetic interference by double-stranded RNA in Caenorhabditis elegans', *Nature*, 391: 806-11.

Fischer, T., B. Cui, J. Dhakshnamoorthy, M. Zhou, C. Rubin, M. Zofall, T. D. Veenstra, and S. I. Grewal. 2009. 'Diverse roles of HP1 proteins in heterochromatin assembly and functions in fission yeast', *Proc Natl Acad Sci U S A*, 106: 8998-9003.

Fischle, W., B. S. Tseng, H. L. Dormann, B. M. Ueberheide, B. A. Garcia, J. Shabanowitz, D. F. Hunt, H. Funabiki, and C. D. Allis. 2005. 'Regulation of HP1-chromatin binding by histone H3 methylation and phosphorylation', *Nature*, 438: 1116-22.

Fischle, W., Y. Wang, S. A. Jacobs, Y. Kim, C. D. Allis, and S. Khorasanizadeh. 2003. 'Molecular basis for the discrimination of repressive methyl-lysine marks in histone H3 by Polycomb and HP1 chromodomains', *Genes Dev*, 17: 1870-81.

Foltz, D. R., L. E. Jansen, A. O. Bailey, J. R. Yates, 3rd, E. A. Bassett, S. Wood, B. E. Black, and D. W. Cleveland. 2009. 'Centromere-specific assembly of CENP-a nucleosomes is mediated by HJURP', *Cell*, 137: 472-84.

Forsburg, S. L. 2001. 'The art and design of genetic screens: yeast', *Nat Rev Genet*, 2: 659-68.

Funabiki, H., I. Hagan, S. Uzawa, and M. Yanagida. 1993. 'Cell cycle-dependent specific positioning and clustering of centromeres and telomeres in fission yeast', *J Cell Biol*, 121: 961-76.

Ghildiyal, M., and P. D. Zamore. 2009. 'Small silencing RNAs: an expanding universe', *Nat Rev Genet*, 10: 94-108.

Goldberg AD1, Banaszynski LA, Noh KM, Lewis PW, Elsaesser SJ, Stadler S, Dewell S, Law M, Guo X, Li X, Wen D, Chapgier A, DeKelver RC, Miller JC, Lee YL, Boydston EA, Holmes MC, Gregory PD, Greally JM, Rafii S, Yang C, Scambler PJ, Garrick D, Gibbons RJ, Higgs DR, Cristea IM, Urnov FD, Zheng D, Allis CD. Distinct factors control histone variant H3.3 localization at specific genomic regions. Cell. 2010 Mar 5;140(5):678-91. doi: 10.1016/j.cell.2010.01.003.

Gonzalez-Perez, A., A. Jene-Sanz, and N. Lopez-Bigas. 2013. 'The mutational landscape of chromatin regulatory factors across 4,623 tumor samples', *Genome Biol*, 14: r106.

Gradolatto, A., R. S. Rogers, H. Lavender, S. D. Taverna, C. D. Allis, J. D. Aitchison, and A. J. Tackett. 2008. 'Saccharomyces cerevisiae Yta7 regulates histone gene expression', *Genetics*, 179: 291-304.

Gradolatto, A., S. K. Smart, S. Byrum, L. P. Blair, R. S. Rogers, E. A. Kolar, H. Lavender, S. K. Larson, J. D. Aitchison, S. D. Taverna, and A. J. Tackett. 2009. 'A noncanonical bromodomain in the AAA ATPase protein Yta7 directs chromosomal positioning and barrier chromatin activity', *Mol Cell Biol*, 29: 4604-11.

Grewal, S. I., and S. Jia. 2007. 'Heterochromatin revisited', *Nat Rev Genet*, 8: 35-46.

Grewal, S. I., and A. J. Klar. 1997. 'A recombinationally repressed region between mat2 and mat3 loci shares homology to centromeric repeats and regulates directionality of mating-type switching in fission yeast', *Genetics*, 146: 1221-38.

Gullerova, M., D. Moazed, and N. J. Proudfoot. 2011. 'Autoregulation of convergent RNAi genes in fission yeast', *Genes Dev*, 25: 556-68.

Gullerova, M., and N. J. Proudfoot. 2008. 'Cohesin complex promotes transcriptional termination between convergent genes in S. pombe', *Cell*, 132: 983-95.

Gullerova, M., and N. J. Proudfoot.. 2012. 'Convergent transcription induces transcriptional gene silencing in fission yeast and mammalian cells', *Nat Struct Mol Biol*, 19: 1193-201.

Gunjan, A., and A. Verreault. 2003. 'A Rad53 kinase-dependent surveillance mechanism that regulates histone protein levels in S. cerevisiae', *Cell*, 115: 537-49.

Guo, Y., and H. L. Levin. 2010. 'High-throughput sequencing of retrotransposon integration provides a saturated profile of target activity in Schizosaccharomyces pombe', *Genome Res*, 20: 239-48.

Gurard-Levin, Z. A., J. P. Quivy, and G. Almouzni. 2014. 'Histone chaperones: assisting histone traffic and nucleosome dynamics', *Annu Rev Biochem*, 83: 487-517.

Halic, M., and D. Moazed. 2010. 'Dicer-independent primal RNAs trigger RNAi and heterochromatin formation', *Cell*, 140: 504-16.

Hall, I. M., K. Noma, and S. I. Grewal. 2003. 'RNA interference machinery regulates chromosome dynamics during mitosis and meiosis in fission yeast', *Proc Natl Acad Sci U S A*, 100: 193-8.

Hansen, K. R., G. Burns, J. Mata, T. A. Volpe, R. A. Martienssen, J. Bahler, and G. Thon. 2005. 'Global effects on gene expression in fission yeast by silencing and RNA interference machineries', *Mol Cell Biol*, 25: 590-601.

Hansen, K. R., P. T. Ibarra, and G. Thon. 2006. 'Evolutionary-conserved telomere-linked helicase genes of fission yeast are repressed by silencing factors, RNAi components and the telomere-binding protein Taz1', *Nucleic Acids Res*, 34: 78-88.

Harigaya, Y., H. Tanaka, S. Yamanaka, K. Tanaka, Y. Watanabe, C. Tsutsumi, Y. Chikashige, Y. Hiraoka, A. Yamashita, and M. Yamamoto. 2006. 'Selective elimination of messenger RNA prevents an incidence of untimely meiosis', *Nature*, 442: 45-50.

Harner, M. J., B. A. Chauder, J. Phan, and S. W. Fesik. 2014. 'Fragment-based screening of the bromodomain of ATAD2', *J Med Chem*, 57: 9687-92.

Hayashi, A., M. Ishida, R. Kawaguchi, T. Urano, Y. Murakami, and J. Nakayama. 2012. 'Heterochromatin protein 1 homologue Swi6 acts in concert with Ers1 to regulate RNAi-directed heterochromatin assembly', *Proc Natl Acad Sci U S A*, 109: 6159-64.

Hayles, J., V. Wood, L. Jeffery, K. L. Hoe, D. U. Kim, H. O. Park, S. Salas-Pino, C. Heichinger, and P. Nurse. 2013. 'A genome-wide resource of cell cycle and cell shape genes of fission yeast', *Open Biol*, 3: 130053.

Hickey, A., C. Esnault, A. Majumdar, A. G. Chatterjee, J. R. Iben, P. G. McQueen, A. X. Yang, T. Mizuguchi, S. I. Grewal, and H. L. Levin. 2015. 'Single Nucleotide Specific Targeting of the Tf1 Retrotransposon Promoted by the DNA-Binding Protein Sap1 of Schizosaccharomyces pombe', *Genetics*.

Hime, G. R., and W. G. Somers. 2009. 'Micro-RNA mediated regulation of proliferation, self-renewal and differentiation of mammalian stem cells', *Cell Adh Migr*, 3: 425-32.

Hiriart, E., A. Vavasseur, L. Touat-Todeschini, A. Yamashita, B. Gilquin, E. Lambert, J. Perot, Y. Shichino, N. Nazaret, C. Boyault, J. Lachuer, D. Perazza, M. Yamamoto, and A. Verdel. 2012. 'Mmi1 RNA surveillance machinery directs RNAi complex RITS to specific meiotic genes in fission yeast', *EMBO J*, 31: 2296-308.

Hiriart, E., and A. Verdel. 2013. 'Long noncoding RNA-based chromatin control of germ cell differentiation: a yeast perspective', *Chromosome Res*, 21: 653-63.

Hoff, E. F., H. L. Levin, and J. D. Boeke. 1998. 'Schizosaccharomyces pombe retrotransposon Tf2 mobilizes primarily through homologous cDNA recombination', *Mol Cell Biol*, 18: 6839-52.

Hogan, C. J., S. Aligianni, M. Durand-Dubief, J. Persson, W. R. Will, J. Webster, L. Wheeler, C. K. Mathews, S. Elderkin, D. Oxley, K. Ekwall, and P. D. Varga-Weisz. 2010. 'Fission yeast Iec1-ino80-mediated nucleosome eviction regulates nucleotide and phosphate metabolism', *Mol Cell Biol*, 30: 657-74.

Holoch, D., and D. Moazed. 2015. 'RNA-mediated epigenetic regulation of gene expression', *Nat Rev Genet*, 16: 71-84.

Horn, P. J., J. N. Bastie, and C. L. Peterson. 2005. 'A Rik1-associated, cullin-dependent E3 ubiquitin ligase is essential for heterochromatin formation', *Genes Dev*, 19: 1705-14.

Hsia, E. Y., M. L. Goodson, J. X. Zou, M. L. Privalsky, and H. W. Chen. 2010. 'Nuclear receptor coregulators as a new paradigm for therapeutic targeting', *Adv Drug Deliv Rev*, 62: 1227-37.

Irvine, D. V., M. Zaratiegui, N. H. Tolia, D. B. Goto, D. H. Chitwood, M. W. Vaughn, L. Joshua-Tor, and R. A. Martienssen. 2006. 'Argonaute slicing is required for heterochromatic silencing and spreading', *Science*, 313: 1134-7.

Isaac, S., J. Walfridsson, T. Zohar, D. Lazar, T. Kahan, K. Ekwall, and A. Cohen. 2007. 'Interaction of Epe1 with the heterochromatin assembly pathway in Schizosaccharomyces pombe', *Genetics*, 175: 1549-60.

Ishida, M., H. Shimojo, A. Hayashi, R. Kawaguchi, Y. Ohtani, K. Uegaki, Y. Nishimura, and J. Nakayama. 2012. 'Intrinsic nucleic acid-binding activity of Chp1 chromodomain is required for heterochromatic gene silencing', *Mol Cell*, 47: 228-41.

Ivanova, T., I. Alves-Rodrigues, B. Gomez-Escoda, C. Dutta, J. A. DeCaprio, N. Rhind, E. Hidalgo, and J. Ayte. 2013. 'The DNA damage and the DNA replication checkpoints converge at the MBF transcription factor', *Mol Biol Cell*, 24: 3350-7.

Jacobs, J. Z., J. D. Rosado-Lugo, S. Cranz-Mileva, K. M. Ciccaglione, V. Tournier, and M. Zaratiegui. 2015. 'Arrested replication forks guide retrotransposon integration', *Science*, 349: 1549-53.

Jae Yoo, E., Y. Kyu Jang, M. Ae Lee, P. Bjerling, J. Bum Kim, K. Ekwall, R. Hyun Seong, and S. Dai Park. 2002. 'Hrp3, a chromodomain helicase/ATPase DNA binding protein, is required for heterochromatin silencing in fission yeast', *Biochem Biophys Res Commun*, 295: 970-4.

Jain, D., A. K. Hebden, T. M. Nakamura, K. M. Miller, and J. P. Cooper. 2010. 'HAATI survivors replace canonical telomeres with blocks of generic heterochromatin', *Nature*, 467: 223-7.

Jambunathan, N., A. W. Martinez, E. C. Robert, N. B. Agochukwu, M. E. Ibos, S. L. Dugas, and D. Donze. 2005. 'Multiple bromodomain genes are involved in restricting the spread of heterochromatic silencing at the Saccharomyces cerevisiae HMR-tRNA boundary', *Genetics*, 171: 913-22.

Jia, S., R. Kobayashi, and S. I. Grewal. 2005. 'Ubiquitin ligase component Cul4 associates with Clr4 histone methyltransferase to assemble heterochromatin', *Nat Cell Biol*, 7: 1007-13.

Jia, S., K. Noma, and S. I. Grewal. 2004. 'RNAi-independent heterochromatin nucleation by the stress-activated ATF/CREB family proteins', *Science*, 304: 1971-6.

Jia, S., T. Yamada, and S. I. Grewal. 2004. 'Heterochromatin regulates cell type-specific long-range chromatin interactions essential for directed recombination', *Cell*, 119: 469-80.

Johnson, S. J., D. Close, H. Robinson, I. Vallet-Gely, S. L. Dove, and C. P. Hill. 2008. 'Crystal structure and RNA binding of the Tex protein from Pseudomonas aeruginosa', *J Mol Biol*, 377: 1460-73.

Jolly, C., A. Metz, J. Govin, M. Vigneron, B. M. Turner, S. Khochbin, and C. Vourc'h. 2004. 'Stress-induced transcription of satellite III repeats', *J Cell Biol*, 164: 25-33.

Kalashnikova, E. V., A. S. Revenko, A. T. Gemo, N. P. Andrews, C. G. Tepper, J. X. Zou, R. D. Cardiff, A. D. Borowsky, and H. W. Chen. 2010. 'ANCCA/ATAD2 overexpression identifies breast cancer patients with poor prognosis, acting to drive proliferation and survival of triple-negative cells through control of B-Myb and EZH2', *Cancer Res*, 70: 9402-12.

Kanke, M., K. Nishimura, M. Kanemaki, T. Kakimoto, T. S. Takahashi, T. Nakagawa, and H. Masukata. 2011. 'Auxin-inducible protein depletion system in fission yeast', *BMC Cell Biol*, 12: 8.

Kanoh, J., and F. Ishikawa. 2001. 'spRap1 and spRif1, recruited to telomeres by Taz1, are essential for telomere function in fission yeast', *Curr Biol*, 11: 1624-30.

Kanoh, J., M. Sadaie, T. Urano, and F. Ishikawa. 2005. 'Telomere binding protein Taz1 establishes Swi6 heterochromatin independently of RNAi at telomeres', *Curr Biol*, 15: 1808-19.

Kato, H., K. Okazaki, T. Iida, J. Nakayama, Y. Murakami, and T. Urano. 2013. 'Spt6 prevents transcription-coupled loss of posttranslationally modified histone H3', *Sci Rep*, 3: 2186.

Kato, H., K. Okazaki, and T. Urano. 2013. 'Spt6: two fundamentally distinct functions in the regulation of histone modification', *Epigenetics*, 8: 1249-53.

Kazazian, H. H., Jr. 2004. 'Mobile elements: drivers of genome evolution', *Science*, 303: 1626-32.

Keller, C., R. Adaixo, R. Stunnenberg, K. J. Woolcock, S. Hiller, and M. Buhler. 2012. 'HP1(Swi6) mediates the recognition and destruction of heterochromatic RNA transcripts', *Mol Cell*, 47: 215-27.

Kemena, C., and C. Notredame. 2009. 'Upcoming challenges for multiple sequence alignment methods in the high-throughput era', *Bioinformatics*, 25: 2455-65.

Kepper, N., D. Foethke, R. Stehr, G. Wedemann, and K. Rippe. 2008. 'Nucleosome geometry and internucleosomal interactions control the chromatin fiber conformation', *Biophys J*, 95: 3692-705.

Kiely, C. M., S. Marguerat, J. F. Garcia, H. D. Madhani, J. Bahler, and F. Winston. 2011. 'Spt6 is required for heterochromatic silencing in the fission yeast Schizosaccharomyces pombe', *Mol Cell Biol*, 31: 4193-204.

Kim, H. S., E. S. Choi, J. A. Shin, Y. K. Jang, and S. D. Park. 2004. 'Regulation of Swi6/HP1-dependent heterochromatin assembly by cooperation of components of the mitogen-activated protein kinase pathway and a histone deacetylase Clr6', *J Biol Chem*, 279: 42850-9.

Kimura, A., and M. Horikoshi. 2004. 'Partition of distinct chromosomal regions: negotiable border and fixed border', *Genes Cells*, 9: 499-508.

Kimura, M., and A. Ishihama. 2004. 'Tfg3, a subunit of the general transcription factor TFIIF in Schizosaccharomyces pombe, functions under stress conditions', *Nucleic Acids Res*, 32: 6706-15.

Klar, A. J. 1992. 'Developmental choices in mating-type interconversion in fission yeast', *Trends Genet*, 8: 208-13.

Klar, A. J., K. Ishikawa, and S. Moore. 2014. 'A Unique DNA Recombination Mechanism of the Mating/Cell-type Switching of Fission Yeasts: a Review', *Microbiol Spectr*, 2.

Klattenhoff, C., and W. Theurkauf. 2008. 'Biogenesis and germline functions of piRNAs', *Development*, 135: 3-9.

Kleinjan, D. A., and L. A. Lettice. 2008. 'Long-range gene control and genetic disease', *Adv Genet*, 61: 339-88.

Kloc, A., M. Zaratiegui, E. Nora, and R. Martienssen. 2008. 'RNA interference guides histone modification during the S phase of chromosomal replication', *Curr Biol*, 18: 490-5.

Knutsen, J. H., I. D. Rein, C. Rothe, T. Stokke, B. Grallert, and E. Boye. 2011. 'Cell-cycle analysis of fission yeast cells by flow cytometry', *PLoS One*, 6: e17175.

Kornberg, R. D. 1977. 'Structure of chromatin', *Annu Rev Biochem*, 46: 931-54.

Kouzarides, T. 2007. 'Chromatin modifications and their function', *Cell*, 128: 693-705.

Kurat, C. F., J. P. Lambert, D. van Dyk, K. Tsui, H. van Bakel, S. Kaluarachchi, H. Friesen, P. Kainth, C. Nislow, D. Figeys, J. Fillingham, and B. J. Andrews. 2011. 'Restriction of histone gene transcription to S phase by phosphorylation of a chromatin boundary protein', *Genes Dev*, 25: 2489-501.

Lambert, J. P., J. Fillingham, M. Siahbazi, J. Greenblatt, K. Baetz, and D. Figeys. 2010. 'Defining the budding yeast chromatin-associated interactome', *Mol Syst Biol*, 6: 448.

Lambert, J. P., L. Mitchell, A. Rudner, K. Baetz, and D. Figeys. 2009. 'A novel proteomics approach for the discovery of chromatin-associated protein networks', *Mol Cell Proteomics*, 8: 870-82.

Lan, F., M. Zaratiegui, J. Villen, M. W. Vaughn, A. Verdel, M. Huarte, Y. Shi, S. P. Gygi, D. Moazed, R. A. Martienssen, and Y. Shi. 2007. 'S. pombe LSD1 homologs regulate heterochromatin propagation and euchromatic gene transcription', *Mol Cell*, 26: 89-101.

Langst, G., and L. Manelyte. 2015. 'Chromatin Remodelers: From Function to Dysfunction', *Genes (Basel)*, 6: 299-324.

Latham, J. A., and S. Y. Dent. 2007. 'Cross-regulation of histone modifications', *Nat Struct Mol Biol*, 14: 1017-24.

Leachman, N. T., F. Brellier, J. Ferralli, R. Chiquet-Ehrismann, and R. P. Tucker. 2010. 'ATAD2B is a phylogenetically conserved nuclear protein expressed during neuronal differentiation and tumorigenesis', *Dev Growth Differ*, 52: 747-55.

Lee, J. T., and M. S. Bartolomei. 2013. 'X-inactivation, imprinting, and long noncoding RNAs in health and disease', *Cell*, 152: 1308-23.

Leeb, M., D. Pasini, M. Novatchkova, M. Jaritz, K. Helin, and A. Wutz. 2010. 'Polycomb complexes act redundantly to repress genomic repeats and genes', *Genes Dev*, 24: 265-76.

Lejeune, E., M. Bortfeld, S. A. White, A. L. Pidoux, K. Ekwall, R. C. Allshire, and A. G. Ladurner. 2007. 'The chromatin-remodeling factor FACT contributes to centromeric heterochromatin independently of RNAi', *Curr Biol*, 17: 1219-24.

Levin, H. L. 1995. 'A novel mechanism of self-primed reverse transcription defines a new family of retroelements', *Mol Cell Biol*, 15: 3310-7.

Lewis, P. W., S. J. Elsaesser, K. M. Noh, S. C. Stadler, and C. D. Allis. 2010. 'Daxx is an H3.3-specific histone chaperone and cooperates with ATRX in replication-independent chromatin assembly at telomeres', *Proc Natl Acad Sci U S A*, 107: 14075-80.

Lippman, Z., and R. Martienssen. 2004. 'The role of RNA interference in heterochromatic silencing', *Nature*, 431: 364-70.

Litt, M., Y. Qiu, and S. Huang. 2009. 'Histone arginine methylations: their roles in chromatin dynamics and transcriptional regulation', *Biosci Rep*, 29: 131-41.

Lombardi, L. M., M. D. Davis, and J. Rine. 2015. 'Maintenance of nucleosomal balance in cis by conserved AAA-ATPase Yta7', *Genetics*, 199: 105-16.

Lombardi, L. M., A. Ellahi, and J. Rine. 2011. 'Direct regulation of nucleosome density by the conserved AAA-ATPase Yta7', *Proc Natl Acad Sci U S A*, 108: E1302-11.

Lorenz, D. R., I. V. Mikheyeva, P. Johansen, L. Meyer, A. Berg, S. I. Grewal, and H. P. Cam. 2012. 'CENP-B cooperates with Set1 in bidirectional transcriptional silencing and genome organization of retrotransposons', *Mol Cell Biol*, 32: 4215-25.

Luger, K., A. W. Mader, R. K. Richmond, D. F. Sargent, and T. J. Richmond. 1997. 'Crystal structure of the nucleosome core particle at 2.8 A resolution', *Nature*, 389: 251-60.

Macfarlan TS1, Gifford WD, Driscoll S, Lettieri K, Rowe HM, Bonanomi D, Firth A, Singer O, Trono D, Pfaff SL. Embryonic stem cell potency fluctuates with endogenous retrovirus activity. Nature. 2012 Jul 5;487(7405):57-63. doi: 10.1038/nature11244.Mandell, J. G., K. J. Goodrich, J. Bahler, and T. R. Cech. 2005. 'Expression of a RecQ helicase homolog affects progression through crisis in fission yeast lacking telomerase', *J Biol Chem*, 280: 5249-57.

Marasovic, M., M. Zocco, and M. Halic. 2013. 'Argonaute and Triman generate dicer-independent priRNAs and mature siRNAs to initiate heterochromatin formation', *Mol Cell*, 52: 173-83.

Martienssen, R. A., M. Zaratiegui, and D. B. Goto. 2005. 'RNA interference and heterochromatin in the fission yeast Schizosaccharomyces pombe', *Trends Genet*, 21: 450-6.

Marzluff, W. F., P. Gongidi, K. R. Woods, J. Jin, and L. J. Maltais. 2002. 'The human and mouse replication-dependent histone genes', *Genomics*, 80: 487-98.

Mathys, H., J. Basquin, S. Ozgur, M. Czarnocki-Cieciura, F. Bonneau, A. Aartse, A. Dziembowski, M. Nowotny, E. Conti, and W. Filipowicz. 2014. 'Structural and biochemical insights to the role of the CCR4-NOT complex and DDX6 ATPase in microRNA repression', *Mol Cell*, 54: 751-65.

Matsuda, A., Y. Chikashige, D. Q. Ding, C. Ohtsuki, C. Mori, H. Asakawa, H. Kimura, T. Haraguchi, and Y. Hiraoka. 2015. 'Highly condensed chromatins are formed adjacent to subtelomeric and decondensed silent chromatin in fission yeast', *Nat Commun*, 6: 7753.

Matsuda, E., R. Sugioka-Sugiyama, T. Mizuguchi, S. Mehta, B. Cui, and S. I. Grewal. 2011. 'A homolog of male sex-determining factor SRY cooperates with a transposon-derived CENP-B protein to control sex-specific directed recombination', *Proc Natl Acad Sci U S A*, 108: 18754-9.

Matsui, T., D. Leung, H. Miyashita, I. A. Maksakova, H. Miyachi, H. Kimura, M. Tachibana, M. C. Lorincz, and Y. Shinkai. 2010. 'Proviral silencing in embryonic stem cells requires the histone methyltransferase ESET', *Nature*, 464: 927-31.

Matsuyama, A., R. Arai, Y. Yashiroda, A. Shirai, A. Kamata, S. Sekido, Y. Kobayashi, A. Hashimoto, M. Hamamoto, Y. Hiraoka, S. Horinouchi, and M. Yoshida. 2006. 'ORFeome cloning and global analysis of protein localization in the fission yeast Schizosaccharomyces pombe', *Nat Biotechnol*, 24: 841-7.

McClintock, B. 1953. 'Induction of Instability at Selected Loci in Maize', *Genetics*, 38: 579-99.

McDonald, S. M., D. Close, H. Xin, T. Formosa, and C. P. Hill. 2010. 'Structure and biological importance of the Spn1-Spt6 interaction, and its regulatory role in nucleosome binding', *Mol Cell*, 40: 725-35.

McWilliam, H., W. Li, M. Uludag, S. Squizzato, Y. M. Park, N. Buso, A. P. Cowley, and R. Lopez. 2013. 'Analysis Tool Web Services from the EMBL-EBI', *Nucleic Acids Res*, 41: W597-600.

Meneghini, M. D., M. Wu, and H. D. Madhani. 2003. 'Conserved histone variant H2A.Z protects euchromatin from the ectopic spread of silent heterochromatin', *Cell*, 112: 725-36.

Mersman, D. P., H. N. Du, I. M. Fingerman, P. F. South, and S. D. Briggs. 2009. 'Polyubiquitination of the demethylase Jhd2 controls histone methylation and gene expression', *Genes Dev*, 23: 951-62.

Messner, S., and M. O. Hottiger. 2011. 'Histone ADP-ribosylation in DNA repair, replication and transcription', *Trends Cell Biol*, 21: 534-42.

Milligan, L., L. Decourty, C. Saveanu, J. Rappsilber, H. Ceulemans, A. Jacquier, and D. Tollervey. 2008. 'A yeast exosome cofactor, Mpp6, functions in RNA surveillance and in the degradation of noncoding RNA transcripts', *Mol Cell Biol*, 28: 5446-57.

Mizuguchi, G., X. Shen, J. Landry, W. H. Wu, S. Sen, and C. Wu. 2004. 'ATP-driven exchange of histone H2AZ variant catalyzed by SWR1 chromatin remodeling complex', *Science*, 303: 343-8.

Monahan, B. J., J. Villen, S. Marguerat, J. Bahler, S. P. Gygi, and F. Winston. 2008. 'Fission yeast SWI/SNF and RSC complexes show compositional and functional differences from budding yeast', *Nat Struct Mol Biol*, 15: 873-80.

Morgunova, V., N. Akulenko, E. Radion, I. Olovnikov, Y. Abramov, L. V. Olenina, S. Shpiz, D. V. Kopytova, S. G. Georgieva, and A. Kalmykova. 2015. 'Telomeric repeat silencing in germ cells is essential for early development in Drosophila', *Nucleic Acids Res*, 43: 8762-73.

Morozumi, Y., F. Boussouar, M. Tan, A. Chaikuad, M. Jamshidikia, G. Colak, H. He, L. Nie, C. Petosa, M. de Dieuleveult, S. Curtet, A. L. Vitte, C. Rabatel, A. Debernardi, F. L. Cosset, E. Verhoeyen, A. Emadali, N. Schweifer, D. Gianni, M. Gut, P. Guardiola, S. Rousseaux, M. Gerard, S. Knapp, Y. Zhao, and S. Khochbin. 2015. 'Atad2 is a generalist facilitator of chromatin dynamics in embryonic stem cells', *J Mol Cell Biol*.

Morris, S. A., Y. Shibata, K. Noma, Y. Tsukamoto, E. Warren, B. Temple, S. I. Grewal, and B. D. Strahl. 2005. 'Histone H3 K36 methylation is associated with transcription elongation in Schizosaccharomyces pombe', *Eukaryot Cell*, 4: 1446-54.

Mosammaparast, N., C. S. Ewart, and L. F. Pemberton. 2002. 'A role for nucleosome assembly protein 1 in the nuclear transport of histones H2A and H2B', *EMBO J*, 21: 6527-38.

Moser, B. A., and T. M. Nakamura. 2009. 'Protection and replication of telomeres in fission yeast', *Biochem Cell Biol*, 87: 747-58.

Motamedi, M. R., E. J. Hong, X. Li, S. Gerber, C. Denison, S. Gygi, and D. Moazed. 2008. 'HP1 proteins form distinct complexes and mediate heterochromatic gene silencing by nonoverlapping mechanisms', *Mol Cell*, 32: 778-90.

Motamedi, M. R., A. Verdel, S. U. Colmenares, S. A. Gerber, S. P. Gygi, and D. Moazed. 2004. 'Two RNAi complexes, RITS and RDRC, physically interact and localize to noncoding centromeric RNAs', *Cell*, 119: 789-802.

Muller, S., P. Filippakopoulos, and S. Knapp. 2011. 'Bromodomains as therapeutic targets', *Expert Rev Mol Med*, 13: e29.

Murawska, M., and A. Brehm. 2011. 'CHD chromatin remodelers and the transcription cycle', *Transcription*, 2: 244-53.

Nakagawa, H., J. K. Lee, J. Hurwitz, R. C. Allshire, J. Nakayama, S. I. Grewal, K. Tanaka, and Y. Murakami. 2002. 'Fission yeast CENP-B homologs nucleate centromeric heterochromatin by promoting heterochromatin-specific histone tail modifications', *Genes Dev*, 16: 1766-78.

Nakamura, T. M., J. P. Cooper, and T. R. Cech. 1998. 'Two modes of survival of fission yeast without telomerase', *Science*, 282: 493-6.

Nicolas, E., T. Yamada, H. P. Cam, P. C. Fitzgerald, R. Kobayashi, and S. I. Grewal. 2007. 'Distinct roles of HDAC complexes in promoter silencing, antisense suppression and DNA damage protection', *Nat Struct Mol Biol*, 14: 372-80.

Nimmo, E. R., A. L. Pidoux, P. E. Perry, and R. C. Allshire. 1998. 'Defective meiosis in telomere-silencing mutants of Schizosaccharomyces pombe', *Nature*, 392: 825-8.

Nishimura, K., T. Fukagawa, H. Takisawa, T. Kakimoto, and M. Kanemaki. 2009. 'An auxin-based degron system for the rapid depletion of proteins in nonplant cells', *Nat Methods*, 6: 917-22.

Nishioka, K., S. Chuikov, K. Sarma, H. Erdjument-Bromage, C. D. Allis, P. Tempst, and D. Reinberg. 2002. 'Set9, a novel histone H3 methyltransferase that facilitates transcription by precluding histone tail modifications required for heterochromatin formation', *Genes Dev*, 16: 479-89.

Noguchi, C., and E. Noguchi. 2007. 'Sap1 promotes the association of the replication fork protection complex with chromatin and is involved in the replication checkpoint in Schizosaccharomyces pombe', *Genetics*, 175: 553-66.

Noma, K., H. P. Cam, R. J. Maraia, and S. I. Grewal. 2006. 'A role for TFIIIC transcription factor complex in genome organization', *Cell*, 125: 859-72.

Noma, K., T. Sugiyama, H. Cam, A. Verdel, M. Zofall, S. Jia, D. Moazed, and S. I. Grewal. 2004. 'RITS acts in cis to promote RNA interference-mediated transcriptional and post-transcriptional silencing', *Nat Genet*, 36: 1174-80.

Octobre, G., A. Lorenz, J. Loidl, and J. Kohli. 2008. 'The Rad52 homologs Rad22 and Rti1 of Schizosaccharomyces pombe are not essential for meiotic interhomolog recombination, but are required for meiotic intrachromosomal recombination and mating-type-related DNA repair', *Genetics*, 178: 2399-412.

Oliva, A., A. Rosebrock, F. Ferrezuelo, S. Pyne, H. Chen, S. Skiena, B. Futcher, and J. Leatherwood. 2005. 'The cell cycle-regulated genes of Schizosaccharomyces pombe', *PLoS Biol*, 3: e225.

Olsen, C. A. 2012. 'Expansion of the lysine acylation landscape', *Angew Chem Int Ed Engl*, 51: 3755-6.

Ong, C. T., and V. G. Corces. 2014. 'CTCF: an architectural protein bridging genome topology and function', *Nat Rev Genet*, 15: 234-46.

Orsi, G. A., P. Couble, and B. Loppin. 2009. 'Epigenetic and replacement roles of histone variant H3.3 in reproduction and development', *Int J Dev Biol*, 53: 231-43.

Pai, C. C., R. S. Deegan, L. Subramanian, C. Gal, S. Sarkar, E. J. Blaikley, C. Walker, L. Hulme, E. Bernhard, S. Codlin, J. Bahler, R. Allshire, S. Whitehall, and T. C. Humphrey. 2014. 'A histone H3K36 chromatin switch coordinates DNA double-strand break repair pathway choice', *Nat Commun*, 5: 4091.

Pal-Bhadra, M., B. A. Leibovitch, S. G. Gandhi, M. R. Chikka, U. Bhadra, J. A. Birchler, and S. C. Elgin. 2004. 'Heterochromatic silencing and HP1 localization in Drosophila are dependent on the RNAi machinery', *Science*, 303: 669-72.

Papamichos-Chronakis, M., S. Watanabe, O. J. Rando, and C. L. Peterson. 2011. 'Global regulation of H2A.Z localization by the INO80 chromatin-remodeling enzyme is essential for genome integrity', *Cell*, 144: 200-13.

Partridge, J. F., K. S. Scott, A. J. Bannister, T. Kouzarides, and R. C. Allshire. 2002. 'cis-acting DNA from fission yeast centromeres mediates histone H3 methylation and recruitment of silencing factors and cohesin to an ectopic site', *Curr Biol*, 12: 1652-60.

Paull, T. T., E. P. Rogakou, V. Yamazaki, C. U. Kirchgessner, M. Gellert, and W. M. Bonner. 2000. 'A critical role for histone H2AX in recruitment of repair factors to nuclear foci after DNA damage', *Curr Biol*, 10: 886-95.

Pchelintsev, N. A., T. McBryan, T. S. Rai, J. van Tuyn, D. Ray-Gallet, G. Almouzni, and P. D. Adams. 2013. 'Placing the HIRA histone chaperone complex in the chromatin landscape', *Cell Rep*, 3: 1012-9.

Penny, G. D., G. F. Kay, S. A. Sheardown, S. Rastan, and N. Brockdorff. 1996. 'Requirement for Xist in X chromosome inactivation', *Nature*, 379: 131-7.

Phillips, D. M. 1963. 'The presence of acetyl groups of histones', *Biochem J*, 87: 258-63.

Plohl, M., A. Luchetti, N. Mestrovic, and B. Mantovani. 2008. 'Satellite DNAs between selfishness and functionality: structure, genomics and evolution of tandem repeats in centromeric (hetero)chromatin', *Gene*, 409: 72-82.

Polo, S. E., S. E. Theocharis, L. Grandin, L. Gambotti, G. Antoni, A. Savignoni, B. Asselain, E. Patsouris, and G. Almouzni. 2010. 'Clinical significance and prognostic value of chromatin assembly factor-1 overexpression in human solid tumours', *Histopathology*, 57: 716-24.

Pritham, E. J., and C. Feschotte. 2007. 'Massive amplification of rolling-circle transposons in the lineage of the bat Myotis lucifugus', *Proc Natl Acad Sci U S A*, 104: 1895-900.

Probst, A. V., I. Okamoto, M. Casanova, F. El Marjou, P. Le Baccon, and G. Almouzni. 2010. 'A strand-specific burst in transcription of pericentric satellites is required for chromocenter formation and early mouse development', *Dev Cell*, 19: 625-38.

Quivy, J. P., P. Grandi, and G. Almouzni. 2001. 'Dimerization of the largest subunit of chromatin assembly factor 1: importance in vitro and during Xenopus early development', *EMBO J*, 20: 2015-27.

Raeder, M. B., E. Birkeland, J. Trovik, C. Krakstad, S. Shehata, S. Schumacher, T. I. Zack, A. Krohn, H. M. Werner, S. E. Moody, E. Wik, I. M. Stefansson, F. Holst, A. M. Oyan, P. Tamayo, J. P. Mesirov, K. H. Kalland, L. A. Akslen, R. Simon, R. Beroukhim, and H. B. Salvesen. 2013. 'Integrated genomic analysis of the 8q24 amplification in endometrial cancers identifies ATAD2 as essential to MYC-dependent cancers', *PLoS One*, 8: e54873.

Ray-Gallet, D., J. P. Quivy, C. Scamps, E. M. Martini, M. Lipinski, and G. Almouzni. 2002. 'HIRA is critical for a nucleosome assembly pathway independent of DNA synthesis', *Mol Cell*, 9: 1091-100.

Recht, J., T. Tsubota, J. C. Tanny, R. L. Diaz, J. M. Berger, X. Zhang, B. A. Garcia, J. Shabanowitz, A. L. Burlingame, D. F. Hunt, P. D. Kaufman, and C. D. Allis. 2006. 'Histone chaperone Asf1 is required for histone H3 lysine 56 acetylation, a modification associated with S phase in mitosis and meiosis', *Proc Natl Acad Sci U S A*, 103: 6988-93.

Revenko, A. S., E. V. Kalashnikova, A. T. Gemo, J. X. Zou, and H. W. Chen. 2010. 'Chromatin loading of E2F-MLL complex by cancer-associated coregulator ANCCA via reading a specific histone mark', *Mol Cell Biol*, 30: 5260-72.

Reyes-Turcu, F. E., K. Zhang, M. Zofall, E. Chen, and S. I. Grewal. 2011. 'Defects in RNA quality control factors reveal RNAi-independent nucleation of heterochromatin', *Nat Struct Mol Biol*, 18: 1132-8.

Richmond, T. J., and C. A. Davey. 2003. 'The structure of DNA in the nucleosome core', *Nature*, 423: 145-50.

Rigaut, G., A. Shevchenko, B. Rutz, M. Wilm, M. Mann, and B. Seraphin. 1999. 'A generic protein purification method for protein complex characterization and proteome exploration', *Nat Biotechnol*, 17: 1030-2.

Rinn, J. L., and H. Y. Chang. 2012. 'Genome regulation by long noncoding RNAs', *Annu Rev Biochem*, 81: 145-66.

Rougemaille, M., S. Braun, S. Coyle, P. A. Dumesic, J. F. Garcia, R. S. Isaac, D. Libri, G. J. Narlikar, and H. D. Madhani. 2012. 'Ers1 links HP1 to RNAi', *Proc Natl Acad Sci U S A*, 109: 11258-63.

Sadaie, M., R. Kawaguchi, Y. Ohtani, F. Arisaka, K. Tanaka, K. Shirahige, and J. Nakayama. 2008. 'Balance between distinct HP1 family proteins controls heterochromatin assembly in fission yeast', *Mol Cell Biol*, 28: 6973-88.

Sakabe, K., Z. Wang, and G. W. Hart. 2010. 'Beta-N-acetylglucosamine (O-GlcNAc) is part of the histone code', *Proc Natl Acad Sci U S A*, 107: 19915-20.

Saksouk, N., E. Simboeck, and J. Dejardin. 2015. 'Constitutive heterochromatin formation and transcription in mammals', *Epigenetics Chromatin*, 8: 3.

Sasai, N., and P. A. Defossez. 2009. 'Many paths to one goal? The proteins that recognize methylated DNA in eukaryotes', *Int J Dev Biol*, 53: 323-34.

Schalch, T., G. Job, V. J. Noffsinger, S. Shanker, C. Kuscu, L. Joshua-Tor, and J. F. Partridge. 2009. 'High-affinity binding of Chp1 chromodomain to K9 methylated histone H3 is required to establish centromeric heterochromatin', *Mol Cell*, 34: 36-46.

Schalch, T., G. Job, S. Shanker, J. F. Partridge, and L. Joshua-Tor. 2011. 'The Chp1-Tas3 core is a multifunctional platform critical for gene silencing by RITS', *Nat Struct Mol Biol*, 18: 1351-7.

Schueler, M. G., and B. A. Sullivan. 2006. 'Structural and functional dynamics of human centromeric chromatin', *Annu Rev Genomics Hum Genet*, 7: 301-13.

Schulze, J. M., A. Y. Wang, and M. S. Kobor. 2009. 'YEATS domain proteins: a diverse family with many links to chromatin modification and transcription', *Biochem Cell Biol*, 87: 65-75.

Shain, A. H., and J. R. Pollack. 2013. 'The spectrum of SWI/SNF mutations, ubiquitous in human cancers', *PLoS One*, 8: e55119.

Shankaranarayana, G. D., M. R. Motamedi, D. Moazed, and S. I. Grewal. 2003. 'Sir2 regulates histone H3 lysine 9 methylation and heterochromatin assembly in fission yeast', *Curr Biol*, 13: 1240-6.

Smit, A. F., and A. D. Riggs. 1996. 'Tiggers and DNA transposon fossils in the human genome', *Proc Natl Acad Sci U S A*, 93: 1443-8.

Soler, G., I. Radford, C. Meyer, R. Marschalek, C. Brouzes, D. Ghez, S. Romana, and R. Berger. 2008. 'MLL insertion with MLL-MLLT3 gene fusion in acute leukemia: case report and review of the literature', *Cancer Genet Cytogenet*, 183: 53-9.

Stoilov, P., I. Rafalska, and S. Stamm. 2002. 'YTH: a new domain in nuclear proteins', *Trends Biochem Sci*, 27: 495-7.

Stralfors, A., J. Walfridsson, H. Bhuiyan, and K. Ekwall. 2011. 'The FUN30 chromatin remodeler, Fft3, protects centromeric and subtelomeric domains from euchromatin formation', *PLoS Genet*, 7: e1001334.

Sugiyama, T., H. P. Cam, R. Sugiyama, K. Noma, M. Zofall, R. Kobayashi, and S. I. Grewal. 2007. 'SHREC, an effector complex for heterochromatic transcriptional silencing', *Cell*, 128: 491-504.

Sugiyama, T., and R. Sugioka-Sugiyama. 2011. 'Red1 promotes the elimination of meiosis-specific mRNAs in vegetatively growing fission yeast', *EMBO J*, 30: 1027-39.

Tackett, A. J., D. J. Dilworth, M. J. Davey, M. O'Donnell, J. D. Aitchison, M. P. Rout, and B. T. Chait. 2005. 'Proteomic and genomic characterization of chromatin complexes at a boundary', *J Cell Biol*, 169: 35-47.

Taddei, A., F. Hediger, F. R. Neumann, and S. M. Gasser. 2004. 'The function of nuclear architecture: a genetic approach', *Annu Rev Genet*, 38: 305-45.

Tagami, H., D. Ray-Gallet, G. Almouzni, and Y. Nakatani. 2004. 'Histone H3.1 and H3.3 complexes mediate nucleosome assembly pathways dependent or independent of DNA synthesis', *Cell*, 116: 51-61.

Takahashi, K., S. Murakami, Y. Chikashige, O. Niwa, and M. Yanagida. 1991. 'A large number of tRNA genes are symmetrically located in fission yeast centromeres', *J Mol Biol*, 218: 13-7.

Talbert, P. B., and S. Henikoff. 2010. 'Histone variants--ancient wrap artists of the epigenome', *Nat Rev Mol Cell Biol*, 11: 264-75.

Teysset, L., V. D. Dang, M. K. Kim, and H. L. Levin. 2003. 'A long terminal repeat-containing retrotransposon of Schizosaccharomyces pombe expresses a Gag-like protein that assembles into virus-like particles which mediate reverse transcription', *J Virol*, 77: 5451-63.

Thon, G., P. Bjerling, C. M. Bunner, and J. Verhein-Hansen. 2002. 'Expression-state boundaries in the mating-type region of fission yeast', *Genetics*, 161: 611-22.

Trewick, S. C., E. Minc, R. Antonelli, T. Urano, and R. C. Allshire. 2007. 'The JmjC domain protein Epe1 prevents unregulated assembly and disassembly of heterochromatin', *EMBO J*, 26: 4670-82.

Trojer, P., and D. Reinberg. 2007. 'Facultative heterochromatin: is there a distinctive molecular signature?', *Mol Cell*, 28: 1-13.

Tseng, R. J., K. R. Armstrong, X. Wang, and H. M. Chamberlin. 2007. 'The bromodomain protein LEX-1 acts with TAM-1 to modulate gene expression in C. elegans', *Mol Genet Genomics*, 278: 507-18.

Turner, B. M. 2005. 'Reading signals on the nucleosome with a new nomenclature for modified histones', *Nat Struct Mol Biol*, 12: 110-2.

UniProt, Consortium. 2014. 'Activities at the Universal Protein Resource (UniProt)', *Nucleic Acids Res*, 42: D191-8.

Vaishnaw, A. K., J. Gollob, C. Gamba-Vitalo, R. Hutabarat, D. Sah, R. Meyers, T. de Fougerolles, and J. Maraganore. 2010. 'A status report on RNAi therapeutics', *Silence*, 1: 14.

Venkatesh, S., and J. L. Workman. 2015. 'Histone exchange, chromatin structure and the regulation of transcription', *Nat Rev Mol Cell Biol*, 16: 178-89.

Verdaasdonk, J. S., and K. Bloom. 2011. 'Centromeres: unique chromatin structures that drive chromosome segregation', *Nat Rev Mol Cell Biol*, 12: 320-32.

Verdel, A., S. Jia, S. Gerber, T. Sugiyama, S. Gygi, S. I. Grewal, and D. Moazed. 2004. 'RNAi-mediated targeting of heterochromatin by the RITS complex', *Science*, 303: 672-6.

Verdel, A., and D. Moazed. 2005. 'RNAi-directed assembly of heterochromatin in fission yeast', *FEBS Lett*, 579: 5872-8.

Verdel, A., A. Vavasseur, M. Le Gorrec, and L. Touat-Todeschini. 2009. 'Common themes in siRNA-mediated epigenetic silencing pathways', *Int J Dev Biol*, 53: 245-57.

Vidler, L. R., N. Brown, S. Knapp, and S. Hoelder. 2012. 'Druggability analysis and structural classification of bromodomain acetyl-lysine binding sites', *J Med Chem*, 55: 7346-59.

Volpe, T. A., C. Kidner, I. M. Hall, G. Teng, S. I. Grewal, and R. A. Martienssen. 2002. 'Regulation of heterochromatic silencing and histone H3 lysine-9 methylation by RNAi', *Science*, 297: 1833-7.

Volpe, T., and R. A. Martienssen. 2011. 'RNA interference and heterochromatin assembly', *Cold Spring Harb Perspect Biol*, 3: a003731.

Wan, W. N., Y. X. Zhang, X. M. Wang, Y. J. Liu, Y. Q. Zhang, Y. H. Que, and W. J. Zhao. 2014. 'ATAD2 is highly expressed in ovarian carcinomas and indicates poor prognosis', *Asian Pac J Cancer Prev*, 15: 2777-83.

Wang, J., S. T. Lawry, A. L. Cohen, and S. Jia. 2014. 'Chromosome boundary elements and regulation of heterochromatin spreading', *Cell Mol Life Sci*, 71: 4841-52.

Wang, J., B. D. Reddy, and S. Jia. 2015. 'Rapid epigenetic adaptation to uncontrolled heterochromatin spreading', *Elife*, 4.

Wang, J., X. Tadeo, H. Hou, P. G. Tu, J. Thompson, J. R. Yates, 3rd, and S. Jia. 2013. 'Epe1 recruits BET family bromodomain protein Bdf2 to establish heterochromatin boundaries', *Genes Dev*, 27: 1886-902.

Wang, Y., S. P. Kallgren, B. D. Reddy, K. Kuntz, L. Lopez-Maury, J. Thompson, S. Watt, C. Ma, H. Hou, Y. Shi, J. R. Yates, 3rd, J. Bahler, M. J. O'Connell, and S. Jia. 2012. 'Histone H3 lysine 14 acetylation is required for activation of a DNA damage checkpoint in fission yeast', *J Biol Chem*, 287: 4386-93.

Wang, Y., B. Reddy, J. Thompson, H. Wang, K. Noma, J. R. Yates, 3rd, and S. Jia. 2009. 'Regulation of Set9-mediated H4K20 methylation by a PWWP domain protein', *Mol Cell*, 33: 428-37.

Watanabe, Y., and M. Yamamoto. 1994. 'S. pombe mei2+ encodes an RNA-binding protein essential for premeiotic DNA synthesis and meiosis I, which cooperates with a novel RNA species meiRNA', *Cell*, 78: 487-98.

Wei, G. H., D. P. Liu, and C. C. Liang. 2005. 'Chromatin domain boundaries: insulators and beyond', *Cell Res*, 15: 292-300.

Williams, S. K., and J. K. Tyler. 2007. 'Transcriptional regulation by chromatin disassembly and reassembly', *Curr Opin Genet Dev*, 17: 88-93.

Winkler, D. D., U. M. Muthurajan, A. R. Hieb, and K. Luger. 2011. 'Histone chaperone FACT coordinates nucleosome interaction through multiple synergistic binding events', *J Biol Chem*, 286: 41883-92.

Wong, L. H. 2010. 'Epigenetic regulation of telomere chromatin integrity in pluripotent embryonic stem cells', *Epigenomics*, 2: 639-55.

Wood, V., R. Gwilliam, M. A. Rajandream, M. Lyne, R. Lyne, A. Stewart, J. Sgouros, N. Peat, J. Hayles, S. Baker, D. Basham, S. Bowman, K. Brooks, D. Brown, S. Brown, T. Chillingworth, C. Churcher, M. Collins, R. Connor, A. Cronin, P. Davis, T. Feltwell, A. Fraser, S. Gentles, A. Goble, N. Hamlin, D. Harris, J. Hidalgo, G. Hodgson, S. Holroyd, T. Hornsby, S. Howarth, E. J. Huckle, S. Hunt, K. Jagels, K. James, L. Jones, M. Jones, S. Leather, S. McDonald, J. McLean, P. Mooney, S. Moule, K. Mungall, L. Murphy, D. Niblett, C. Odell, K. Oliver, S. O'Neil, D. Pearson, M. A. Quail, E. Rabbinowitsch, K. Rutherford, S. Rutter, D. Saunders, K. Seeger, S. Sharp, J. Skelton, M. Simmonds, R. Squares, S. Squares, K. Stevens, K. Taylor, R. G. Taylor, A. Tivey, S. Walsh, T. Warren, S. Whitehead, J. Woodward, G. Volckaert, R. Aert, J. Robben, B. Grymonprez, I. Weltjens, E. Vanstreels, M. Rieger, M. Schafer, S. Muller-Auer, C. Gabel, M. Fuchs, A. Dusterhoft, C. Fritzc, E. Holzer, D. Moestl, H. Hilbert, K. Borzym, I. Langer, A. Beck, H. Lehrach, R. Reinhardt, T. M. Pohl, P. Eger, W. Zimmermann, H. Wedler, R. Wambutt, B. Purnelle, A. Goffeau, E. Cadieu, S. Dreano, S. Gloux, V. Lelaure, S. Mottier, F. Galibert, S. J. Aves, Z. Xiang, C. Hunt, K. Moore, S. M. Hurst, M. Lucas, M. Rochet, C. Gaillardin, V. A. Tallada, A. Garzon, G. Thode, R. R. Daga, L. Cruzado, J. Jimenez, M. Sanchez, F. del Rey, J. Benito, A. Dominguez, J. L. Revuelta, S. Moreno, J. Armstrong, S. L. Forsburg, L. Cerutti, T. Lowe, W. R. McCombie, I. Paulsen, J. Potashkin, G. V. Shpakovski, D. Ussery, B. G. Barrell, and P. Nurse. 2002. 'The genome sequence of Schizosaccharomyces pombe', *Nature*, 415: 871-80.

Wu, G., H. Liu, H. He, Y. Wang, X. Lu, Y. Yu, S. Xia, X. Meng, and Y. Liu. 2014. 'miR-372 down-regulates the oncogene ATAD2 to influence hepatocellular carcinoma proliferation and metastasis', *BMC Cancer*, 14: 107.

Wu, S. Y., G. Lopez-Berestein, G. A. Calin, and A. K. Sood. 2014. 'RNAi therapies: drugging the undruggable', *Sci Transl Med*, 6: 240ps7.

Yamada, T., W. Fischle, T. Sugiyama, C. D. Allis, and S. I. Grewal. 2005. 'The nucleation and maintenance of heterochromatin by a histone deacetylase in fission yeast', *Mol Cell*, 20: 173-85.

Yamanaka, S., S. Mehta, F. E. Reyes-Turcu, F. Zhuang, R. T. Fuchs, Y. Rong, G. B. Robb, and S. I. Grewal. 2013. 'RNAi triggered by specialized machinery silences developmental genes and retrotransposons', *Nature*, 493: 557-60.

Yamanaka, S., A. Yamashita, Y. Harigaya, R. Iwata, and M. Yamamoto. 2010. 'Importance of polyadenylation in the selective elimination of meiotic mRNAs in growing S. pombe cells', *EMBO J*, 29: 2173-81.

Yamane, K., T. Mizuguchi, B. Cui, M. Zofall, K. Noma, and S. I. Grewal. 2011. 'Asf1/HIRA facilitate global histone deacetylation and associate with HP1 to promote nucleosome occupancy at heterochromatic loci', *Mol Cell*, 41: 56-66.

Yamashita, A., Y. Shichino, H. Tanaka, E. Hiriart, L. Touat-Todeschini, A. Vavasseur, D. Q. Ding, Y. Hiraoka, A. Verdel, and M. Yamamoto. 2012. 'Hexanucleotide motifs mediate recruitment of the RNA elimination machinery to silent meiotic genes', *Open Biol*, 2: 120014.

Yang, J., J. Huang, L. Luo, Z. Chen, Y. Guo, and L. Guo. 2014. 'Significance of PRO2000/ANCCA expression, a novel proliferation-associated protein in hepatocellular carcinoma', *Cancer Cell Int*, 14: 33.

Ye, J., X. Ai, E. E. Eugeni, L. Zhang, L. R. Carpenter, M. A. Jelinek, M. A. Freitas, and M. R. Parthun. 2005. 'Histone H4 lysine 91 acetylation a core domain modification associated with chromatin assembly', *Mol Cell*, 18: 123-30.

Yu, C., M. J. Bonaduce, and A. J. Klar. 2012. 'Going in the right direction: mating-type switching of Schizosaccharomyces pombe is controlled by judicious expression of two different swi2 transcripts', *Genetics*, 190: 977-87.

Zamudio, N., and D. Bourc'his. 2010. 'Transposable elements in the mammalian germline: a comfortable niche or a deadly trap?', *Heredity (Edinb)*, 105: 92-104.

Zaratiegui, M., S. E. Castel, D. V. Irvine, A. Kloc, J. Ren, F. Li, E. de Castro, L. Marin, A. Y. Chang, D. Goto, W. Z. Cande, F. Antequera, B. Arcangioli, and R. A. Martienssen. 2011. 'RNAi promotes heterochromatic silencing through replication-coupled release of RNA Pol II', *Nature*, 479: 135-8.

Zaratiegui, M., M. W. Vaughn, D. V. Irvine, D. Goto, S. Watt, J. Bahler, B. Arcangioli, and R. A. Martienssen. 2011. 'CENP-B preserves genome integrity at replication forks paused by retrotransposon LTR', *Nature*, 469: 112-5.

Zentner, G. E., and S. Henikoff. 2013. 'Regulation of nucleosome dynamics by histone modifications', *Nat Struct Mol Biol*, 20: 259-66.

Zhang, K., K. Mosch, W. Fischle, and S. I. Grewal. 2008. 'Roles of the Clr4 methyltransferase complex in nucleation, spreading and maintenance of heterochromatin', *Nat Struct Mol Biol*, 15: 381-8.

Zhang, Y., Y. Sun, Y. Li, Z. Fang, R. Wang, Y. Pan, H. Hu, X. Luo, T. Ye, H. Li, L. Wang, H. Chen, and H. Ji. 2013. 'ANCCA protein expression is a novel independent poor prognostic marker in surgically resected lung adenocarcinoma', *Ann Surg Oncol*, 20 Suppl 3: S577-82.

Zheng, L., T. Li, Y. Zhang, Y. Guo, J. Yao, L. Dou, and K. Guo. 2015. 'Oncogene ATAD2 promotes cell proliferation, invasion and migration in cervical cancer', *Oncol Rep*, 33: 2337-44.

Zilberman, D., X. Cao, and S. E. Jacobsen. 2003. 'ARGONAUTE4 control of locus-specific siRNA accumulation and DNA and histone methylation', *Science*, 299: 716-9.

Zofall, M., and S. I. Grewal. 2006. 'Swi6/HP1 recruits a JmjC domain protein to facilitate transcription of heterochromatic repeats', *Mol Cell*, 22: 681-92.

Zofall, M., S. Yamanaka, F. E. Reyes-Turcu, K. Zhang, C. Rubin, and S. I. Grewal. 2012. 'RNA elimination machinery targeting meiotic mRNAs promotes facultative heterochromatin formation', *Science*, 335: 96-100.

Zou, J. X., L. Guo, A. S. Revenko, C. G. Tepper, A. T. Gemo, H. J. Kung, and H. W. Chen. 2009. 'Androgen-induced coactivator ANCCA mediates specific androgen receptor signaling in prostate cancer', *Cancer Res*, 69: 3339-46.

Zou, J. X., A. S. Revenko, L. B. Li, A. T. Gemo, and H. W. Chen. 2007. 'ANCCA, an estrogen-regulated AAA+ ATPase coactivator for ERalpha, is required for coregulator occupancy and chromatin modification', *Proc Natl Acad Sci U S A*, 104: 18067-72.

Zunder, R. M., and J. Rine. 2012. 'Direct interplay among histones, histone chaperones, and a chromatin boundary protein in the control of histone gene expression', *Mol Cell Biol*, 32: 4337-49. Cell. 2009 May 1;137(3):485-97. doi: 10.1016/j.cell.2009.02.040.

Acknowledgements

I am grateful to André Verdel who accepted me in his laboratory for my PhD, for his useful advises, his guide and involvement in my project. I would like to express my gratitude to Daniel Perazza for his daily supervision, for his support and for all he taught me.

I would like to thank Aline Probst, Benjamin Loppin, Angela Taddei and Saadi Khochbin for being members of my jury and for evaluating my PhD project. Thanks to the members of my thesis committee, Saadi Khochbin and Jérôme Govin, for their precious suggestions.

Special thanks to all members of Team 13 for all they have done for me in these years: Benoit, Cyril, Edwige, Emeline, Frédérique, Leila, Louis, Mathieu, Maxime and Mina. Thanks for their suggestions, our discussions, their daily support and sincere friendship. They made me feel at home even at work; there are so many good memories with them that I will keep forever in my heart. Thanks also to Claire, Sivan, Solenne and Virginie for their suggestions and support in the last months of my PhD. Thanks to Emilie and Otana: sharing the office with them was a wonderful moment.

I would like to thank all members of Team 6, their suggestions and our discussions were important for the advancement of my work.

I am grateful to a lot of people in the Albert Bonniot Institute: all members of the administrative team and many other friends who helped me a lot during these years. A special thanks to all students for our funny moments, in particular the 'old' PhD students: we sailed together in this sea, taking care of each other…good luck for your next steps!

I am grateful to the Fondation Arc for the financial support during my last year of PhD.

Thanks to all great friends I met in Grenoble and all people close to me during my PhD, in particular the 'Italians' and friends from CUG: they made these years perfect! I would like to thank all dear friends always present in my life, the ones who studied with me in the University of Milano-Bicocca and Paris7, in the high school and my unique 'coiresi'. They always show me that real friendship is not limited by space and time.

Thanks to my family for their constant support, in particular my brother Giacomo, Chiara and my beloved granny Piera. Special thanks to my parents Cesare and Maria Teresa who are my models and the constant source of my strength.