

Studying the function and regulation of non-coding transcription in fission yeast by the exosome complex

A dissertation presented for the degree of D. Phil.

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Abstract

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Recent studies have revealed multiple functions of non-coding (nc) transcripts, challenging our current understanding of RNA biology. In this study, I uncover the molecular mechanism involved in repression of the *pho*1 gene by the overlapping transcription of *prt* ncRNA, in the presence of inorganic phosphate (Pi). Upon Pi starvation, nc transcription is abolished and full induction of gene expression takes place. *prt* transcription results in formation of a transient heterochromatin at this locus. The heterochromatin may play a role in ensuring slow induction of the mRNA upon Pi starvation. The heterochromatin machinery is recruited via the binding of Red1 and Mmi1, RNA binding proteins, suggesting that these proteins are not exclusively restricted to meiotic RNAs. Interestingly, nuclear exosome is also recruited via Mmi1 to the nc transcript. It is required for transcription termination and degradation of *prt*. These data imply that coupling between 3' end formation and degradation of unstable nc RNAs is similar to that in *S. cerevisiae*.

I conclude that nc transcription in response to presence of nutrients can fine-tune gene expression and might also regulate activation kinetics of the inducible gene, *pho1*. Furthermore, I demonstrate the presence of a transcription termination coupled decay mechanisms relying on the nuclear exosome.

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These years of my D. Phil will remain to be my most formative years as a researcher.

"doing what little one can to increase the general stock of knowledge is as respectable an object of life, as one can in any likelihood pursue".

- Charles Darwin

Abbreviations

ChIP	Chromatin Immunoprecipitation
RNA	Ribonucleic acid
DNA	Deoxyribonucleic acid
Pol II	RNA Polymerase II
TI	Transcription interference
h	hour/hours
Pi	Inorganic phosphate
Nc	Non-coding
ncRNA	non-coding RNA
lncRNA	long non-coding RNA
phosphate-responsive signalling pathway	PHO pathway
prt	(*pho1* repressing transcript)
snRNA	Small nuclear RNA
snoRNA	Small nucleolar RNA
priRNA	Primal RNA
eRNA	Enhancer RNA
PROMPTs	Promoter associated transcripts
TSS	Transcription Start site
TSSa-RNAs	Transcription Start site associated RNAs
Poly (A)	Polyadenylation and cleavage site
CPF	Cleavage and polyadenylation factor
CPA	Cleavage and Polyadenylation
YES	Yeast Extract media with Supplements
EMMG	Edinburgh Minimal Media Glutamate
WT	wild type
tRNA	Transfer RNA
rRNA	Ribosomal RNA
S. cerevisiae	*Saccharomyces cerevisiae*
S. pombe	*Schizosaccharomyces pombe*
A. thaliana	*Arabidopsis thaliana*
H3	Histone 3
H4	Histone 4
TAP	Tandem Affinity purification
mRNA	Messenger RNA
PARs	promoter-associated RNAs
DTT	Dithiothreitol
cRACE	circularized rapid amplification of cDNA ends
μ M	micro molar
μ g	micro gram
μ l	micro litre
CTD	C-terminal domain
Bp/kb	base pairs/kilobases
Nt	nucleotides
kDa	Kilo Dalton
PNPP	*para*-Nitrophenylphosphate

Gfr	gene free region
PCR	Polymerase chain reaction
RT-PCR	Reverse transcriptase PCR
qRT-PCR	quantitative Reverse transcriptase PCR
pmol	pico mole
UV	ultra violet
RNAi	RNA interference
RISC	RNA-induced silencing complex
RITS	RNA induced transcriptional silencing
RDRC	RNA dependent RNA Pol complex
RNAse	Ribonuclease
SAP	shrimp alkaline phosphatase
TAP	tobacco acid pyrophosphatase
TRAMP	Trf4-Air1/2-Mtr4 polymerase complex

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Chapter 1

INTRODUCTION

Regulation of gene expression is essential for cell division, meiosis, growth, maintenance and adaptability of a cell. Gene expression can be regulated at multiple levels including transcription, RNA processing and export, translation and post-translational modification as well as turnover of a protein. Although extensive research has been performed to understand gene regulatory mechanisms, we are continuing to discover new players. With the advances in technology, in the past decade we have stumbled upon non-protein coding RNAs (ncRNAs). These are emerging as important players in the field of gene regulation.

A primary tool for the discovery of ncRNAs was the understanding that some ncRNAs are unstable. They can be visualised only when the RNA degradation machineries in the cell are compromised, the nuclear RNA exosome or the cytoplasmic RNA decay machinery, Xrn1. To our surprise, these studies revealed that 90% of the genome is transcriptionally active and encodes for ncRNAs (94% in *Schizosaccharomyces pombe (S. pombe)* (Bitton et al., 2011)). Techniques such as high resolution tiling arrays, RNA-seq and SAGE, have been key for the discovery of these transcripts.

It has become apparent that roles of RNA are not limited to coding for proteins. A subset of nc transcripts are constitutively synthesized at high levels and have house-

keeping functions in the cell, for example, tRNA, rRNA, sn/snoRNA etc. Apart from these ncRNAs, the cell transcribes a large number of nc transcripts whose functional role is still not evident. Recently, a few studies have shown that some of these ncRNAs can play an important role by affecting gene expression patterns (for review, see (Nie et al., 2012; Tisseur et al., 2011)). These results argue against ncRNAs being a by-product of low transcription fidelity. But, the number of cases studied is a minor fraction of the actual pervasive transcription of ncRNAs, showing that this field is far from exhaustion. We still don't understand completely the full impact of ncRNA transcription on genome function.

1.1 ncRNA: Types and genomic location

NcRNAs can be classified in a number of ways based on their, size, location, function, degradation, localization, 3' end formation mechanisms etc. Based on size, ncRNAs can be classified as-

Category	**Size (bp)**	**Example**
Short	17-34	miRNA, piRNA, siRNA
Small	60-300	snoRNA, PROMPTs, TSSa-RNAs
Large	>300	lincRNAs, CUTs, other lncRNAs

Table 1. Classification of ncRNA based on size

NcRNAs can be classified depending on the length of the transcripts into three broad categories of Short, small or large ncRNAs.

Some ncRNAs are found to be associated with specific cellular functions. For example, small nuclear RNA (snRNA; which performs intron splicing), tRNA and rRNA (which are involved in protein synthesis), telomerase RNA (which serves as template during DNA replication), microRNA (regulating translation and mRNA stability) and small interfering RNA (siRNA) (gene silencing). Other categories of ncRNAs include piwi interacting RNAs (piRNAs) (Transposon repression, DNA methylation), promoter-associated RNAs (PARs) and enhancer RNAs (eRNA) (may affect gene transcription). In wild-type (WT), *S. pombe* cells, 22% of total genes contribute to ncRNA transcripts other than those with housekeeping functions (Fig. 1) (Wood et al., 2012).

Large ncRNAs (>200 bp) are found to be transcribed from a number of genomic regions- (a) sense or antisense direction (the ncRNA partially or completely overlaps the mRNA gene in the sense or antisense direction); (b) bidirectional (when the ncRNA and mRNA use the same promoter); (iii) intronic (when the ncRNA is derived from an intron of a second transcript); and (iv) intergenic (when the ncRNA is transcribed from a region between two genes) (Da Sacco et al., 2012).

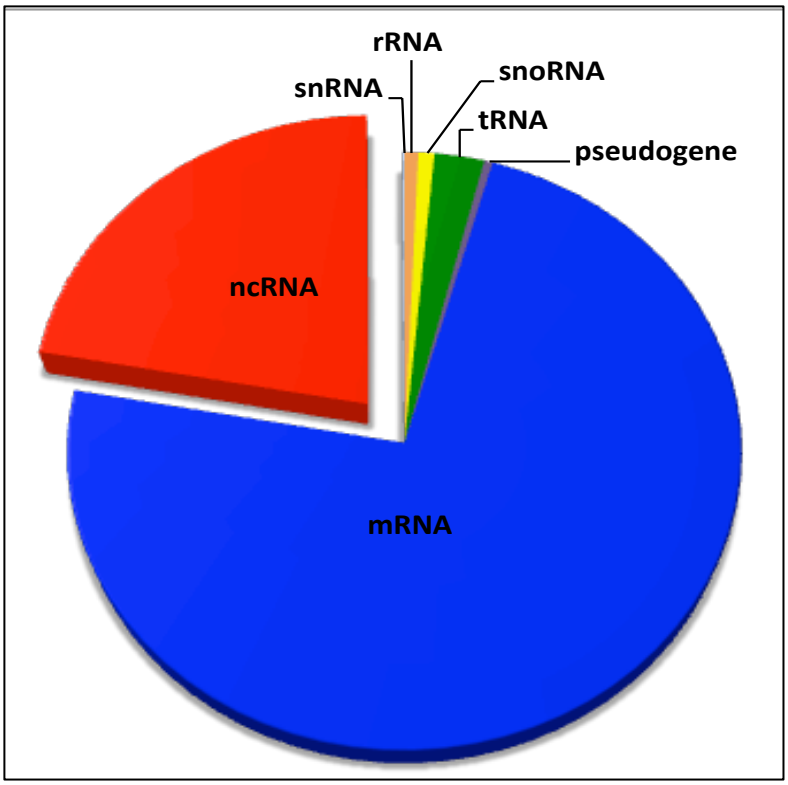


Figure 1—1. Transcript breakdown.

Summary of the gene counts for transcript categories based on data from *S. pombe* genome database (Wood et al., 2012). The number of genes encoding a particular class of ncRNA is calculated as a percent of the total number of genes in the *S. pombe* genome.

Transcript	Gene Count	Percentage
rRNA	47	0.7%
snoRNA	55	0.8%
tRNA	171	2.5%
pseudogene	29	0.4%
mRNA	5123	73.5%
ncRNA	1538	22.1%
snRNA	7	0.1%

Table 2. Number of genes encoding different classes of transcripts in *S. pombe* (Wood et al., 2012).

1.2 Role of 3' end formation in production and turnover of ncRNA

What distinguishes ncRNAs from mRNAs is not fully understood. Most ncRNAs are transcribed by Pol II (RNA Polymerase II). They bear features similar to mRNA transcripts i.e. they are capped, polyadenylated and can be spliced. The identification of uORFs (upstream Open Reading frames) argues against the lack of a substantial ORF as a criteria enough to classify transcripts as ncRNAs (Dinger et al., 2008). Interestingly, the mechanism of transcription termination of the ncRNAs and their tendency of rapid decay was found to play an important role in identifying some ncRNAs (for example, CUTs (Cryptic unstable transcripts) in *S. cerevisiae)*.

1.2.1 Transcription termination

Transcription termination for Pol II transcribed mRNA genes is the process by which the pre-mRNA attached to the elongating Pol II is released to form the 3' end of the mature RNA. This is followed by the transcribing Pol II detaching from the DNA template leading to termination of transcription. The pre-mRNA is capped at the 5' end during transcription. As the Pol II transcribes past the cleavage site, the RNA is cut by the cleavage and polyadenylation factor (CPF). The endonuclease *Ysh1* (Brr5 in budding yeast or CPSF-73 in mammals) performs endonucleolytic cleavage at the poly (A) (pA) site to generate the 3' end. The RNA 3' end is then polyadenylated by the Poly (A) polymerase. After cleavage of the mRNA, the Pol II still continues to transcribe (Kuehner et al., 2011). The release of the Pol II from the DNA template can occur until a few kilobases (Proudfoot, 1989). The nascent transcript being generated, with an exposed 5' end, is recognized by the 5' to 3' exonuclease Dhp1 in *S. pombe* (Rat1 in *S. cerevisiae* and Xrn2 in mammalian cells) (Kim et al., 2004, 2006). It was

proposed that, Dhp1 competes with the Pol II for its substrate. This results in release of the Pol II from the DNA template and termination of transcription (Torpedo model). The release of Pol II may also be a consequence of allosteric changes in the conformation of the enzyme after transcribing past the cleavage site (Allosteric model). Current ideas in the field suggest a unified model comprising of both torpedo and the allosteric model (for review, (Buratowski, 2005; Kuehner et al., 2011; Richard and Manley, 2009). Failure of proper transcription termination results in production of read-through transcripts. These are degraded by the cellular exonucleases and thus not exported to the cytoplasm for translation. It is speculated that improper termination results in decreased initiation of gene transcription forming a negative feedback loop (Richard and Manley, 2009). However, to ensure proper termination, cells possess a number of fail-safe termination mechanisms. These mechanisms can be studied upon compromised function of the canonical transcription termination pathway (Grzechnik and Kufel, 2008; Rondón et al., 2009).

Interestingly, as opposed to mRNAs, which have well defined 3' ends (due to CPF), CUTs are shown to have heterogeneous 3'ends. Similar to mRNAs, CUTs are transcribed by Pol II and are capped (Thiebaut et al., 2006). A defining feature for identification of CUTs is the rapid degradation of these transcripts by the exosome complex. Interestingly, RNA decay is coupled to the manner in which their transcription is terminated (Fig. 1-2) (Arigo et al., 2006; Thiebaut et al., 2006; Vasiljeva and Buratowski, 2006). Transcription termination of CUTs relies on the Nrd1-Nab3-Sen1 complex. The Nrd1-Nab3-Sen1 complex interacts with both Pol II and the RNA. This complex acts in a sequence specific manner and is recruited soon after transcription initiation. Nrd1 binds to the C-terminal domain (CTD) of the Pol II

largest subunit (Rpb1) phosphorylated in the beginning of the transcription cycle when it is phosphorylated on Serine 5 (Vasiljeva et al., 2008). This directs early transcription termination based on the presence of the Nrd1 binding sites on the nascent RNA (Gudipati et al., 2008). This determines a size limits for CUTs, which are typically short transcripts of 200-500 bp.

Although the CUTs are rapidly degraded, it is becoming evident that their transcription may not be a mere by-product of open chromatin (Neil et al., 2009; Xu et al., 2009). But this pervasive transcription may have a regulatory role (Colin et al., 2011; Xu et al., 2009). A few examples of CUTs regulating gene expression have been described *SRG1* (regulating the *SER3* gene) and the *PHO84* antisense RNA (regulating the *PHO84* gene) in *S. cerevisiae* (Camblong et al., 2009a; Martens et al., 2004). The distinction between stable and unstable transcripts may not be very discrete since CUTs may be stable under specific physiological conditions (eg. PHO84 CUT is stabilized in aging cells (Camblong et al., 2007)).

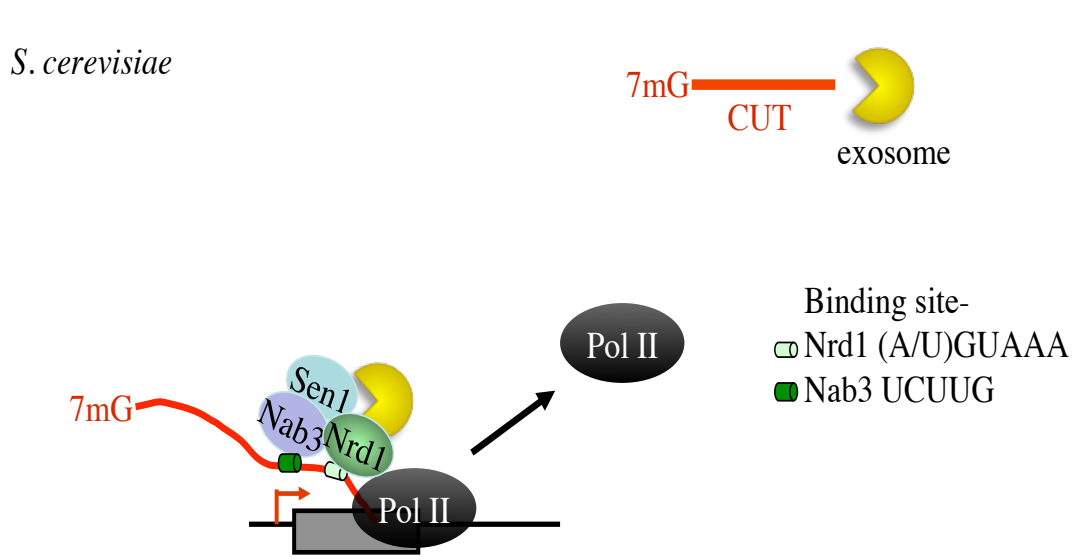


Figure 1—2. Transcription termination of CUTs in *S. cerevisiae* is linked to RNA decay.

Nrd1, Nab3 and Sen1 complex is recruited to the Pol II transcribed CUTs by the presence of the binding site sequences. This leads to the release of the Pol II via the process of transcription termination and recruitment of the exosome complex. The CUTs are degraded by the exosome. Thus recruitment of the Nrd1/Nab3/Sen1 complex is important for the transcription termination coupled RNA decay of these transcripts found in *S. cerevisiae*.

1.2.2 The exosome complex

The exosome is one of the major 3'-5' RNA processing/degradation machinery in the cell. Exosomes were originally found in yeast as an essential protein complex with 3′ → 5′ exonuclease activity and subsequently identified in humans; previously known as the PM-Scl (polymyositis–scleroderma overlap syndrome) complex (Allmang et al., 1999; Mitchell et al., 1997; Morrissey and Tollervey, 1995). The yeast exosome core consists of a barrel like structure formed by a hexameric ring and a trimeric cap. The catalytic subunit Rrp44 (Dis3) attaches at the bottom of this structure (Fig. 1-3). The trimeric cap comprises of proteins with SI and KH RNA

binding domains (Csl4, Rrp4, Rrp40). The hexameric ring is composed of RNAse PH domain containing inactive proteins (Rrp 41/42/43/45/46 and Mtr3). Although the archael and plant exosome core subunits do possess catalytic activity, the yeast exosome core harbours its catalytic activity in the Rrp44 subunit that has both a 3' to 5' exonuclease activity along with an endonuclease activity. The above 10 proteins form the core exosome. Furthermore the nuclear exosome has two additional subunits the exonuclease Rrp6 as well as Rrp47, a protein that is suspected to have a role as an RNA binding protein (Butler and Mitchell, 2010). Both Rrp6 and Dis3 are thus the main catalytic subunits of the yeast exosome.

Double mutants of Rrp6 and Dis3 are found to be lethal. However the temperature sensitive mutants of Dis3 and deletion of Rrp6 individually are sufficient to compromise exosome activity. Crystal structures of exosome bound to substrate RNA have helped understand how the substrates are recognized and channelled through the exosome to aid degradation or processing (Wasmuth et al., 2014). The focus of many studies has been to identify cofactors bound to the exosome to aid in recognition of substrates.

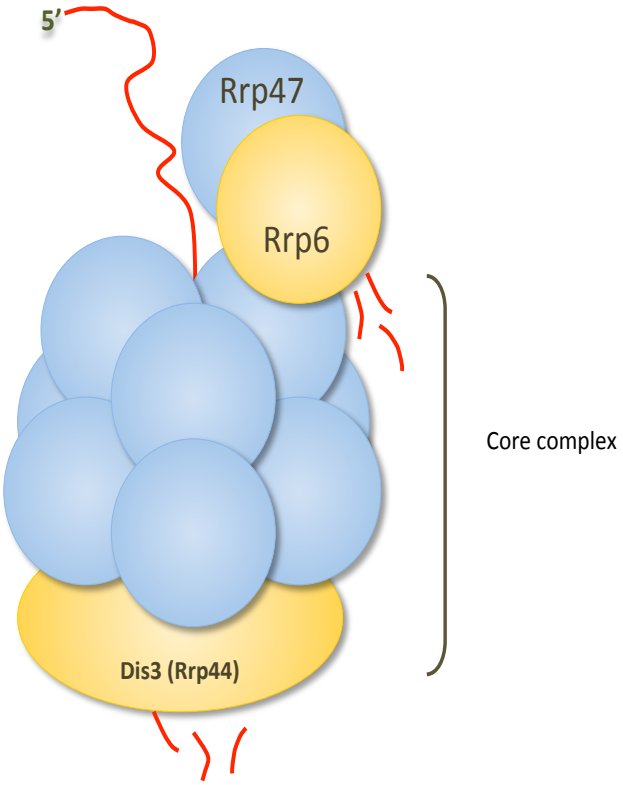


Figure 1—3. Exosome complex.

The yeast exosome complex comprises of a 9 inactive subunits forming a heterohexameric core and a trimeric ring structure. The activity to the core is provided by the Dis3 (Rrp44) subunit at the base of the structure. Additional proteins such as Rrp6 (catalytic subunit) and Rrp47 are found in the nuclear form of the exosome. The active subunits are represented in yellow and inactive in blue.

1.3 Mechanisms for regulation of gene expression by large ncRNAs

Long non-coding RNA classification however is not as forthright as in the case of CUTs. Similar to mRNAs, most lncRNAs are transcribed by Pol II. Thus, they possess a 5' terminal methylguanosine cap and are polyadenylated and can be spliced. However, several lncRNAs overlap with mRNA genes and hence may possess a functional ORF. Also, short peptides (uORFs) have been found, thus creating ambiguity in distinguishing lncRNAs (Dinger et al., 2008). Mechanisms involved in 3' end formation of lncRNAs have not been studied. It is possible that similar to CUTs, lncRNA termination may also rely on additional factors, so far unknown.

However, in contrast to CUTs, whose functions are not well defined, more examples of lncRNAs are known, which perform a number of cellular functions. Interestingly, it is also evident that no two lncRNAs have a similar mode of action. The functional data for lncRNAs in *S. pombe* is available only for a few genes such as *fbp1*, *ade6-M26* (Hirota and Ohta, 2009; Hirota et al., 2008). However, lncRNA functions are extensively investigated in *S. cerevisiae* and higher eukaryotes (Lee, 2012). A complete overview of known lncRNAs and their functions can be found in a recent review (Cech and Steitz, 2014). NcRNAs exert RNA mediated as well as transcription dependent regulatory effects.

1.3.1 RNA mediated regulation

The mode of RNA-mediated regulation of genes can be classified in four main categories: signalling, decoys, guides and scaffolds (Baker, 2011). Acting as signalling molecules or decoys, lncRNAs may activate or suppress gene expression. Alternatively, they may affect chromatin modifications by functioning as guides or scaffolds for chromatin modifying enzymes.

1) Signalling- The RNA molecule may signal proteins by binding to the gene. Thus recruiting transcription factors or silencing factors to activate or repress gene transcription respectively. Eg. XIST, which plays a role in mammalian X chromosome inactivation in females), although the mechanism is still unclear.

2) Decoys- Acting as decoys lncRNAs may bind to the transcription activators thereby suppressing gene expression. Eg. *TUG1* and *MALAT1/NEAT2* lncRNAs that regulate methylation/demethylation of Polycomb 2 protein (Pc2) and regulate a subset of genes

3) Guides- lncRNAs might be transcribed in *cis* (lncRNA transcribed from the same genomic locus as the gene it regulates) or in *trans* (lncRNA transcribed from a genomic locus/ chromosome distant to the gene it regulates). They can regulate gene expression by acting as guides to recruit chromatin-modifying enzymes. Thus creating a genomic environment that may be favourable or inhibiting for gene expression. (Eg. *PHO5* ncRNA)

4) Scaffolds- lncRNA can act as scaffolds for binding of ribonucleoprotein complexes to aid histone modifications (eg. HOTAIR)

1.3.2 Transcription mediated gene regulation

Apart from the RNA mediated regulatory functions of nc transcripts, the transcription of ncRNAs in *cis* can itself mediate gene silencing by a phenomenon called transcriptional interference (TI). Previously, TI was thought to be restricted to upstream ncRNA transcription physically interfering with transcription initiation machinery at the downstream mRNA gene (Greger et al., 2000; Martens et al., 2005). However, TI could also influence chromatin architecture including nucleosome remodelling, enhancer or promoter activity or block RNAPII elongation, splicing or

polyadenylation (Gullerova and Proudfoot, 2010; Kornienko et al., 2013; Palmer et al., 2011). It is essential for the transcription of the ncRNA to overlap with a regulatory region of the other gene to exert its regulatory function. This region could be an activator/ repressor sequence, gene promoter or a region of active collision between transcribing polymerases on convergent transcripts.

Nonetheless, it is apparent that each ncRNA regulates gene expression in a different manner and may rely on RNA mediated or transcription mediated mechanisms or a combination of these strategies to affect gene expression (Cech and Steitz, 2014).

1.4 ncRNA mediated regulation of heterochromatic locus

Based on the degree of chromatin condensation, nuclear domains are classified into euchromatin and heterochromatin. Euchromatin comprises actively transcribed regions whereas heterochromatin is transcriptionally silent. Heterochromatic structures are required for proper centromere function, sister chromatid cohesion, repression of recombination and the maintenance of telomere stability. They also play an essential role in heritable gene silencing in a variety of organisms from yeast to humans (Grewal and Jia, 2007). The initiation, propagation and spreading of heterochromatin is mainly controlled by mono, di or tri methylation of lysine 9 residue on histone H3 (H3K9me). H3K9me also results in repression of gene transcription by antagonizing lysine 9 acetylation, serine 10 phosphorylation and tri-methylation of lysine 4 on histone H3, which are all marks of active transcription. Different enzymes in mammals, drosophila and plants mediate H3k9me methylation.

The first member to be identified amongst this group of enzymes was Su(var)3-9 in Drosophila. In mammals, the H3K9me2 and H3K9me3 in pericentromeric heterochromatin are mediated via Suv39h1/h2 isoforms. The H3k9me and H3K9me2 marks in euchromatic regions are mediated by the G9a/GLP complex (Fritsch et al., 2010). Additionally another protein, SETDB1 might mediate all forms of H3K9 methylation. 10 putative H3K9MTs (SUV1–10) are found in plants (Vaillant and Paszkowski, 2007). However, in *S. pombe*, Clr4 methyltransferase (Suv39h) is the sole methyltransferase responsible for all the different forms of methylation of H3K9 which so far have not been shown to represent different forms of heterochromatin (Nakayama et al., 2001; Zhang et al., 2008, 2011). In *S.pombe*, genome-wide study of H3K9me2 revealed these marks to be present in almost all heterochromatic regions and on some euchromatic genes that are repressed (Yamanaka et al., 2013). I used antibodies against H3K9me2 to detect potential heterochromatin formation.

Importantly, it was found that heterochromatin formation relies on the roles of long and short ncRNAs. These ncRNAs may act as guides, scaffolds, or signalling molecules acting in *cis* or *trans*.

1.4.1 Types of heterochromatin

Constitutive heterochromatin

Initially, heterochromatin was studied at compact repetitive regions of eukaryotic chromosomes (centromeres, peri centromeres, mating type locus and subtelomeres). All these loci have dg and/or dh repeats in common. Transcription of

these loci gives rise to long ncRNAs (eg. cenRNAs from centromeric outermost (dg and dh) and innermost (tRNA clusters) repeats. The centromeric repeats are transcribed in both forward and reverse direction. Reverse strand transcription is found to be constitutive and maybe required for the maintenance of heterochromatin. However, the transcription from the forward strand is dependent on chromatin status and is required for heterochromatin initiation (Kurdistani and Grunstein, 2003). The constitutive heterochromatin is essential for maintaining genome integrity. It silences regions that are hotspots of DNA damage and recombination and preventing abnormal chromatid segregation. Transcription in these regions occurs only in the S phase in a cell cycle dependent manner which is needed for nucleation of heterochromatin (Chen et al., 2008).

Facultative heterochromatin

On the other hand, facultative heterochromatin assembles at developmentally regulated loci or rRNA genes in response to developmental cues (Trojer and Reinberg, 2007). Facultative heterochromatin is essential for normal cell development and differentiation by inactivation of germ-line specific genes. In higher eukaryotes it is responsible for allelic exclusion, genomic imprinting, and inactivation of X chromosome etc. In higher eukaryotes this type of silencing occurs over large regions of the genome. However, in *S. pombe,* apart from regions of constitutive heterochromatin, H3K9me2 is found at additional 30 loci including meiotic genes that are silenced during vegetative growth, ncRNAs and a few LTRs (Zofall et al., 2012). In *S. pombe*, dedicated cellular machinery is employed for the formation of facultative heterochromatin at meiotic loci (described in section 4.B).

Transient/Intermediate heterochromatin

Unlike constitutive or facultative heterochromatin, this type of heterochromatin is not dependent completely on cell cycle stage. The heterochromatin may be formed for a short time period on an actively transcribed gene, which might be regulated depending on environmental signals mainly stress. However, so far very few examples of this type of heterochromatin have been found. The biochemical details are not very well understood. Intermediate heterochromatin mainly occurs at single gene loci and the genes bear marks of both active and repressive chromatin (Tittel-Elmer et al., 2010). Marks such as H3K4me3, H3K9Ac that signify active transcription and inhibiting chromatin marks H3K9me3, H3K9me2 and H3K27me3 are found to be present. It is believed that this type of heterochromatin is used to merely fine tune gene expression by modulating a few histones rather than leading to complete transcriptional silencing. An example of this type of heterochromatin is found in plants. In *A. thaliana*, it was found that transient heterochromatin modulates expression of flowering genes in response to heat stress (Tittel-Elmer et al., 2010).

1.4.2 lncRNAs, siRNAs and priRNAs are required for targeting of silencing machinery to heterochromatic loci.

NcRNAs transcribed from heterochromatic regions are processed into small RNAs via the two ribonuclease III enzymes Dicer (Dcr1) and Drosha, components of the RNAi (RNA interference) pathway (Kim et al., 2009b). Drosha is involved in generation of small ncRNAs called miRNAs (micro RNAs) from hairpin structures in RNA. However the existence of miRNA in *S. pombe* remains unproven. The RNAi

pathway in *S. pombe* relies on Dcr1 to generate siRNAs (silencing RNAs). siRNAs are transported to the cytoplasm by shuttling of Dcr1 and are incorporated into RISC (RNA-induced silencing complex that promotes RNA degradation and translational inhibition. In *S. pombe*, siRNAs also result in transcriptional gene silencing in the nucleus by loading onto the Argonaute family protein, Ago1, component of the RNA induced transcriptional silencing (RITS) complex. RITS also comprises of the chromodomain protein Chp1 and Tas3 (a WG/GW containing protein that links Ago1 to Chp1) (Verdel et al., 2004). Ago1 and Chp1 subunits of RITS have been shown to bind to chromatin using ChIP (Chromatin immunoprecipitation) (Hiriart et al., 2012). Although it has been difficult to ChIP Dcr1; using Dam ID technique, chromatin association of Dcr1has been demonstrated (Woolcock et al., 2011). With the help of siRNAs bound to Ago1, RITS recruits the Rik1/CLRC (recombination in K, cryptic locus regulator) complex to silence the targeted loci (Reinhart and Bartel, 2002; Volpe et al., 2002).

In *S. pombe*, heterochromatic silencing is regulated via actions of Clr4/Suv39h and Lid2 which methylate H3K9 and demethylate histone H3K4 respectively (Kloc et al., 2008). H3K9 methylation results in compaction of the chromatin, thereby disallowing Pol II transcription (Kato et al., 2005). Paradoxically, a basal level of transcription is required to initiate and maintain the heterochromatin. Indeed mutations in Pol II lead to defective heterochromatin silencing at centromeres. Nucleation of heterochromatin also relies on another class of small ncRNAs called the priRNA (primal RNAs) (Halic and Moazed, 2010). Thus, a concerted effort comprising of long and short ncRNAs is required for heterochromatin formation (Fig. 4).

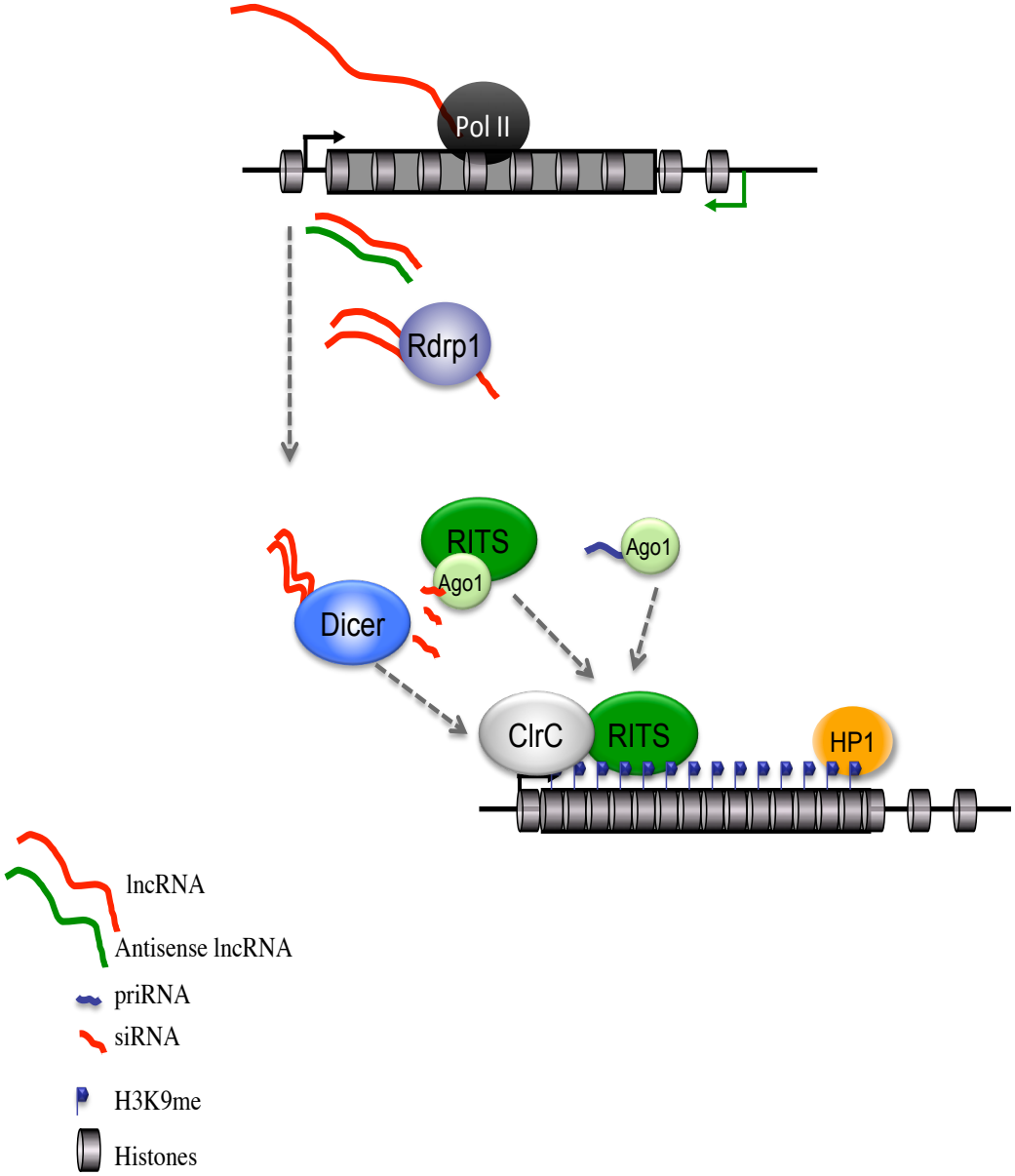


Figure 1—4. Roles of ncRNA in heterochromatin formation

In *S. pombe*, most constitutive heterochromatic regions are transcribed by Pol II to generate lncRNA. Some genes comprise of convergent antisense transcription units, which results in dsRNA structures. These RNAs are bound by Dicer to generate siRNAs. Ago1, a component of the RITS complex, binds siRNAs. In case of genes with no antisense transcription, dsRNAs are generated by the action of Rdrp1. Degradation products (in the case of centromeric transcripts) result in formation of priRNA, which can also feed into Ago1. These Ago1 bound siRNA guide the heterochromatic machinery to the target gene and result in recruitment of ClrC complex that methylates H3K9 residues via Clr4 methyltransferase. This in turn recruits the HP1-like proteins resulting in heterochromatin establishment at the target genes. Adapted from (Halic and Moazed, 2010)

H3k9me2 is important for stabilizing RITS association with chromatin by creating a binding site for the Chp1 protein (Noma et al., 2004). Binding of Chp1 further facilitates recruitment of the RNA-dependent RNA polymerase complex (RDRC) and production the double-stranded RNA (dsRNA), that are processed by Dcr1 into siRNA (Motamedi et al., 2004; Sugiyama et al., 2005). Thus leading to amplification of the siRNAs signal. Interestingly, Dcr1 can also result in production of siRNAs from stem loop structures in cenRNAs or from convergent transcription and can recruit Clr4 independent of RITS complex (Yu et al., 2014). Another mechanism for heterochromatin formation depends on read-through transcription from convergent loci, producing dsRNAs. Processing of the dsRNA into siRNAs results in H3k9me2 at the locus and heterochromatin assembly (Gullerova et al., 2011).

The H3k9me2 histones are bound by HP1 homologs, Chp2 and Swi6, which result in heterochromatin condensation and spreading. However, heterochromatin is restricted from spilling into euchromatic regions by nucleosome turnover (Zofall et al., 2009) as against constitutive heterochromatic regions where histone H3 turnover is low (Aygün et al, 2013). Chp2 and Swi6 also associate with the Snf2-HDAC repressor complex (SHREC), Asf1-HIRA chaperone and Clr6 deacetylase which aid transcriptional silencing (Anderson et al., 2009; Fischer et al., 2009; Motamedi et al., 2008; Sugiyama et al., 2007). Another fascinating aspect is that Swi6 recruits an anti-silencing factor, Epe1 that allows transcription in heterochromatin regions. In wild type cells, heterochromatin islands are found at around 30 genes in euchromatic loci. However, in Epe1 mutants more than 100 genes display H3k9me2 (Zofall et al., 2012). It is speculated that Swi6 with the help of Epe1 balances the amount of

transcriptional silencing at the heterochromatic loci. Another mechanism that regulates transcriptional silencing at mRNAs where heterochromatin assembly occurs has recently been described. The presence of polyadenylation signals in 3' UTR of mRNAs prevents the heterochromatin from silencing the gene. These sequences result in the release and export of the mRNA before it can be targeted by the RITS complex (Yu et al., 2014).

Interestingly, work from the Bühler lab identified a novel lncRNA, BORDERLINE, which helps in maintaining the boundary between heterochromatic and euchromatic regions restricting the spread of silencing. BORDERLINE RNA is transcribed from pericentromeric regions on chromosome I at the border of heterochromatin. Dcr1, to form short RNAs called brdrRNAs, cleaves the long ncRNA. Surprisingly, instead of loading onto Ago1, like other siRNAs, the brdrRNAs bind to Swi6 protein. Swi6 is required for maintenance and spreading of silencing in heterochromatin loci. Binding of the ncRNA to Swi6 changes its conformation and reduces its affinity to the chromatin. Thus resulting in loss of heterochromatin. Thus, ncRNA helps to prevent spreading of heterochromatin into euchromatic regions. It acts as a decoy causing an allosteric change in the protein conformation of Swi6 (Keller & Bühler 2013).

Long and short ncRNAs, both in *cis* and in *trans,* use a number of RNA and transcription dependent mechanisms to regulate heterochromatin nucleation, spreading and maintenance. Thus, ncRNAs can serve as scaffolds to recruit the entire machinery for constitutive heterochromatin formation at a locus. Moreover, it is also becoming increasingly evident that the act of transcription of the ncRNA is also

important for heterochromatin formation.

1.4.3 Role of ncRNAs and facultative heterochromatin in regulating meiosis

In yeast, gene expression patterns, including protein coding and ncRNAs, are greatly altered between mitotic (vegetative) and meiotic (sexual differentiation) cells. In *S. cerevisiae*, meiosis specific ncRNAs are termed Meiotic Unannotated Transcripts (MUTs) that are degraded by the exosome complex in mitotic cells (Lardenois et al., 2011).

Interestingly, in *S. pombe*, greater than 600 loci show change in ncRNA expression upon sexual differentiation (Bitton et al., 2011). Indeed, meiRNA (a ncRNA encoded by the *sme2* gene) plays a critical role in sexual differentiation in *S. pombe*. During meiosis, meiRNA forms a complex with Mei2 (regulator of meiotic commitment) visualized in cells as the 'Mei dot' (Fig. 5) (Harigaya et al., 2006). Importantly this Mei dot structure sequesters the Mmi1 protein (a YTH family RNA binding protein). Mmi1 along with Pab2, the poly(A) mRNA binding protein and cleavage factors Rna15 and Pcf11, is responsible for degrading meiotic transcripts during mitotic growth, thereby blocking sexual differentiation (McPheeters et al., 2009; St-André et al., 2010; Yamanaka et al., 2010). Thus, sequestering Mmi1 via meiRNA and Mei2 is essential for progression into meiosis. Conversely, during mitosis, Mei2 is inhibited via phosphorylation by Pat1 kinase (Watanabe and Yamamoto, 1997). Along with additional proteins such as Red1 (RNA elimination defective protein), Mmi1 and Pab2 recruit the exosome complex for degradation of meiotic transcripts inappropriately expressed during mitosis (Harigaya et al., 2006;

Lemay et al., 2010; Sugiyama and Sugioka-Sugiyama, 2011; Yamanaka et al., 2010). Mmi1 recognizes meiotic transcripts via presence of a sequence motif U(U/C)AAAC called DSR (Determinant of Selective-Removal) elements (Fig. 5) (Yamashita et al., 2012).

Meiotic gene expression during mitosis is not only restricted via post-transcriptional decay of transcripts but also via formation of facultative heterochromatin (for review, (Holm and Thon, 2012)) which is shown to be developmentally regulated in other organisms (Trojer and Reinberg, 2007). This heterochromatin is lost in response to nutritional signals that result in sexual differentiation. Components of the RITS complex, Ago1, Tas3 and Chp1 were shown to bind meiotic genes (Hiriart et al., 2012). Despite RITS being recruited to meiotic loci, mutants of the RITS complex have no major effects on H3K9me. Thus suggesting RNAi independent mechanisms also play a role in heterochromatin formation. Indeed, Clr4 can be recruited by the RNA binding protein, Red1 directly (Fig. 5) ((Hiriart et al., 2012; Tashiro et al., 2013; Zofall et al., 2012).

Loss of Mmi1, Red1 or Pla1 (Poly (A) polymerase) abolishes H3k9me2 at meiotic loci (Yamanaka et al., 2013; Zofall et al., 2012). Additionally deletion of DSR also abolishes heterochromatin nucleation. In fact, artificially expressed DSR can result in heterochromatin formation. Moreover, upon sexual differentiation (which mimics Mmi1 loss), H3k9me2 is lost from meiotic genes (Hiriart et al., 2012; Tashiro et al., 2013). Thus, Mmi1 and Red1 mediate heterochromatin assembly at meiotic genes in RNAi dependent and independent manner. Hence effectively connect RNA processing to facultative heterochromatin formation in response to sexual

differentiation signals (Fig. 5) (Hiriart et al., 2012; Tashiro et al., 2013; Yamanaka et al., 2013; Zofall et al., 2012).

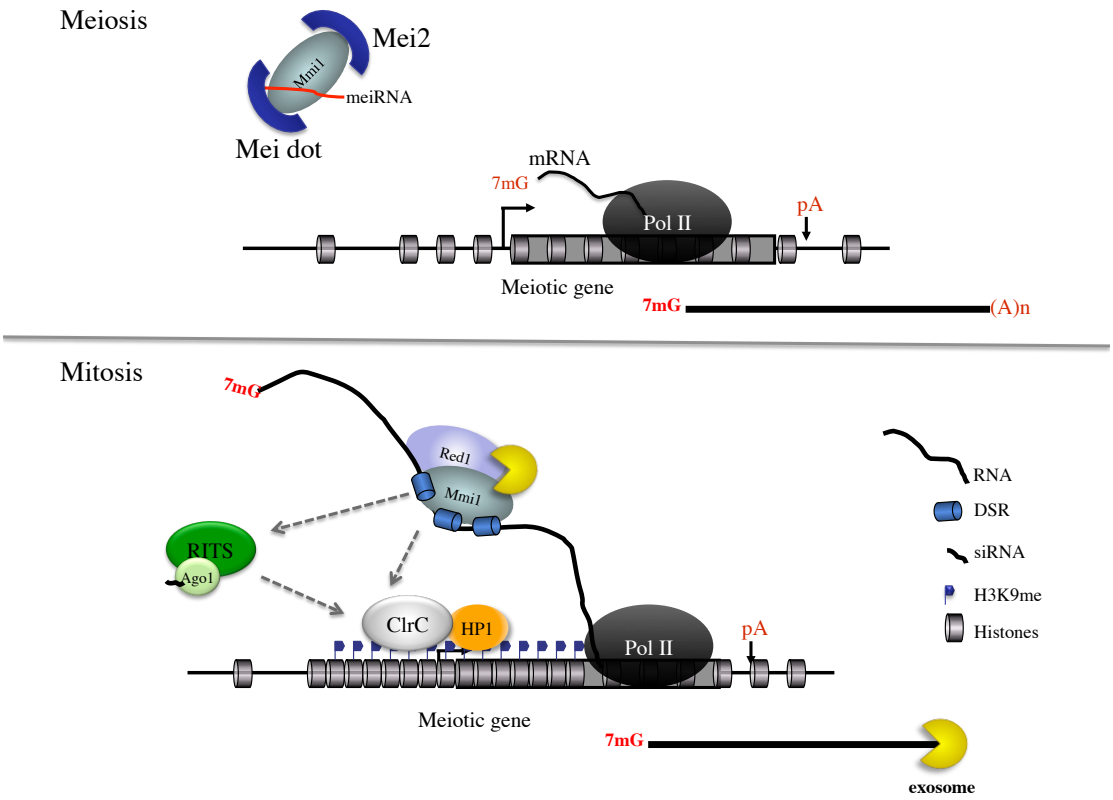


Figure 1—5. Mmi1 mediated degradation of meiotic transcripts during vegetative growth.

To allow sexual differentiation, meiotic RNAs need to be expressed during meiotic division. This is achieved by sequestering Mmi1 in a Mei dot structure with the help of Mei2 protein and meiRNA. Thus the exosome complex does not degrade the meiotic RNAs. However, during mitotic growth, free expression of meiotic RNA is deleterious to the cell. Thus, the Mei dot structure disassembles to allow Mmi1 binding to meiotic RNAs via the DSR element. Simultaneously other proteins such as Red1 also bind these RNAs. This results in recruitment of the exosome complex, which degrades the transcripts. Also, the Mmi1 and Red1 can result in heterochromatin formation at the locus via either recruiting the RNAi machinery or by directly recruiting Clr4 and HP1 proteins. Adapted from (Tashiro et al., 2013; Yamamoto, 2010; Yamashita et al., 2012)

Although genome-wide maps of H3k9me2 revealed low levels of heterochromatic marks on most meiotic loci, mainly *mei4* and *ssm4* genes were studied. Further studies at other loci, revealed that, DSRs are insufficient for heterochromatic silencing (Tashiro et al., 2013), suggesting other mechanisms might

be important. Interestingly, a recent study (Egan et al., 2014), has revealed additional proteins that mediate heterochromatic silencing in a complex with Red1 called, nuclear RNA silencing (NURS) complex. NURS comprises of an Mtr4-like helicase Mtl1 (SPAC17H9.02), two zinc finger proteins, Red5 (SPBC337.12) and Ars2 (SPBC725.08), the RNA recognition motif (RRM)-containing protein Rmn1 (SPBC902.04), and a serine- and proline-rich protein Iss10 (SPAC7D4.14c). The NURS is responsible for interaction of Red1 with the exosome and Mmi1 and also for H3k9me2 formation. It is therefore involved in both RNA degradation and heterochromatin formation. Surprisingly, Egan et al. revealed that the H3k9me2 at meiotic loci might not play a functional role. The Pol II access to the genes was not interrupted as in the case of canonical heterochromatic regions. Thus, using mutants of Clr4 or NURS, they showed that Pol II levels across the gene remain unchanged.

1.4.4 Role of RNA processing/degradation machineries in heterochromatin formation

Exosome mediated decay of the heterochromatic RNA requires polyadenylation by Cid14 (member of the Trf4 family of poly A polymerases), which is a component of the conserved TRAMP complex. Mlo3 (Yra1 in *S.cerevisiae,* TREX component*)* is responsible for determining whether the RNA is to be exported to the cytoplasm or degraded in the nucleus. Interestingly, Mlo3 was also found to interact with the RITS complex via direct binding to Clr4 (Zhang et al., 2011). In Mlo3 and Cid14 mutants, it has been found that lncRNAs from centromeric regions are stabilized and levels of siRNAs are reduced. Thus, Mlo3 may aid in channelling ncRNAs into the RNAi pathway for processing into siRNAs and/or into the exosome/TRAMP mediated degradation pathway acting as hub between the decay

and silencing pathways. Interestingly, mutants of Cid14 or Mlo3 can restore loss of heterochromatic silencing in RNAi mutants. However, Clr4 is required for the silencing in *mlo3*Δ and *cid14*Δ background (Reyes-Turcu et al., 2011). This may suggest RNAi independent mechanisms, which require Clr4 to mediate heterochromatin formation and are suppressed by the Mlo3 and the TRAMP complex. Another finding supporting this view is that in *mlo3*Δ, heterochromatic silencing can occur even in the absence of RNAi pathway, at a few euchromatic loci. Mutants of Mlo3 accumulate antisense transcripts for some genes overlapping with sense transcripts and these convergent gene pairs show H3k9me2 (Reyes-Turcu et al., 2011).

Surprisingly, the exosome complex shows an effect opposite to that of the TRAMP complex at centromeric regions. Although the exosome is thought to act post-transcriptionally, chromatin association of Rrp6 has been demonstrated on specific genes using ChIP (Zofall et al., 2012) and genome-wide using Dam-ID (Woolcock et al., 2011). In a double mutant of Rrp6 and the RNAi pathway, heterochromatic silencing is completely abolished at centromeric and pericentromeric repeats as opposed to partial loss in single RNAi mutants. Thus, the exosome may act in a parallel pathway to the RNAi at the centromeric repeats (Reyes-Turcu et al., 2011). Interestingly, at meiotic loci and retrotransposons as well as transporter genes, loss of Rrp6 results in a dramatic increase in siRNAs and H3k9me2 (Yamanaka et al., 2013).

1.5 ncRNA mediated regulation of inducible genes

In *S. cerevisiae,* ncRNA transcription regulates expression of inducible genes associated with nucleotide biosynthetic pathways. Inducible genes are specifically expressed in the presence or absence of a substance (an inducer) in the environment. Examples of ncRNAs regulating inducible genes include *SER3, PHO5, PHO84, GAL1-10, URA2*, *URA8*, *IMD3* or *ADE12* etc. (Thiebaut et al., 2008). In *S. pombe,* transcriptomes were analysed from cells exposed to alternative growth and stress conditions such as shift from rich media to minimal medium, heat stress and DNA damage (methyl methanesulfonate, MMS) etc. Results showed a dynamic shift in ncRNA expression. Indeed, changes in expression of ncRNAs is 24% in heat stress, 12% in minimal media and 15% in the presence of MMS (Dutrow et al., 2008). For example, ncRNAs were shown to regulate expression of Fbp1 in a glucose dependent manner in *S. pombe* (Hirota et al., 2008). Thus ncRNAs mediated regulation of inducible genes might be conserved to fine tune gene expression.

A. *SRG1* transcription results in downregulation of *SER3* gene.

First example of ncRNA regulating gene expression was the *SRG1* ncRNA regulating the *SER3,* inducible gene in *S. cerevisiae*. In the presence of serine, *SER3* is repressed by transcription of an upstream CUT, *SRG1* (*SER3* Regulatory Gene 1). *SRG1* transcription in *cis*, overlaps with the first nucleotides of the *SER3* coding region. It was proposed that nc transcription may act by preventing binding of the transcription machinery at the *SER3* promoter (Martens et al., 2004). However,

further studies showed that *SRG1* transcription mediates assembly of nucleosomes across the *SER3* promoter by the action of Spt6p and Spt16p proteins. In the absence of serine, *SRG1* transcription is abolished resulting in a nucleosome free region (NFR) at the *SER3* promoter thereby allowing full induction (Thebault et al., 2011).

B. mlonRNAs positively regulate *fbp1* expression.

Interestingly, in *S. pombe,* ncRNA transcription in *cis,* upstream of the *fbp1* gene results in a stepwise cascade for activation of *fbp1*. These ncRNAs are termed as mlonRNAs (metabolic stress-induced lncRNAs). Under repressive conditions (glucose rich media), two proteins Tup11 and Tup12 ("Groucho" like global co-repressors) suppress chromatin remodelling and therefore full activation of *fbp1* expression is hindered. However, upon shift to glucose deficient media, mlonRNAs are transcribed from upstream of *fbp1*. Atf1 and Rst2 proteins bind to the promoter region of *fbp1* and antagonize Tup mediated repression. This results in stepwise remodelling of the chromatin possibly with the help of histone acetylases to form an open chromatin structure at the TATA box, resulting in massive induction of *fbp1* gene (Hirota and Ohta, 2009; Hirota et al., 2008).

C. ncRNA mediated regulation of PHO pathway

Phosphate (Pi) homeostasis is essential for cell survival and function. In *S. pombe* and *S. cerevisiae*, both a Pi signal transduction (PHO) pathway is de-repressed in response to low inorganic Pi levels as well as low adenine levels. The main

function of the PHO pathway is to scavenge Pi from extracellular sources with the help of acid phosphatases that hydrolyse Pi containing compounds and phosphatase transporters that mediate transport of Pi. A genome-wide screen has been performed to identify a number of positive and negative regulators have been identified that might be important to regulate timely expression, potential repression and fine-tuning of this cascade (Henry et al., 2011). Interestingly, in both yeast it has been found that ncRNAs play a role in regulating essential genes of the PHO pathway.

S. cerevisiae :

In response to Pi, the genes de-repressed in *S. cerevisiae*, comprise *PHO5*, which encodes the repressible acid phosphatase (rAPase), and *PHO84*, encoding a high affinity Pi transporter. The transcription factor Pho4 is responsible for activation of the PHO pathway genes. In the presence of Pi (repressive conditions), Pho4 is phosphorylated by the cyclin-dependent kinase (CDK) complex Pho80/ Pho81/Pho85. Phosphorylated Pho4 is exported to the cytoplasm thereby rendering it unavailable for *PHO5* and *PHO84* gene activation. Conversely, in the absence of Pi (inducible conditions), the CDK inhibitor Pho81 inactivates Pho80-Pho85. Thus, Pho4 is not phosphorylated and therefore active in the nucleus. Pho4 can now induce expression of *PHO5* and *PHO84*. Pho84 is a high affinity Pi transporter that is activated upon low intracellular Pi. However, when intracellular Pi levels are high, two low-affinity Pi transporters, Pho87 and Pho90 are also activated (Fig. 6). Thus, a constant influx of Pi is maintained, by modulating protein expression depending on intracellular Pi levels (Auesukaree et al., 2003, 2004; Gilliquet et al., 1990; Wykoff et al., 2007).

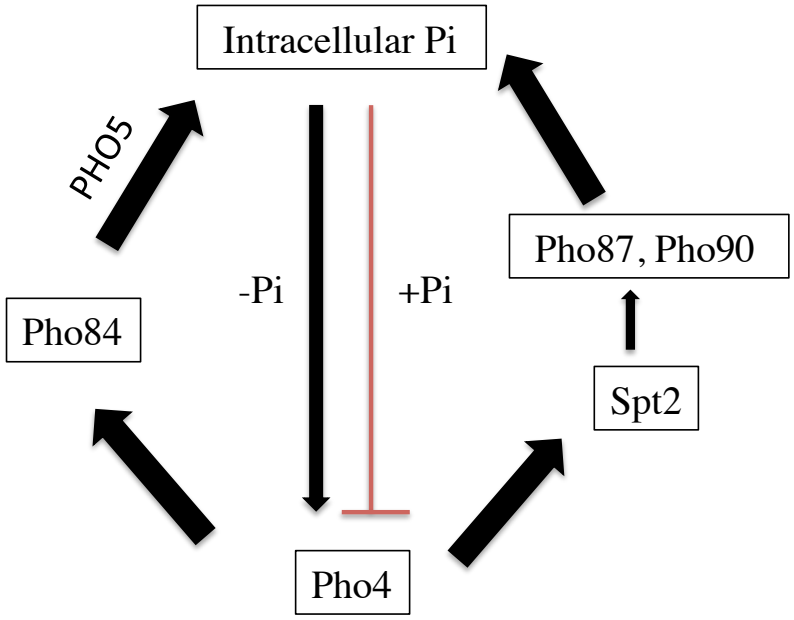


Figure 1—6. The PHO pathway in *S. cerevisiae*.

In the presence of inorganic phosphate (Pi), the Pho80/Pho85 complex phosphorylates Pho4, prevents it from entering the nucleus thereby enabling Spt2 to allow expression of low affinity transporters Pho87/Pho90. However, when Pi levels are low, the unphosphorylated Pho4 is transported into the nucleus and allows expression of Pho84 and PHO5, which leads to influx of Pi via acid phosphatases. Adapted from (Secco et al., 2012).

NcRNAs play an important role in regulation of components of the PHO response in *S. cerevisiae* including *PHO84* and *PHO5*. The *PHO84* gene is regulated in ageing cells (when the exosome subunit Rrp6 dissociates from the gene) via stabilization of a long antisense RNA. Set1 contributes to antisense RNA production by placing H3K4me3 marks across antisense RNA promoter region. The ncRNA spans the entire *PHO84* gene upto the UAS sequence of *PHO84* mRNA. Interestingly, the ncRNA acts not only in *cis* but also in *trans*. The antisense RNA in *cis* results in de-acetylation of histones at the *PHO84* promoter via recruitment of the histone deacetylases, Hda1/2/3. Conversely in *trans*, the *PHO84* antisense RNA can also mediate repression however, the mechanism is unknown (Camblong et al., 2007, 2009b).

At *PHO5*, antisense transcription across the gene body plays an activating role by positively affecting nucleosome remodelling. The *PHO5* promoter harbours two upstream activating sequences p1 and p2 (UASp1 and UASp2), which are major binding sites for the transcription activators Pho2 and Pho4. In the presence of high Pi, Pho4 is localized in the cytoplasm and is therefore unable to activate transcription of *PHO5*. However, in low Pi, Pho4 localizes mainly in the nucleus, where it interacts with another activator, Pho2 and together they bind the *PHO5* promoter to allow gene expression. Additionally, chromatin remodelling complexes, such as, SWI/SNF, SAGA, INO80 are also found to be involved in regulating *PHO5* expression (Wongwisansri and Laybourn, 2005). Interestingly, the activation of this gene is also regulated at the chromatin level. Under repressive conditions, the chromatin condenses and the UASp2 and the TATA box for *PHO5* are unavailable for binding. However, in low Pi conditions the nucleosomes disassemble and expose the binding sites allowing for activation. Interestingly, it was shown that the nucleosome remodelling is a result of transcription of a 2.4-kb noncoding exosome-degraded transcript that originates near the PHO5 termination site and is transcribed in the antisense direction. Transcription of the ncRNA results in nucleosome eviction and thus aids transcription of the *PHO5* gene (Uhler et al., 2007).

S. pombe :

The PHO pathway is conserved in *S. pombe* and a number of studies have been undertaken to identify the components and regulators of the pathway. It comprises the Pho1 protein (major acid phosphatase, product of SPBP4G3.02 gene, orthologous to ScPHO5) and the Pi transporters are encoded by SPBC8E4.01c (orthologous to

ScPHO84), and SPBC1271.09. However, differences do exist between the PHO pathways in both the yeasts. In *S. pombe*, no clear homologues of Pho4, Pho2, Pho80, Pho81 and Pho85 exist (Henry et al., 2011). Also, in *S. pombe* Pho7 (transcription factor) acts as the positive regulator whereas Csk1 (CDK-activating kinase- activating kinase) serves as the negative regulator of *pho1* expression. SWI/SNF chromatin remodelers also participate in regulating PHO pathway genes. Unlike Pho4 that is regulated by the Pho80-85 kinases, Pho7 action is primarily independent of the kinase Csk1. Indeed, high throughput microarray analysis indicated that the main targets of Pho7 and Csk1 are the core Pi harvesting and transport genes, *pho1*, SPBC8E4.01c and SPBC1271.09 (Carter-O'Connell et al., 2012). It has been shown that activation of *pho1* relies on Pho7 binding to an UAS (Upstream Activating sequence (-280 to -180 bp)) in the *pho1* promoter region.

What comprises the UAS was so far unknown. Moreover, studies also showed that Pho7 alone is insufficient for full *pho1* induction implying that additional regulators are involved. It was speculated that the region upto -2kb upstream of *pho1* called the Pi sensing element might be important for *pho1* repression but the mechanism was so far not understood (Fig. 7). Furthermore, the Pi sensing element when placed upstream of an ORF could drive transcription thus indicating it might harbour a promoter element (Carter-O'Connell et al., 2012; Henry et al., 2011). Thus, although the basic structure of the PHO pathway has been described, the molecular mechanisms and some key components of the pathway essential for its regulation remain elusive. Another, interesting aspect is that the *pho84* gene which encodes for the Pi transporter is situated upstream of the *pho1* gene on the chromosome (Fig. 7).

However, the relation between expression of both these genes with respect to each other and upon deletion of the Rrp6 protein has not been investigated so far.

Interestingly, the region upstream of the *pho1* promoter shows differential expression in WT and exosome mutants in microarray analysis data, demonstrating that this region is transcriptionally active (Fig. 7, (Wilhelm et al., 2008)). Also, H3k9me2 and small RNA reads have been mapped at the *pho1* locus (Yamanaka et al., 2013). Indeed, a high accumulation of small RNA reads was found at the Pi sensing element. However, the activity of the Pi sensing element was detected only in Rrp6 mutant background and these studies failed to detect transcription or heterochromatic marks in WT background.

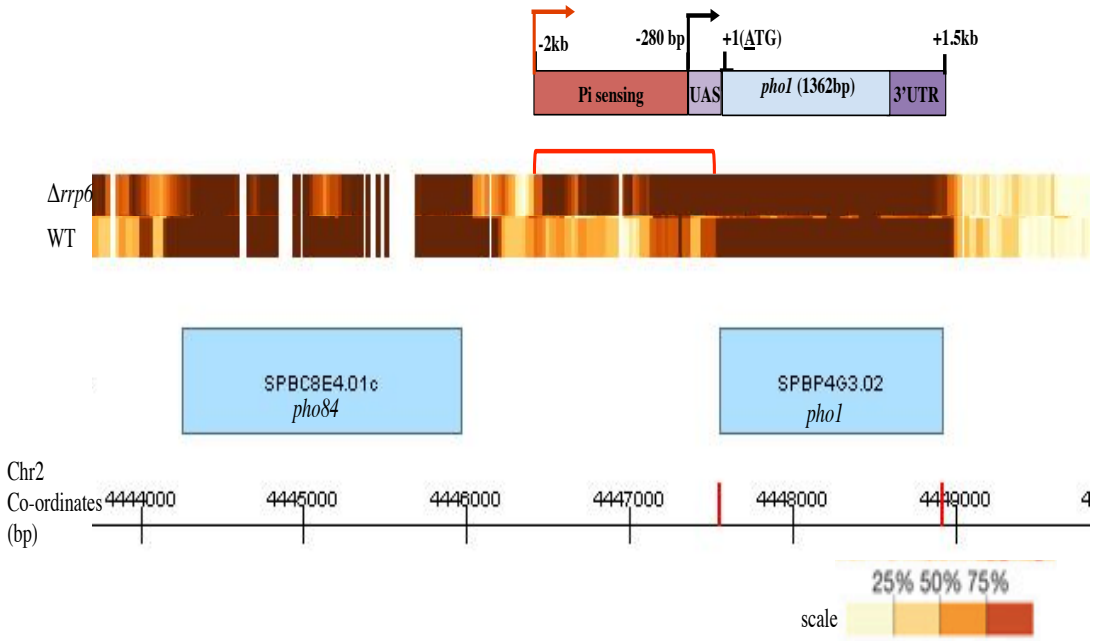


Figure 1—7. RNA analysis at the *pho1* locus.

Tiling-chip hybridization signals (in which the strength of colour reflects the signal-distribution quartile) across the for the *pho1* (SPBP4G3.02) gene and *pho84* (SPBC8e4.01c) on Chromosome II, in Wt. and Rrp6 mutants (Wilhelm et al., 2008). Above the tiling array data is a schematic depicting all the different regions of the pho1 gene studied so far (Carter-O'Connell et al., 2012; Henry et al., 2011). The region marked with a red bracket shows difference in transcript signal between the two strains. The key below the figure shows the scale demonstrating signal intensity with yellow to red in increasing order of intensity.

1.6 Scope of the thesis

My aim was to understand how the *pho1* gene is regulated in response to Pi. I set out to study the composition of the Phosphate sensing element. Interestingly, H3k9me2 and small RNAs were also found at the *pho1* locus, however, the role of heterochromatin at this locus has so far remained unknown. I aim to study the heterochromatin formation in the presence and absence of Pi to ascertain whether there is a functional co-relation between heterochromatin formation and gene expression. Also, I will look at mutants for the RNAi pathway to find whether the heterochromatin is RNAi dependent or independent. Furthermore, the tiling array clearly showed a difference in expression pattern of the locus between WT and rrp6 mutants. I will probe further to understand whether Rrp6 plays a role in regulation at the locus.

With these aims in mind, the data discussed in the following chapters helps understanding gene regulation at the *pho1* locus. Surprisingly, analysing the *pho1* locus in exosome mutants, I find that the Pi sensing element encodes an lncRNA, which I term as *prt* (*pho1* repressive transcript) RNA. *prt* fully overlaps with the *pho1* gene. Importantly, *prt* is expressed specifically under repressive nutrient conditions (presence of Pi) as opposed to inducible conditions. Thus, the lncRNA *prt* may be associated with repression of *pho1* RNA in the presence of Pi.

Interestingly, I find that the nuclear exosome is co-transcriptionally recruited via RNA binding protein Mmi1 and appears to regulate transcription termination and degradation of *prt*. This raises the question as to how the exosome recognises only the

lncRNA and not the mRNA it overlaps. Indeed, we find DSR elements present in *prt* that mediate specific recognition of the lncRNA by the Mmi1 protein and the exosome.

Studies have shown H3k9me2at the *pho1* locus and the presence of small RNAs (Lee et al., 2013; Yamanaka et al., 2013). I find that similar to meiotic loci, transcription of *prt* also results in H3K9me2 across the locus via Red1 and Mmi1 dependent recruitment of RITS complex. While my work was in progress an independent study showed evidence for Red1 mediated regulation of *pho1* by an upstream RNA (Lee et al., 2013). Although, H3k9me2was observed, the study did not address the role of heterochromatin at this locus. Also, additional components that are involved in regulation of this locus were not uncovered. Thus, to study the *pho1* locus in more detail, I first mapped the 5' and 3' ends of the ncRNA and show that in the absence of the exosome, levels of H3K9me2 are enhanced across this locus causing further repression of mRNA and protein levels. Interestingly, blocking nc transcription by deleting nc promoter or growing cells under nutrient depletion conditions, leads to loss of H3k9me2 as well as loss of Ago1 recruitment. Although, loss of H3k9me2 in Clr4 mutants does not de-repress transcription, I find that the induction of *pho1* is faster. I show that the previously unknown Pi sensing element might comprise an ncRNA, which allows for dynamic regulation of gene expression by creating a transient heterochromatic state in response to environmental cues.

Chapter 2

MATERIALS AND METHODS

3.1 Media and growth conditions

Cells were grown either in EMMG in the presence of phosphate (10mM KH_2PO_4) or EMMG without phosphate (Henry et al., 2011). The cells were incubated for 12 hrs in no phosphate media to allow full induction. Standard YES medium (contains phosphate) was used for some experiments. Typically cells were grown to OD (A_{600})=0.5 before they were harvested.

3.2 Yeast transformation

Yeast strains were grown in YES media. Cells where harvested while in exponential growth phase and washed with cold water. For the transformation reaction LiCl was added at a concentration of 100mM, ssDNA, PEG-3000 and the desired DNA product/plasmid. Cells where left incubating for 1 hour at 30^0C and then heat shocked for 20 minutes at 42^0C. Transformants were selected using selective media plates according to their antibiotic or auxotrophic markers.

3.3 Site specific mutagenesis

Point mutations were generated by a PCR method. The desired gene was cloned into plasmid vectors and PCR was performed using primers bearing the desired base change mutation.

3.4 Strain construction

The DSR element in *prt* was deleted by 2-step PCR using primers 2574 and 3409 or 3408 and 3601. The 68bp deletion was introduced in YP 213 using homologous recombination to replace the *ura4+* marker with the cassette containing endogenous *prt* promoter and region spanning deleted DSR elements.

3.5 Construction of plasmids

The fragment containing the entire *prt* transcription unit was amplified from YP 223 strain where the *pho1* promoter was deleted, using primers 3599 and 3601. The PCR product was cloned into TOPO using the Zero Blunt® TOPO® PCR Cloning Kit (Invitrogen). The DSR element was deleted from the plasmid by using primers 3409 and 3408. The *prt* containing fragment was sub cloned into pREP41 vector where the nmt1 promoter was removed. For expression of the *prt* RNA in *trans*, the resulting plasmids were transformed into YP 225 strain where endogenous *prt* promoter is deleted and a wild-type strain as a control (YP 4).

3.6 Northern blotting

Total RNA was extracted using hot phenol extraction from cells at exponential growth phase. 8 μ g of total RNA was loaded onto 1.2% agarose (MOPS formaldehyde) gel, or onto denaturing acrylamide gels (5%). RNA was transferred onto nylon membranes, and pre-hybridization was carried out for 4 hours at 42°C in a solution containing 50% formamide, 5% Denhardt's solution, 5% SSPE, 0.1% SDS, and 200 μ g/ml single-stranded DNA. Hybridization was performed in the same conditions with a radiolabeled probe for 12 h, and the membranes were washed in pre-heated at 65°C 2% SSC/0.1% SDS at Room Temperature. After washes, the membranes were exposed to X-ray film or analysed by phosphoimager and AIDA image analyser software. DNA fragments were amplified from genomic loci with specific primers (Table 4) and used as templates for random oligo labelling using Prime-it® labelling kit.

3.7 cRACE analysis

cRACE was performed as described previously (Rissland and Norbury, 2009). For schematic representation see Fig. 3-2. RNA was purified from WT (YP 51) and Rrp6 mutants (YP 52) grown in mid-log phase. 12μg of RNA was combined with 1X SAP buffer and 1U SAP and incubated for 30 mins at 37^{0}C. RNA was then phenol chloroform extracted and combined with 1X TAP buffer and 2.5 U TAP and incubated at 37^{0}C for 1 hr prior to phenol chloroform extraction. The sequential treatment with shrimp alkaline phosphatase (SAP) and tobacco acid pyrophosphatase (TAP) was used to dephosphorylate decapped RNAs from the 5' ends and cleave the pyrophosphate bond of the methyl-7-guanosine cap respectively. The resulting RNA

isolate contained ligatable 5'-monophosphorylated ends only on transcripts that were previously capped and not those that lacked the cap. These were intramolecularly ligated 5' to 3' to form circular RNA molecules. The RNA was combined with 1X T4 ligase I buffer and 1 U T4 ligase in a large volume (400 μL) to favour intramolecular ligations rather than intermolecular ligations. The ligation sites were amplified by reverse transcription of 4μL of the circularized RNA and PCR amplified with divergent primers. Following cloning and sequencing to determine the position of ligation of ends, the exact 5' and 3' ends were identified (Fig. 3-2).

3.8 Purification of HTP-Mmi1

6xHis-TEV-Protein A (HTP) tagged Mmi1 was purified from 8L of yeast culture in YES medium, essentially as described in (Vasiljeva and Buratowski, 2006). Strains expressing HTP tagged Mmi1 in WT *pho1* (YP 241) and *pho1* DSR Δ (YP 322) background were used, as well as a wild-type strain (YP 51) without tagged Mmi1 as a control. In brief, 4 L of yeast culture were grown to an OD_{600} of 0.5, washed once with sterile water, resuspended in TMG buffer (10 mM Tris-HCl pH 8.0, 1 mM $MgCl_2$, 150 mM NaCl, 0.1 mM DTT, 10% Glycerol) containing 1 mM phenylmethylsulfonyl fluoride (PMSF), 1 mM benzamidine, 2.3 μM leupeptin (1 μg/mL), 1.5 μM pepstatin A (1 μg/mL), 81 μM bestatin (25 μg/mL), 1.5 μM aprotinin (10 μg/mL), 1 μL/mL RNasin ribonuclease inhibitor (Promega) and frozen by dripping into liquid nitrogen. All following steps were performed at 4°C using pre-cooled equipment. Cell extracts were obtained by grinding and subsequent vortexing of the cells with glass beads in TMN buffer, followed by ultracentrifugation at 75,000 x *g* for 1.5 h. The tagged protein was immunoprecipitated using 100 μL of IgG

sepharose (VWR) and 100 μL of IgG agarose (Sigma-Aldrich) for 6h. Beads were washed 3 x 10 minutes with TMG buffer (without protease inhibitors) and 1 x 5 minutes with TEV cleavage buffer (10 mM Tris-HCl pH 8.0, 150 mM NaCl, 0.05% NP-40, 0.5 mM DTT, 1 μL/mL RNasin). Elution was performed by cleavage of the Protein A-tag using 2μL of AcTEV protease® (Invitrogen) overnight. Purification of the tagged protein was confirmed by western blotting.

3.9 Meiotic time course

Synchronous meiosis was induced as described in (Chen et al., 2011). Briefly, diploid cells homozygous for *pat1-114* were grown in EMM2 without adenine at 25°C to OD(A_{600})=0.3. Cells were washed with water and then grown in EMM2 without NH_4Cl for 16 h at 25°C to arrest cells in G1. 5mg/ml NH_4Cl were added and the culture shifted to 34°C to inactivate Pat1 and induce meiosis. Every two hours, 20ml of culture was harvested for RNA extraction and 180ml for chromatin preparation.

3.10 Preparation of RNA and RT-PCR

Co-purified RNA extractions and northern blot experiments were performed as previously described (Vasiljeva and Buratowski, 2006). Any DNA contaminations were digested with 2 U DNase I (Promega) and the RNA extraction was repeated as before. Reverse transcription of 100 ng RNA per reaction was performed in a total volume of 20 μL using specific primers (Table 4) with the Transcriptor Reverse Transcriptase® (Roche). A non-RT control was included in each reaction. To

determine DNA amounts, 4 μL of the RT reaction were used for PCR with the Phire Hot start polymerase® (NEB) and specific oligonucleotides in 25 cycles. Products were visualized on a 1% agarose gel.

3.11 Transcriptional Run-On

Transcriptional Run on (TRO) was performed as described in (Birse et al., 1997). Single-stranded RNA probes for TRO analysis were generated by *in vitro*-transcription using T7 or SP6 polymerase from plasmids with fragments against *pho1*, gfr (gene-free-region, Chr. II: 1149380-1149521) and *adh1* that were generated using primer pairs described in Table 4 and cloned into pCR-Blunt II-TOPO vector (Invitrogen).

Strand specific RNAs were blotted onto positively charged nylon membrane and UV cross-linked. The membrane was pre-hybridized for 4 hours at 55°C as described in 1. Exponentially growing yeast cells were harvested and put on ice and they were permeabilized using 10% N-Lauryl sarcosine sulphate. Cells were centrifuged and re-suspended in transcription buffer (50mM Tris/HCL pH 7.7, 50mM KCl, 80mM MgCl2). A mix of rNTPs (A,C,G) was added and 100mM of DTT. After the addition of 4 μl α-32P rUTP transcription was initiated at 30°C for 15 minutes. Total RNA extraction was performed using hot-acid phenol.

Extracted RNA was added to hybridization buffer and added to the membrane as described in Northern blotting protocol.

3.12 ChIP experiments

ChIP procedures and quantification were performed as described (Keogh and Buratowski, 2004; Kim et al., 2004a; Kim et al., 2004b). For temperature-shift experiments, cells were incubated at 25°C until OD=0.5 and cells were further incubated at 37°C for 4 hours. Cells were grown in YES media overnight and shifted to EMMG with no Pi and EMMG with 10mM Pi for 12 hrs. Primers are listed in Table 4. Antibodies against Ago1 (Abcam, 18190), H3K9me2 (Abcam, 1220) and Rpb1 (8WG16) (Millipore) were used. The samples were either run on a 4% PAGE

3.13 PNPP assay

Pho1 protein activity was assessed using a *para*-Nitrophenylphosphate (PNPP) activity assay as previously described (Schweingruber et al., 1986). *S. pombe* cells were grown in EMMG medium to OD600=0.5 (5ml in no Pi, 5ml in high Pi [10mM]). Phosphatase activity was assayed via incubation with 200µl of PNPP reagent (5.6 μ g/1 ml) for 10 minutes at room temperature. The reaction was stopped by addition of 360 μ l saturated Na2CO3 solution. Products of the reaction were measured at OD400 against Na2CO3 whilst normalizing for original OD600 of the sample.

3.14 RNAse H treatment

Total RNA (20ug) or Poly A enriched RNA (12ug) was added to 25pmol of specific primer to make a total volume of 20ul in water. The mixture was heated at 95^0C for 2 min and then at 65^0C for 5 mins. After cooling the reaction down to room temperature, 1X RNAseH buffer (NEB, M0297S) was added. The reaction was

incubated at 37^{0}C for 30 mins after addition of 1 unit of RNAseH enzyme. The total volume was made upto 200 ul with H_2O and 200ul of Phenol/chloroform, 50ul of 7.5M Ammonium acetate, 2ul glycogen and 2.5X ethanol was added. The RNA was precipitated at Precipitate at -20^{0}C for an hour. The ethanol was discarded after spinning for 30 min at 14000 rpm and the pellet was washed with 70% ethanol and dried. The RNA was resuspended in 20ul water and denatured for 20 mins at 80^{0}C before running on a 4% PAGE/8M urea gel. The RNA was blotted onto a nylon membrane and probed as described in the Northern blotting protocol.

Table 3. Yeast strains used in this study

Yeast Strains		
Name	**Properties**	**References**
YP 51	**h+,ura4-Δ18,leu1-32,ade6-M216,imr1R(NcoI)::ura4+**	**(Bühler et al., 2008)**
YP 52	**h+,ura4-Δ18,leu1-32,ade6-M216,imr1R(NcoI)::ura4+, rrp6 Δ ::NAT**	**(Bühler et al., 2008)**
YP 97	**h-, ade6-M210,pho1Δ::KAN MX**	**(Henry et al., 2011)**
YP 217	**h+, ura4-Δ18,leu1-32 oir1,ade6-M216,imr1::Ura4+**	**(Hiriart et al., 2012)**
YP 222	**h+, leu1-32 oir1, ade6-M216,chp1-TAP-KAN MX,mei4 Δ (nt828-1554)::GFP-hph,mmi1 Δ::nat**	
YP 173	**h+,ura4-Δ18,leu1-32,ade6-M216, rrp6Δ::NAT MX**	**This study**
YP 223	**h-,ura4-Δ18,leu1-32,ade6-M210, pro1Δ(-346-(-1))**	**This study**
YP 227	**h-,ura4-Δ18,leu1-32,ade6-M216, pro1Δ(-346-(-1))** **rrp6 Δ::NAT MX**	**This study**
YP 61	**h+, leu1-32,his2, dis3-54**	**(Ohkura et al., 1988)**
YP 112	**h-, dhp1-154**	**(Sajiki et al., 2009)**
YP 126	**h-, dhp1-54, rrp6Δ::NAT MX**	**This study**
YP 94	**h-, pfs2-11**	**(Wang et al., 2005)**

YP 203	**mat/Msmt0, ura4 DS/E; otrlR(Sph1)::ura+,leu1-32, his2, ade6-210, ago1 Δ ::KAN MX**	**(Reyes-Turcu et al., 2011)**
YP 202	**mat/Msmt0, ura4 DS/E; otrlR(Sph1)::ura+,leu1-32, his2, ade6-210, rrp6Δ::KAN MX, ago1Δ::KAN MX**	**(Reyes-Turcu et al., 2011)**
YP 56	**h+ ade6-M210 ura4-Δ18 leu1-32, otr1R(Sph1)::ura4+ clr4 Δ ::NatMX6**	**(Gullerova et al., 2011)**
YP 221	**h+,ura4-Δ18,leu1-32,ade6-M216, imr1R::ura4+, KAN-p3nmt1-TAP::mmi1**	**(Hiriart et al., 2012)**
YP 217	**h+,ura4-Δ18,leu1-32,ade6-M216, imr1R::ura4+**	**(Hiriart et al., 2012)**
YP 213	**h+,ura4-Δ18,leu1-32,ade6-M216,** **pro2::ura4+**	**This study**
YP 207	**h+,ura4-Δ18,leu1-32,ade6-M216,rrp6Δ::NAT MX, pro2::ura4+**	**This study**
YP 225	**h-,ura4-Δ18,leu1-32,ade6-M216,** **pro2 Δ (-1438-(-1204) (ATG=1)**	**This study**
YP38	**h+/h+, ade6-M216/ ade6-M216,pat1-114/pat1-114**	**(Mata et al., 2002)**
YP 241	**h+,ura4-Δ18,leu1-32,ade6-M216,imr1R(NcoI)::ura4+,mmi1-his6-TEV-Prot A::KAN MX**	**This study**
YP 322	**h+,ura4-Δ18,leu1-32,ade6-M216,** **DSR Δ , mmi1-his6-TEV-Prot A::KAN MX**	**This study**
YP 316	**SPY418 h+ otr1R(SphI)::ura4+ ura4-DS/E leu1-32 ade6-M210 ago1 Δ ::Kan**	**(Halic and Moazed, 2010)**
YP 320	**SPY1336 h+ leu1-32 ade6-216 ura4-D18 imr1 R(NcoI)::ura4+ oriI swi6 Δ :: kanR**	**(Motamedi et al., 2008)**

YP 319	**SPY1297 h+ leu1-32 ade6-216 ura4-D18 imr1 R(NcoI)::ura4+ oriI chp2 Δ:: kanR**	**(Motamedi et al., 2008)**
YP 321	**SPY86 h+ leu1-32 ade6-216 ura4DS/E imr1R(NcoI)::ura4+ oriI dcr1Δ::TAP-kanR**	**(Motamedi et al., 2004)**
YP 318	**SPY87 h+ leu1-32 ade6-216 ura4DS/E imr1R(NcoI)::ura4+ oriI rdr1Δ::TAP-kanR**	**(Motamedi et al., 2004)**
YP 310	**PM1011 clr4::kanR, rrp6::natR, smt0, ade6-M210, leu1-32, ura4-D18**	**(Garcia et al., 2010)**
YP 331	**h+ leu1-32, ura4 D18, imr1R(Nco11):ura4, rrp6Δ::NAT, dcr1Δ::HPH**	**(Marasovic et al., 2013)**
YP 311	**otrR(SphI)::ura4 ura4DS/E leu1-32 ade6-M210 pREP1-nmt1-3xFLAG-Ago1D580A**	**(Gullerova et al., 2011)**
YP 312	**leu1-32 ura4D18 ade6-216 p1982 TAPdcr1-D937A**	**(Gullerova et al., 2011)**
YP 332	**otr1R::ura4, ura4-DS/E, ade6-M216; leu1-32, his7-366 rrp6Δ::KAN ago1Δ:: HPH**	**Mario Halic**
YP 305	**h+,ura4-Δ18,leu1-32,ade6-M216,** **DSR Δ (–809-(-740))**	**This study**
YP4	**h-,ura4-Δ18,leu1-32,ade6-M216**	**(Kim et al., 2009)**

Table 4. Oligonucleotides Used in This Study

No.	Name	Sequence (5'-3' direction)	Purpose
2463	Pho1-forward	TAAATTTGATTTCAAGGAACATTTGACTTC	Northern Probe 1
2464	Pho1-reverse	TTGTAAGTACTAGCATTAAAGAGCTCATGG	Northern Probe 1
2469	Adh 1-1 F (Kim et al., 2009a)	CGGAAGCTGGTGAGAAGAAC	ChIP
2470	Adh 1-1 R (Kim et al., 2009a)	CGTTGGAATGCGGAGTAGAG	ChIP
2479	GFR F (ChrII) (Kim et al., 2009a)	GCATCGTTTTTCGCACAATA	ChIP, TRO
2480	GFR R (ChrII) (Kim et al., 2009a)	CATGGCATGGCATTTTGTTA	ChIP, TRO
2521	Pho1_F1	CTGTTTGTTGCTTTCTTTCTACTTATTATC	ChIP, TRO, RT-PCR, cRACE, Northern Probe2
2522	Pho1_R1	AGTTTAACTCAAAAGGTTTAAAGAATTCAG	ChIP, TRO, RT-PCR, cRACE, Northern Probe2
2523	Pho1_F2	GATTAATTAAACAATTATATCTTGGTCTGG	RT-PCR
2525	Pho1_F3	GTTAAAAATCTTAATTTACTATACGTTGAAGC	ChIP, Northern Probe3
2526	Pho1_R3	ACTTGTAAATGCCCTAACCCTGTATTAAG	ChIP, Northern Probe3
2531	Pho1_F6	AACTTTTTAAGGTAGCGTTCAATGTTC	ChIP, TRO
2532	Pho1_R6	AGTATGGACCTGCTTTATCTTACAAGTG	ChIP, TRO,

			cRACE
2537	**Pho1_F9**	**TATGGATATGGTATGTTTGGTGAAGAC**	**ChIP, TRO**
2538	**Pho1_R9**	**AGTAAGGGTTTAAACGTTGACGAATAG**	**ChIP, TRO**
2545	**Pho1_F13**	**TGCTGTTTATCCTTATTAAACGTATATCTC**	**ChIP, TRO, RT-PCR, cRACE**
2546	**Pho1_R13**	**TACATTATCTCCTACATAAACTATCCTCTG**	**ChIP, TRO, RT-PCR**
2547	**pho1-14 F**	**AGCAGAGGATAGTTTATGTAGGAGATAATG**	**ChIP**
2548	**pho1-14 R**	**TTTATATGGTGAGAGTATTGTCAAAGAAAC**	**ChIP**
2549	**Pho1_F15**	**TTTAGAAGAGATGACTAATTACGCGATAC**	**ChIP, TRO, RT-PCR**
2550	**Pho1_R15**	**AGTTACGAAATAAAGGGAATGAAAGAG**	**ChIP, TRO, RT-PCR**
2857	**Pho1_R17**	**AAACTAAGTCTTGACAACTATAACGAAACC**	**ChIP, TRO**
3032	**Fbp1r**	**CAAGTGACGGCATAGGAACC**	**ChIP, TRO**
3052	**pho1-4 F**	**TTTGTACCAACTTGGACTCCTG**	**ChIP**
3053	**pho1-4 R**	**GAGTTATTTGACATGGGACGC**	**ChIP**
3072	**Fbpf**	**ACTGCGATGAAGTCGAACG**	**ChIP, TRO**
3087	**Adh gene F chip**	**CACGTCGGTAACCGTATTGAC**	**ChIP, RT-PCR**
3088	**Adh gene R chip**	**CCTTCTCTACTCTTCCCGACG**	**ChIP, RT-PCR**
3089	**Adh after pa F Chip**	**GTACGACGATCCCTAATCCAAC**	**ChIP, RT-PCR**
3090	**Adh after pa R Chip**	**ACGCAAATCTTGAAAAAGATCC**	**ChIP, RT-PCR**
3129	**Pho1 F17**	**AAAATTCTATGTTTCTATACATGCCTCTG**	**ChIP, TRO**

3245	**Dg Fwd pombe**	**AAGACTGTTGTTGAGTGCTGTGGA**	**ChIP**
3246	**Dg Rev pombe**	**CCATGCTTTTAGTGCGGTCA**	**ChIP**
3408	**DSR deln 2 step F**	**cagcgtctaaaatttttagcatgttgacaacggaaacccagtgatttgtgtatgaca**	**DSR deletion**
3409	**DSR deln 2 step R**	**tgtcatacacaaaatcactgggtttccgttgtcaacatgctaaaaatttttagacgctg**	**DSR deletion**
3545	**sme2-body-fwd**	**TGCATGCAAGATCGCTTTAG**	**ChIP**
3546	**sme2-body-rev**	**ACCGAATCCAGCTTTTTGAA**	**ChIP**
3547	**sme2-down1-fwd**	**GTTGTGCTGTGCTCCCTCTT**	**ChIP**
3548	**sme2-down1-rev**	**AGCTGTTCCTTGCGATTTCA**	**ChIP**
3549	**sme2-down2-fwd**	**GCAGCCCCAATTAAACTGAA**	**ChIP**
3550	**sme2-down2-rev**	**AGGGAAAAATGCTGGGATCT**	**ChIP**
3551	**mug180-ORF1-fwd**	**TTGGCCTAAAGGTGTTGCTT**	**ChIP, Northern**
3552	**mug180-ORF1-rev**	**ATCGTTGGCCCTGTATGAAC**	**ChIP**
3554	**mug180-ORF2-rev**	**AAATCATTTGCAACCATCCA**	**Northern**
3555	**mug180-down1-fwd**	**TGACTAAACATCTGCACCGAAA**	**ChIP**
3556	**mug180-down1-rev**	**CGTTGTCACTGCCTCTTCTCT**	**ChIP**
3557	**mug180-down2-fwd**	**TCACTGCTAAAAGGCGGCTA**	**ChIP**
3558	**mug180-down2-rev**	**TGGAATCGGGGTTTTCAGTA**	**ChIP**
3599	**Pho1 ncProm fwd**	**ACATGATTCCCTTCGTCTTTTGA**	**cloning**
3601	**Pho1 after gene rev**	**TAGGTTTGTACACGCAACGG**	**cloning**
3605	**Rps15-2 fwd**	**GAGGAAAATCACGACGAGGC**	**RT-PCR**
3606	**Rps15-2 rev**	**CCTCAGACTTGGCCTTACGA**	**RT-PCR**
3643	**Pho1_SalI_rev**	**TTATAAgtcgacTAGGTTTGTACACGCAACGG**	**cloning**

Chapter 3

Phosphate dependent transcription of *prt* ncRNA leads to *pho1* gene repression

Introduction:

Maintaining a constant uptake of inorganic phosphate (Pi) is vital for a number of cellular pathways such as cellular differentiation, proliferation, cell death, mobility, metabolism, survival, and organization of the cytoskeleton, which are mainly regulated via phosphorylation of proteins. Availability of Pi varies in different ecosystems and thus to maintain Pi homeostasis, a phosphate-responsive signalling (PHO) pathway is present in all organisms that acquire Pi from the environment. This pathway has been studied across the kingdom in mammals, plants, fungi and bacteria (Auesukaree et al., 2004; Bergwitz and Jüppner, 2011; Okumura et al., 1998).

Interestingly, a number of different strategies are used to regulate Pi responsive genes and for fine-tuning their expression. For example, in *S. cerevisiae*, the *PHO5* gene encodes an acid phosphatase (homologous to *pho1* in *S. pombe*) that is required for Pi uptake and is tightly regulated in response to the intracellular Pi concentration (Auesukaree et al., 2003, 2004). *PHO5* induction relies on availability of two upstream activating sequences p1 and p2 (UASp1 and UASp2) in

the *PHO5* promoter, which provide for the major binding sites for the transcription activators Pho2 and Pho4. Antisense transcription results in nucleosome eviction from the mRNA promoter and exposes the UASp1 and UAS p2, thereby aiding gene transcription (Magbanua et al., 1997; Uhler et al., 2007; Wykoff et al., 2007).

Similar to *PHO5* regulation described above, the *pho1* locus (the major acid phosphatase of the PHO pathway in *S. pombe*) is regulated by several positive and negative regulators. However, details of the mechanism of Pi mediated repression of *pho1* remains elusive. So far, the transcription factor Pho7, Csk1 (a Serine/threonine-protein kinase), an upstream activating sequence (UAS) and a putative Pi sensing element upstream of the *pho1* gene have been implicated in regulating gene expression (Carter-O'Connell et al., 2012; Henry et al., 2011).

In the presence of Pi, *pho1* is repressed, whereas absence of Pi results in induction of the *pho1* gene. The repression of *pho1* is regulated via the action of Csk1, but how Csk1 performs this inhibitory role is currently unknown. It is speculated that Csk1 functions via interfering with the activity of the transcription factor Pho7, at the *pho1* promoter. However, Pho7 function is not regulated directly by binding to Csk1 unlike the kinase regulation of Pho4 transcription factor in *S. cerevisiae*. In high Pi conditions, Pho7 is bound to the UAS and results in basal expression of *pho1*. Full induction of the gene is inhibited via Csk1 interaction near the UAS. When the extracellular levels of Pi drop, more Pho7 is recruited to the promoter, thereby relieving the repression via Csk. Thus resulting in maximum induction of the *pho1* gene. It was also shown that proper regulation is controlled within a Pi sensing module located between -2000 and -280 base pairs (bp) upstream

of the *pho1* gene in an unknown manner. This element is very important for Pi response of the *pho1* gene. Its deletion results in loss of sensitivity to Pi presence and constitutive expression of *pho1*. The levels of gene expression achieved upon loss of the Pi sensing element are much greater than that upon loss of Csk1. Thus this element might not require Csk1 for repression of gene expression. Another important observation was the ability of this element to drive expression of YFP protein when placed before the ORF (Carter-O'Connell et al. 2012). How the Pi sensing element mediates *pho1* repression is so far unknown. Indeed, this region was found to be transcriptionally active in microarray analysis using an exosome mutant (Wilhelm et al., 2008). Furthermore, small RNA reads were also enriched in this region in an exosome mutant (Yamanaka et al., 2013).

I decided to examine the Pi sensing element in detail to understand its function in regulating *pho1* expression. Interestingly, I find that the Pi sensing element produces a novel *prt* lncRNA (long non-coding RNA). My studies provide a clear mechanistic role for lncRNA transcription in regulating *pho1* expression. I speculate that the transcription of lncRNA is crucial for Pi mediated repression of *pho1*.

This chapter will describe the *prt* lncRNA and its response to Pi, followed by the mapping of the 5' and 3' ends of the various *pho1* transcripts. Next, a detailed promoter deletion analysis will be described to understand the effect of the lncRNA on *pho1* repression.

RESULTS:

4.1 Two transcripts are produced from the *pho1* locus in response to Pi presence

The *pho1* gene is known to be induced in the absence of Pi in the growth medium (Henry et al., 2011; Schweingruber et al., 1986, 1992a). In the presence of Pi (10mM KH_2PO_4) the levels of *pho1* mRNA decrease whereas in the absence of Pi (0mM KH_2PO_4), mRNA levels increase (Henry et al. 2011 and Fig. 3-1A and B, lanes 1 and 2) to execute uptake of extracellular Pi.

To investigate regulation of *pho1* expression in more detail, I studied the *pho1* RNA profile in WT and *rrp6* Δ using Northern blot. Indeed, I detected two *pho1* transcripts in *rrp6* Δ and only a shorter transcript in WT (Fig. 3-1B, lanes 2 and 4). The approximate size of the transcripts detected is ~2.8 kb (RNA2) and ~1.5 kb RNA (RNA1). Surprisingly, under the same conditions (presence of Pi) in wild-type (WT) cells, only RNA1 transcript with a shorter 5'UTR was detected.

In agreement with previous studies (Schweingruber et al., 1992b), in the WT, only RNA1 was detected and its levels increase in the absence of Pi (Fig. 3-1B, lane 1 and 2). In *rrp6* Δ, RNA1 was detected in the absence of Pi, however in the presence of Pi, both RNA1 and RNA2 transcripts were detected (Fig. 3-1B, lane 3 and 4). Similar to that in WT, RNA1 is induced in response to no Pi, in *rrp6* Δ. However, the levels of RNA1 were much lower compared to the WT in the presence of Pi (Fig. 3-1B, compare lane 1 with 3 and lane 2 with 4)

Thus, using exosome mutants, two overlapping transcript are detected at the *pho1* locus only in presence of Pi.

4.2 RNA2 is transcribed from the region upstream of RNA1 and is degraded by the exosome complex.

To determine the orientation of RNA2 with respect to the *pho1* gene I used strand specific probes to the region upstream of *pho1*. In Fig. 3-1B, the probe 1 used to detect *pho1* transcripts is not strand specific, thus it was unclear whether RNA2 is transcribed from the sense or antisense direction and whether it corresponds to a 5' or 3' extended *pho1* RNA. Therefore, I used probes 2 and 3 that detect only sense RNA and are specific to the region between -2 kb and -280 bp.

Rrp6 mutants are compromised in post-transcriptional quality control and thus can be used to detect transcripts that are made in the WT cells but rapidly degraded. Indeed, using an *rrp6* Δ strain, I detected RNA2 alone using probe 2 and 3 (Fig. 3-1C lanes 4 and 5). An accumulation of RNA2 was also found in the *dis3*-ts mutant (Dis3 is the catalytic subunit of the exosome core) (Fig. 3-1 C lanes 1, 2 and 3).

To determine whether RNA2 is transcribed in the WT, I used Pol II ChIP to evaluate Pol II occupancy across the *pho1* locus in the presence and absence of Pi. Indeed, in the presence of Pi, Pol II is detected across the region encompassing RNA2 (Fig 3-1A, D and E, primer pairs 1 and 2). In the absence of Pi, Pol II is detected only across *pho1* coding region (Fig 1A, D and E, primer pairs 3 ad 4). I predict that, RNA2 is transcribed in the WT in the presence of Pi but is degraded by the exosome

complex (Fig 1B and 1E).

Taken together this data suggests that RNA1 is induced in the absence of Pi in WT and *rrp6*Δ. In the presence of Pi, another transcript RNA2 is synthesized from upstream of RNA1. The exosome complex rapidly degrades RNA2.

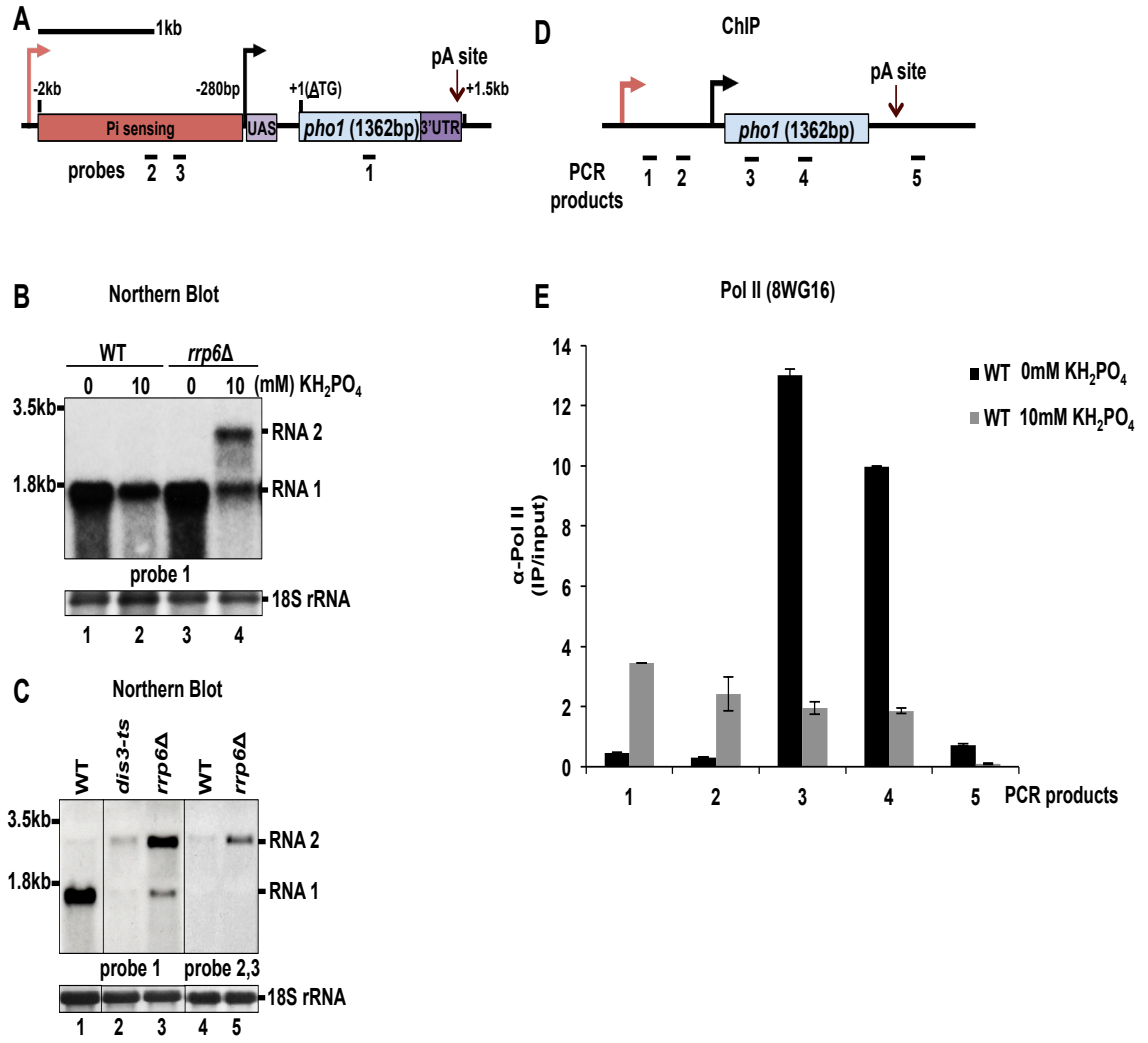


Figure 4—1 Two overlapping transcripts are made at the *pho1* locus.

(A) *pho1* schematic. The coding sequence is depicted in blue, the Upstream Activating Sequence (UAS) in purple, the Pi sensing region in red and the 3'UTR in dark purple. Probes are shown as black bars (B) Northern blot analysis of RNA isolated from WT and *rrp6*Δ strains grown in EMMG medium with 0mM or 10mM KH_2PO_4. (C) Indicated strains were grown in YES for Northern blot analysis using probes indicated. (D) *pho1* schematic showing locations of the PCR products. Pol II ChIP performed in WT cells grown in EMMG (0mM or 10mM KH_2PO_4). Quantification of results from three independent experiments is shown; the error bars indicate standard error.

4.3 Mapping the ends of *pho1* RNAs

To map the exact 5' and 3' ends of RNA1 and RNA2, circularized RACE (cRACE) technique was used. RNA was purified from cells grown in mid-log phase and sequentially treated with shrimp alkaline phosphatase (SAP) and tobacco acid pyrophosphatase (TAP). The enzymatic treatment is used to dephosphorylate decapped RNAs from the 5' ends and cleave the pyrophosphate bond of the methyl-7-guanosine cap respectively, resulting in an RNA isolate containing ligatable 5'-monophosphorylated ends only on transcripts that were previously capped and not those that lacked the cap. These were intramolecularly ligated 5' to 3' to form circular RNA molecules. The ligation sites were amplified by reverse transcription and PCR amplified with divergent primers (Fig. 3-2A and B). Following cloning and sequencing to determine the position of ligation of ends, the exact 5' and 3' ends were identified (Fig. 3-2C and D).

Interestingly, sequencing reads of the cDNA generated from the ligated molecules have identified three *pho1* transcript start sites at -1198nt, -324nt and -52nt respectively, upstream from the start codon (ATG=1). All clones from the WT strain correspond to the transcript produced from the -52 TSS (corresponding to RNA1 in Fig. 3-1B). In *rrp6* Δ , 2 clones mapped to -52 TSS, but the majority of clones (5 out of 7) were derived from the -1198 TSS (corresponding to RNA2 in Fig. 3-1B). This correlates with the Northern blot data, and suggests that the two major *pho1* transcripts are transcribed from two independent promoters and differ only in their 5'UTR. A transcript produced from the -324 TSS was not detected by Northern blot suggesting that transcription from this site is very inefficient. The poly (A) site was found to be at 135nt downstream of the stop codon for all transcripts (Fig. 3-2D).

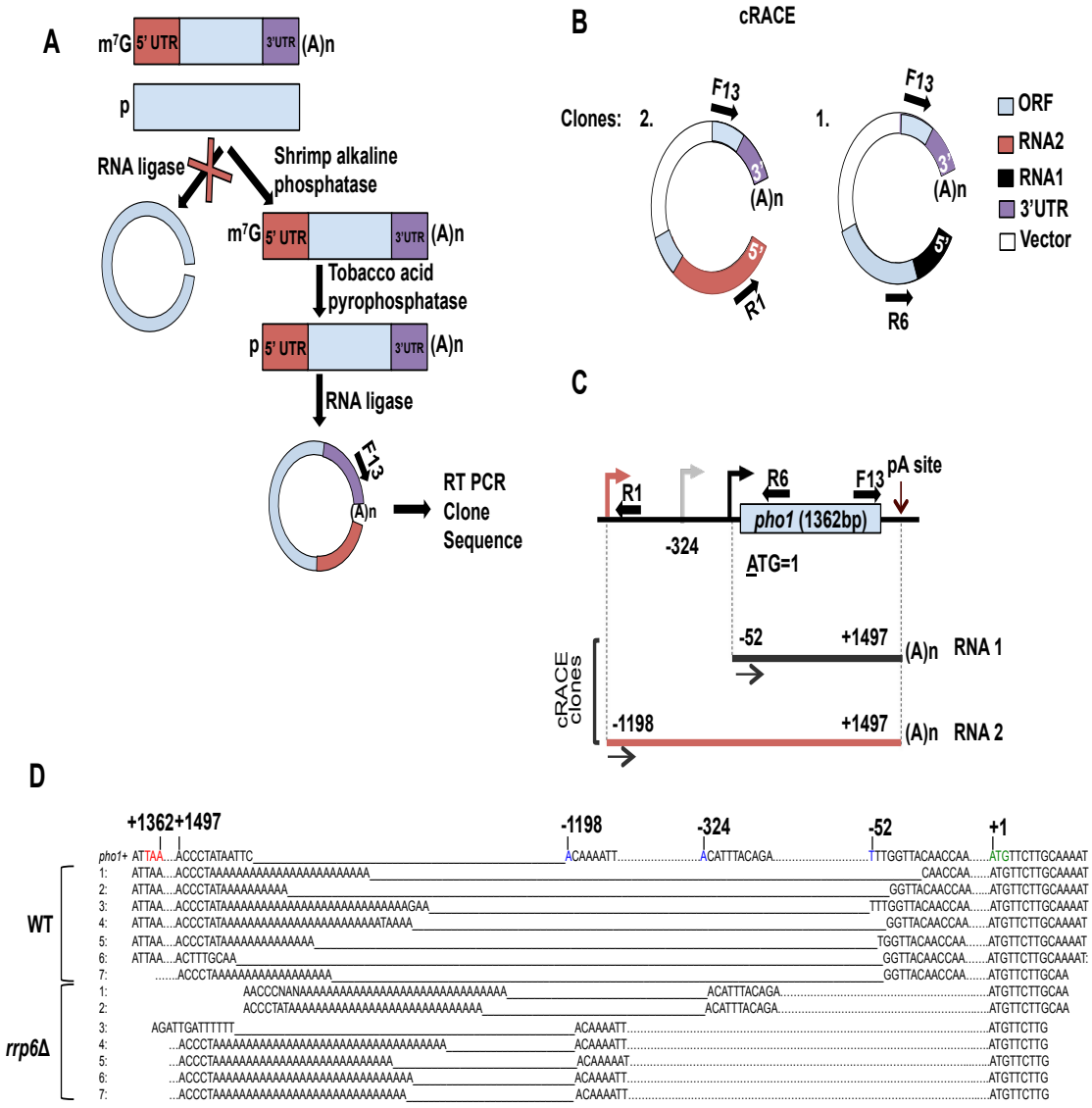


Figure 4—2. cRACE mapping of *pho1* transcripts.

(A) Schematic representing the protocol for cRACE. Total RNA was treated with shrimp alkaline phosphatase to dephosphorylate the ends of uncapped RNAs. Capped messages were then decapped with tobacco acid pyrophosphatase. The RNAs were ligated using T4 RNA ligase to form circularized products. cDNAs were generated from ligated products using primer F13 and then cloned into a vector. (B) Schematic representation of the cRACE clones. Primers R1, R6 and F13 were used for sequencing of 7 clones obtained from the WT and *rrp6* Δ strains. (C) Schematic diagram indicating positions of the mapped 5' and 3' ends from the sequencing reactions. Three TSS were identified at positions -1198, -324 and -52, where +1 defines the start codon. The 3' ends of all transcripts were mapped at +1497. (D) Sequences of the clones obtained from the WT and *rrp6* Δ strains by cRACE. The stop codon (TAA) of the *pho1* gene is marked in red and the start codon (ATG) in green.

4.4 ncRNA transcription overlaps the protein coding *pho1* mRNA

Based on the cRACE (Fig. 3-2D) and Northern Blot data (Fig. 3-1 C) I found that RNA2 overlaps completely with RNA1 and both transcripts (RNA1 and RNA2) contain the entire *pho1* ORF. Thus, it was imperative to determine which of them encodes for the Pho1 protein. Pho1 enzymatic activity was measured using liquid PNPP assays (Henry et al., 2011) and it was found that in the absence of Pi, Pho1 protein activity is maximal. Based on the RNA profile in the presence and absence of Pi, I hypothesized that RNA1 must encode for the Pho1 protein. To verify this, each of the RNA1 and RNA2 promoters were deleted in WT and Rrp6 mutant background. To delete RNA1 promoter, 346 nt of the predicted promoter region was deleted (-346 to -1 nt) (Fig. 3-3A). In parallel, 234 nt of the RNA2 promoter was replaced with an *ura4+* cassette (-1438 to -1284) (Fig. 3-3A). Loss of the respective RNAs was verified using Northern Blot (Fig. 3-3A and B). I determined Pho1 protein activity in these strains using liquid PNPP assays (as described in (Henry et al., 2011)) and compared it to that in the WT cells. Indeed, deletion of RNA1 promoter resulted in complete loss of Pho1 protein activity, whereas loss of RNA2 promoter resulted in an increase in activity in high Pi conditions.

This data suggests that the RNA1 is likely to be the protein encoding mRNA and RNA2 is most probably a long non-coding RNA (Fig. 3-3C).

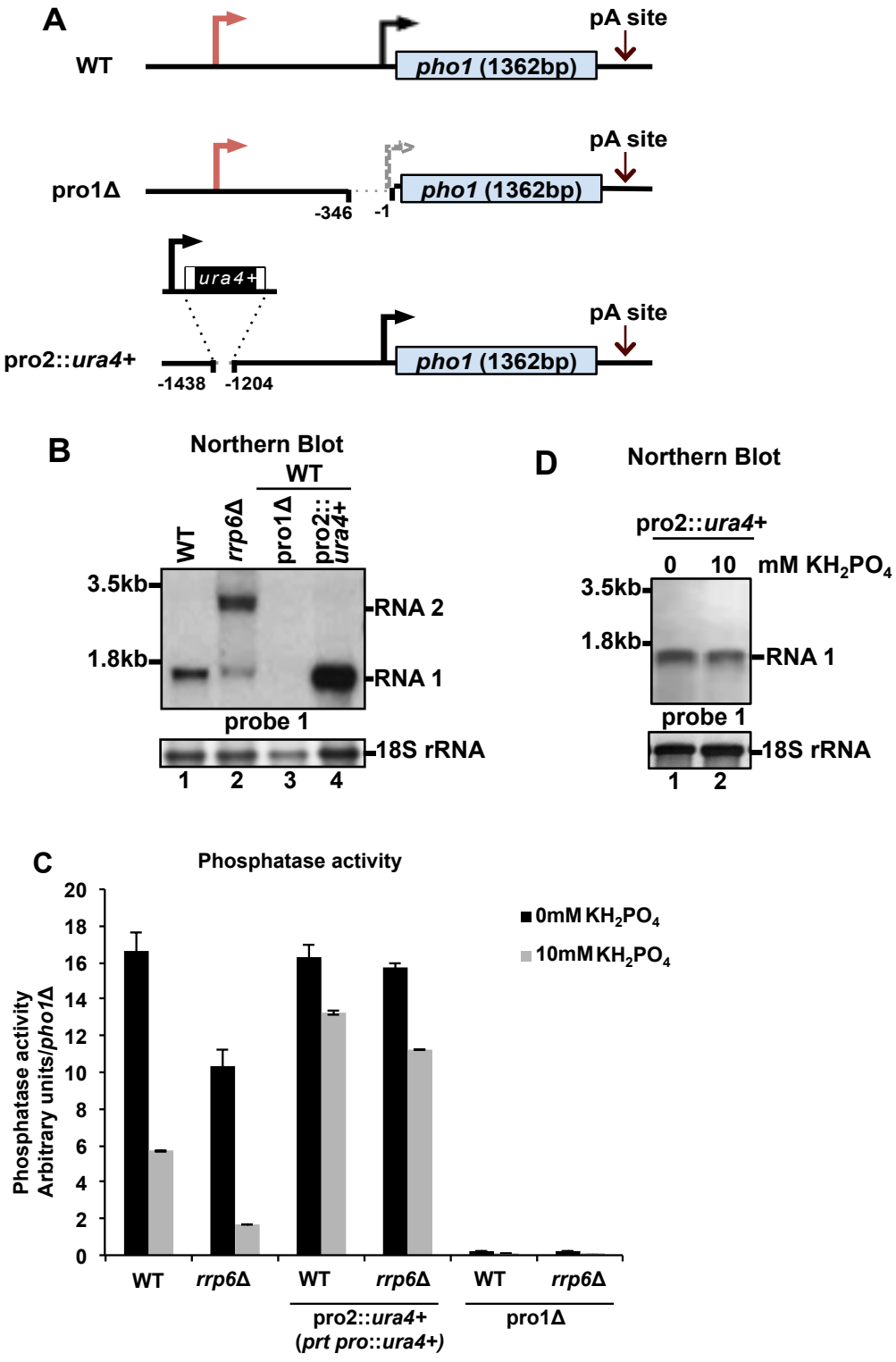


Figure 4—3. Expression of lncRNA negatively affects *pho1* and protein levels.

(A) Schematic representation of the promoter deletion strains. (B,D) Northern blot analysis of RNA levels in indicated strains grown in YES. Probe used is the same as Probe1 indicated in Fig. 3-1A (C) Analysis of phosphatase activity for the strains in (A) Strains were grown in EMMG medium (0mM or 10mM KH_2PO_4). Values were normalized to those from a *pho1*Δ strain.

4.5 Non-coding transcription is involved in repression of *pho1* mRNA expression in response to Pi.

My data indicates an inverse co-relation between RNA1 and RNA2 expression, to test whether expression of RNA2 has a negative impact on RNA1 levels, I analyzed the effect on RNA levels and protein activity upon deletion of RNA2. Strikingly, a substantial increase in *pho1* mRNA levels (~3 fold) (Fig. 3-3B, compare lanes 1 and 4) and in phosphatase activity (Fig. 3C) (3-6 fold) was observed in the strains lacking the RNA2 promoter even in the presence of Pi. These data suggest that RNA2 has a regulatory role to play at the *pho1* locus in fine-tuning expression of the *pho1* mRNA in response to Pi. I thus refer to RNA2 as *prt* (*pho1* repressing transcript).

I also noticed that deletion of *prt* promoter resulted in constitutive expression of *pho1* suggesting a loss of sensitivity to Pi presence (Fig. 3-3C and D). Since *prt* is transcribed only in the presence of Pi (Fig. 3-1 D) when *pho1* expression is repressed (Fig. 3-1B), this indicates that *prt* may have a role in repressing *pho1* expression in response to extracellular Pi.

Taken together this data suggests that *prt*, a lncRNA is associated with negatively regulating expression of *pho1* mRNA and may play an important role in repressing *pho1* in response to presence of Pi.

Discussion:

It is becoming apparent that non-coding RNAs have acquired a fundamental role in gene expression studies in the past decade. This study describes a novel lncRNA, *prt* that negatively regulates expression of a nutrient uptake gene *pho1* in *S. pombe*. *prt* is transcribed from a promoter upstream to *pho1* gene in a tandem orientation and overlaps with the *pho1* ORF. Both the RNA's have the same 3' end and are capped; the exosome complex degrades *prt*. A similar scenario was previously described in *S. cerevisiae* for another nutrient responsive gene, *SER3*. *SRG1* is the ncRNA that is transcribed in-tandem orientation from upstream of *SER3,* but terminates adjacent to the UAS and overlaps only with the first few nt of *SER3* (Thompson and Parker, 2007). A number of detailed studies have been performed to study regulation of *SER3*. They show that, transcription of *SRG1* leads to *SER3* repression. The *SRG1* transcript is classified as a CUT and is targeted by the exosome for degradation (Martens et al., 2005; Nishizawa et al., 2008).

Although these similarities help in understanding the possible regulation at the locus, it is interesting to note that unlike *SRG1*, *prt* does not terminate before the *pho1* gene but transcribes all the way through. How does the exosome complex recognize specifically *prt* but not *pho1* mRNA for degradation? The mechanism of achieving substrate specificity for exosome function is an important long-standing question in the exosome field. I will address exosome targeting at the *pho1* locus in the following chapters. The further reduction of *pho1* levels in Rrp6 mutants is striking.

Importantly, abolishing *prt* expression results in constitutive expression of *pho1*. This clearly indicates the importance of *prt* in mediating repression of *pho1* mRNA in response to Pi. Thus, the Pi sensing element might comprise of a lncRNA that may be responsible for its role. How *prt* regulates *pho1* mRNA expression, will be addressed in the next chapter.

Previous studies implicated binding of Pho7 and Csk1 at the UAS as decisive factors regulating *pho1* levels in response to Pi (Carter-O'Connell et al., 2012; Henry et al., 2011). In light of my new data, it would be interesting to re-assess the binding of these factors upon deletion of the *prt* ncRNA. This would help understand the contribution of *prt,* Pho7 and Csk1 in repressing *pho1* expression in response to Pi. Since the UAS is part of the *prt* RNA it is possible that under repressive conditions the transcription of *prt* hinders binding of Pho7. This might somehow allow Csk1 to repress expression of *pho1*. However, it is also likely that *prt* functions independently of Csk1 and Pho7. Possibly the concerted function of all these factors is important for fine-tuning *pho1* expression. A detailed analysis is required to understand the interplay between these factors.

Chapter 4

ncRNA mediates deposition of transient heterochromatic marks at the *pho1* locus

Introduction:

In recent years, a number of extensively transcribed large non-protein coding RNA transcripts have been discovered. In yeast, genes such as *FLO11* (Voynov et al., 2006), *SER3* (Martens et al., 2005; Thompson and Parker, 2007) and *PHO84* (Camblong et al., 2007, 2009a) are some of the examples of genes regulated via lncRNAs. In fission yeast, much fewer examples of ncRNA mediated regulation of gene expression have been studied, exemplified by ncRNAs regulating *fbp1* and *ade6-M26* (Hirota and Ohta, 2009; Hirota et al., 2008). Thus, careful investigation of ncRNA function and regulation is lacking in this eukaryote and impedes our understanding of the entire breadth of their regulatory roles.

NcRNAs regulate gene expression in a variety of ways (for review, (Wilusz et al., 2009). The act of ncRNA transcription can alter expression of neighbouring genes by transcription interference (TI) (Gullerova and Proudfoot, 2010). NcRNAs also change chromatin architecture and thus affect gene expression (Marina et al., 2013; Uhler et al., 2007) or can bind to proteins and modulate their activity on genes (Rossbach, 2011).

Non coding transcription in *S. pombe*, results *in* stable heterochromatic (constitutive heterochromatin) formation at the pericentric repeats, subtelomeric regions and the silent mating-type locus (Bühler et al., 2007; Marina et al., 2013; Noma et al., 2004). Interestingly, in addition to constitutive heterochromatin there is facultative heterochromatin that was found during mitotic growth at the meiotic genes (Tashiro et al., 2013; Zofall et al., 2012). Although heterochromatin formation at meiotic genes, during mitosis, is dependent on RNAi factors, it also relies on other RNA binding proteins, such as Mmi1 and Red1 (Hiriart et al., 2012; Holm and Thon, 2012). Mmi1 binds to its target RNAs via a specific sequence motif called the DSR (Determinant of Selective removal) element. Mmi1 binds to meiotic RNAs only during mitosis since upon induction of meiosis; Mmi1 is sequestered away from meiotic genes (Harigaya et al., 2006). Meiotic RNAs expressed during mitosis are lethal for the cells. Therefore Mmi1 binding provides for exclusive and timely targeting of meiotic genes to allow assembly of heterochromatin thereby preventing transcription of these RNAs during mitotic growth (Hiriart et al., 2012; Tashiro et al., 2013; Zofall et al., 2012). In plants, intermediate heterochromatin was also identified at temperature-sensitive genes (Tittel-Elmer et al., 2010).

Recent genome wide studies in *S. pombe*, identified H3K9me2 on a number of expressed genes other than the previously identified meiotic genes, however its role at these loci is not clear (Yamanaka et al., 2013). Surprisingly, H3K9me2 and small RNA reads were also detected at the *pho1* locus in mitotically dividing cells. But these heterochromatin marks were detected only in the exosome mutant background and not in the WT cells.

Heterochromatin formation at these loci could be triggered via binding of RNA binding proteins such as Mmi1 or Red1 that recruit the RNAi machinery. Alternatively, large amount of antisense transcription is observed in eukaryotic genomes and can serve as a potential source for siRNAs. In *S. pombe*, overlapping RNAs transcribed in antisense direction which overlap due to failure in terminating their transcription, can also form dsRNA and recruit the RNAi pathway (Gullerova et al., 2011). Alternatively, dsRNA can also be synthesized via the action of RNA-dependent RNA polymerase (Rdrp1) on single stranded RNA templates (Moazed et al., 2006; Motamedi et al., 2004; Verdel and Moazed, 2005).

I have identified an nc transcript at the *pho1* locus and transcription of this RNA coincides with H3K9me. I thus set out to determine whether there is a correlation between these two phenomenon. Next, I checked whether the heterochromatin plays a role at the *pho1* locus by investigating whether loss of H3K9me2 affects *pho1* expression. Does heterochromatin play a functional role in repressing pho1 expression in response to Pi presence? Does *prt* transcription lead to H3K9me2? Furthermore, I investigated the contribution of the RITS complex, HP1 proteins and other proteins like Red1 in heterochromatin formation at *pho1*.

Results:

5.1 Formation of transient heterochromatin at the *pho1* locus in response to Pi presence

Presence or absence of Pi regulates expression of *pho1*. Thus, if H3K9me2 affects *pho1* expression it may also vary with respect to Pi presence. To determine whether H3K9me2 at the *pho1* locus is responsive to Pi, I analysed H3K9me2 in WT and exosome mutant cells grown in presence of Pi and after 12 h of shifting to no Pi media (when *pho1* is completely induced in both strains (Fig. 3-2A and B)). Indeed, my analysis revealed that in the presence of Pi, H3k9me2 is detected at the *pho1* locus in WT and *rrp6*Δ cells (Fig. 4-1A and B). Interestingly, in the absence of Pi, H3k9me2 in WT and *rrp6*Δ cells was found to be as low as the background (Fig. 4-1A and B). H3k9me2 was measured across *adh1*, a highly expressed gene, to provide for background signals. Furthermore, the levels of H3K9me2 in *rrp6*Δ were much higher than in the WT.

This data suggests that the presence of H3k9me2 is responsive to the presence of Pi and hence transient in nature. Thus, H3k9me2 at the *pho1* locus correlates with Pi presence/absence and hence might play a regulatory role.

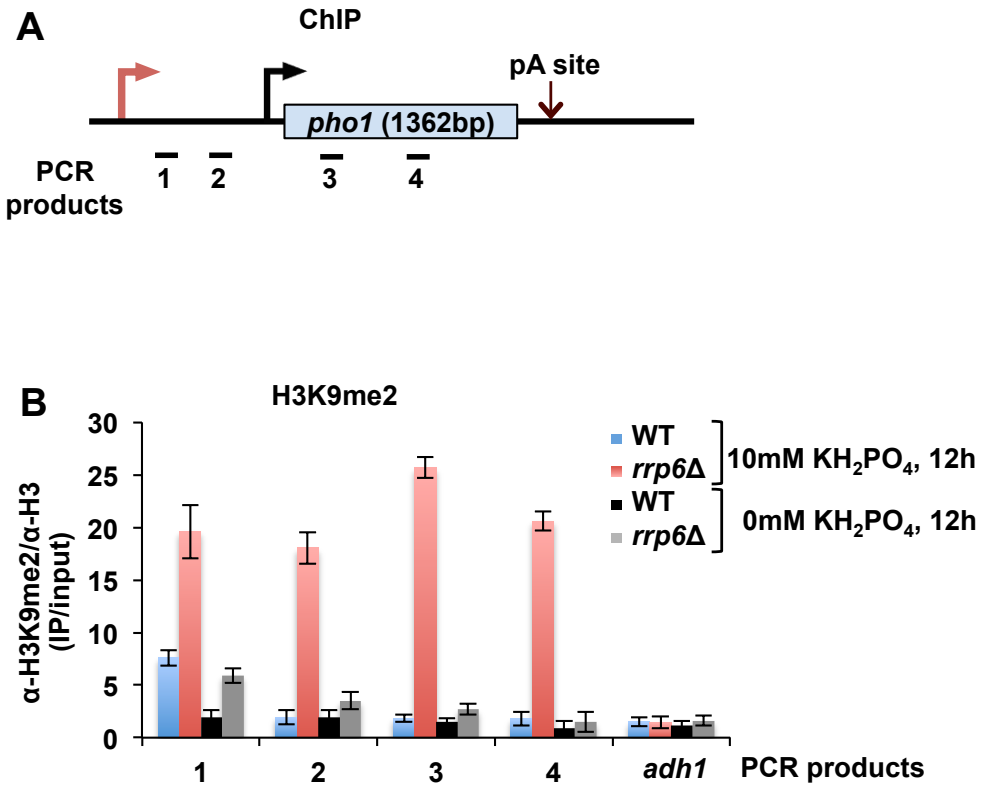


Figure 5—1. Distribution of H3K9me2 across *pho1* in the presence and absence of Pi in the media.

(A) Schematic representation of the pho1 gene. Black bars show locations of the PCR products. (B) H3K9me2 ChIP experiments were performed in the strains indicated. Cells were grown in YES media overnight and then shifted to EMMG media (0mM or 10mM KH_2PO_4) for 12 hrs. Quantification of results from three independent experiments are shown; the error bars indicate standard error.

5.2 Loss of Clr4 results in faster induction of *pho1*.

My data show that H3k9me2 occurs at *pho1* only in the presence of Pi, suggesting that Pi mediated repression of *pho1* maybe linked to H3K9me2. Therefore depending on the contribution of H3k9me2 to *pho1* repression, I hypothesized that, in Clr4 mutants (resulting in loss of H3K9me), *pho1* might be de-repressed (partially or completely) even in the presence of Pi. In case of partial de-repression, the induction kinetics of *pho1* might be affected. Thus, to study whether H3k9me2 has a functional role at the *pho1* locus, I performed a detailed time course to study *pho1* induction kinetics. Cells growing in presence of Pi (10mM KH_2PO_4) were shifted to no Pi media (0mM KH_2PO_4) to induce Pho1 expression. Cells were collected at 0, 2, 4, 6 and 24h and *pho1* RNA and protein levels were monitored (Fig. 4-2 A and B). Northern blot analysis of the RNA levels in the WT cells revealed that *pho1* mRNA levels increase up till 6h of induction and then begin to decrease. Similar to my previous observation (Fig. 3-1 and 3-3), in *rrp6*Δ the steady state *pho1* mRNA levels in presence of Pi is much lower than those in the WT. The induction of *pho1* is also much slower in *rrp6* Δ compared to the WT, resulting in lower mRNA levels and protein activity (Fig. 4-2A and B). However, after 12h of induction the protein and mRNA levels in *rrp6*Δ is comparable to the WT.

Interestingly, in the Clr4 mutant I observe faster induction kinetics of *pho1* compared to the WT in the initial hours of induction whereas after 4h the protein activity is similar to that in the WT (Fig. 4-2A, B and C). Furthermore, in double mutants of Rrp6 and Clr4 the delayed kinetics observed in *rrp6*Δ is completely suppressed. This data clearly implies that Clr4 protein, responsible for H3K9me,

plays a role in delaying induction of *pho1* upon loss of Pi. Also, Rrp6 mutation results in higher H3k9me2 at *pho1* (Fig. 4-1A and B) and delayed induction kinetics, which are restored upon deletion of Clr4 in an Rrp6 mutant background (Fig. 4- 2A and B).

Taken together, these data suggest a role for Clr4 in regulating induction of *pho1,* thus implying a functional role for the transient heterochromatin formed at this locus. Interestingly, H3k9me2 may not contribute to the steady state repression of *pho1* but may result in a change in chromatin architecture that inhibits quick activation of this gene. How, ncRNA results in repression of *pho1* remains an open question.

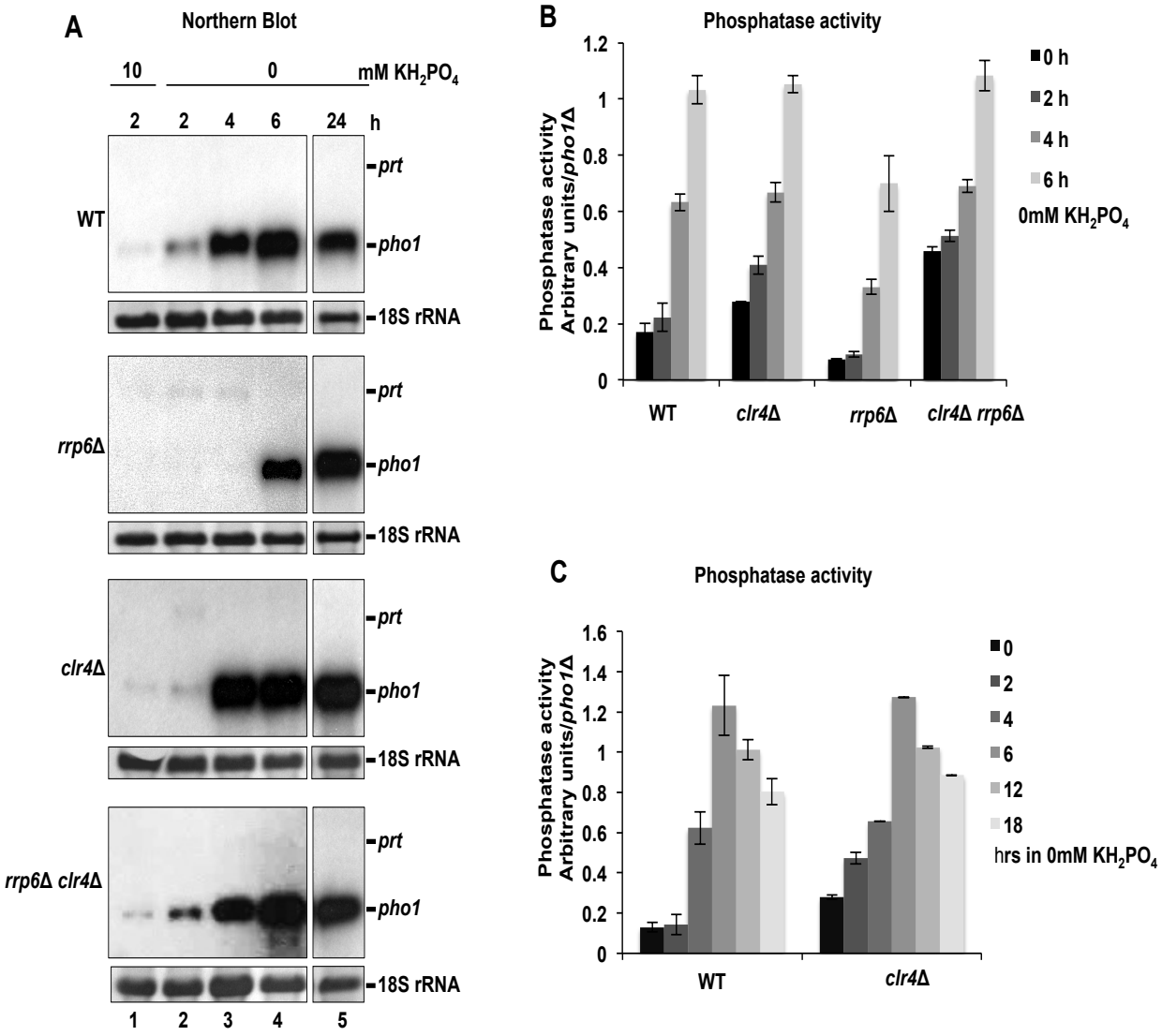


Figure 5—2. *pho1* induction kinetics.

Experiments were performed in indicated strains grown in EMMG with 10mM KH_2PO_4 and then shifted to 0mM KH_2PO_4 for 2, 4, 6 and 24 h. Cells were pelleted for PNPP assays and RNA extraction. (A) Northern blot analysis for *pho1* from samples collected during the time course. The same probe was used as in (Chapter 3, Fig. 1A and B). (B) Phosphatase activity measured for the strains in (A). (C) Phosphatase activity for the indicated strains measured at uptil 18hrs of induction. Samples were taken every two hours.

5.3 RNAi is required for *pho1* silencing in the presence of Pi via methylation of H3K9.

To determine whether the RITS complex plays a role in mediating heterochromatin formation at the *pho1* locus, I analysed H3K9me2 levels in the absence of Ago1, Dcr1 and Rdp1 (Components of the RITS complex). Indeed, a partial loss of H3K9me2 marks was observed in *ago1*Δ and *ago1*Δ*rrp6*Δ strains (Fig. 4-3A and B). Conversely, the recruitment of Ago1 was also analysed using ChIP. My data shows, an enrichment in Ago1 recruitment at the *pho1* locus compared to the background levels at the *adh1* gene (Fig. 4-3C). In support of the involvement of the RNAi machinery, mutants of Dcr1 and Rdp1 also showed partial loss of H3K9me2 marks at *pho1* (Fig. 4-3D). I also analysed catalytic mutants of Dcr1 (*dcr1*D937A) and Ago1 (*ago1*D580A); both mutants displayed the partial loss of H3K9me2 as seen previously with the complete deletion mutants for Ago1 and Dcr1 (Fig. 3D). Importantly, the Ago1 slicer mutant phenotype helps verify that the slicing activity of Ago1 is required for H3K9me2 that along with the Rdp1 mutant data supports the involvement of RITS complex in mediating H3K9me2 at the *pho1* locus. However, I could not detect siRNAs generated from *pho1* locus that were previously detected in *rrp6*Δ by genome-wide small RNA tiling arrays most likely due to lack of sensitivity (Yamanaka et al., 2013).

Interestingly, in strains with deletion of Clr4, H3K9me2 levels were further reduced compared to the levels observed in RITS mutants suggesting that Clr4 can also be recruited independently of the RITS complex (Fig. 4-3D). Previously, interaction between Red1 and Clr4 has been demonstrated, suggesting Red1 involvement in recruiting Clr4 to assemble heterochromatin (Hiriart et al., 2012;

Tashiro et al., 2013). In support of this, loss of H3K9me2 in Red1 mutants was observed for a number of facultative heterochromatic genes (Zofall et al., 2012). I thus checked for H3k9me2 across *pho1* in Red1 mutants and indeed I found a partial loss of H3K9me2 (Fig. 4-3E). Conversely, I also found an increase in *pho1* mRNA levels upon deletion of Red1 (Fig. 4-3F), which could be due to loss of transcriptional repression as a consequence of inefficient heterochromatin formation. RNA analysis also showed stabilization of *prt* similar to levels in the Rrp6 deletion strain (Fig. 4-3F), similar to that shown previously for meiotic RNAs (Sugiyama and Sugioka-Sugiyama, 2011). Involvement of Red1 in heterochromatin formation at the *pho1* locus and exosome mediated decay of *prt* is reminiscent of the role of Red1 at meiotic genes (Tashiro et al., 2013; Zofall et al., 2012). A recent study also showed the involvement of Red1 and Ago1 in heterochromatin formation at the *pho1* locus (Yamanaka et al., 2013).

To analyse whether HP1-like proteins Swi6 and Chp2 are also involved in heterochromatin maintenance at *pho1* locus, I analysed RNA levels in deletion mutants of Swi6 and Chp2. Indeed, I found a modest de-repression of RNA levels in both these strains compared to that in the WT cells (Fig. 4-3G).

Thus, heterochromatin formation at the *pho1* locus relies on RNAi dependent and independent mechanisms and due to the dependence on Red1 might resemble the heterochromatin formation at the meiotic genes (Hiriart et al., 2012; Yamanaka et al., 2013; Zofall et al., 2012).

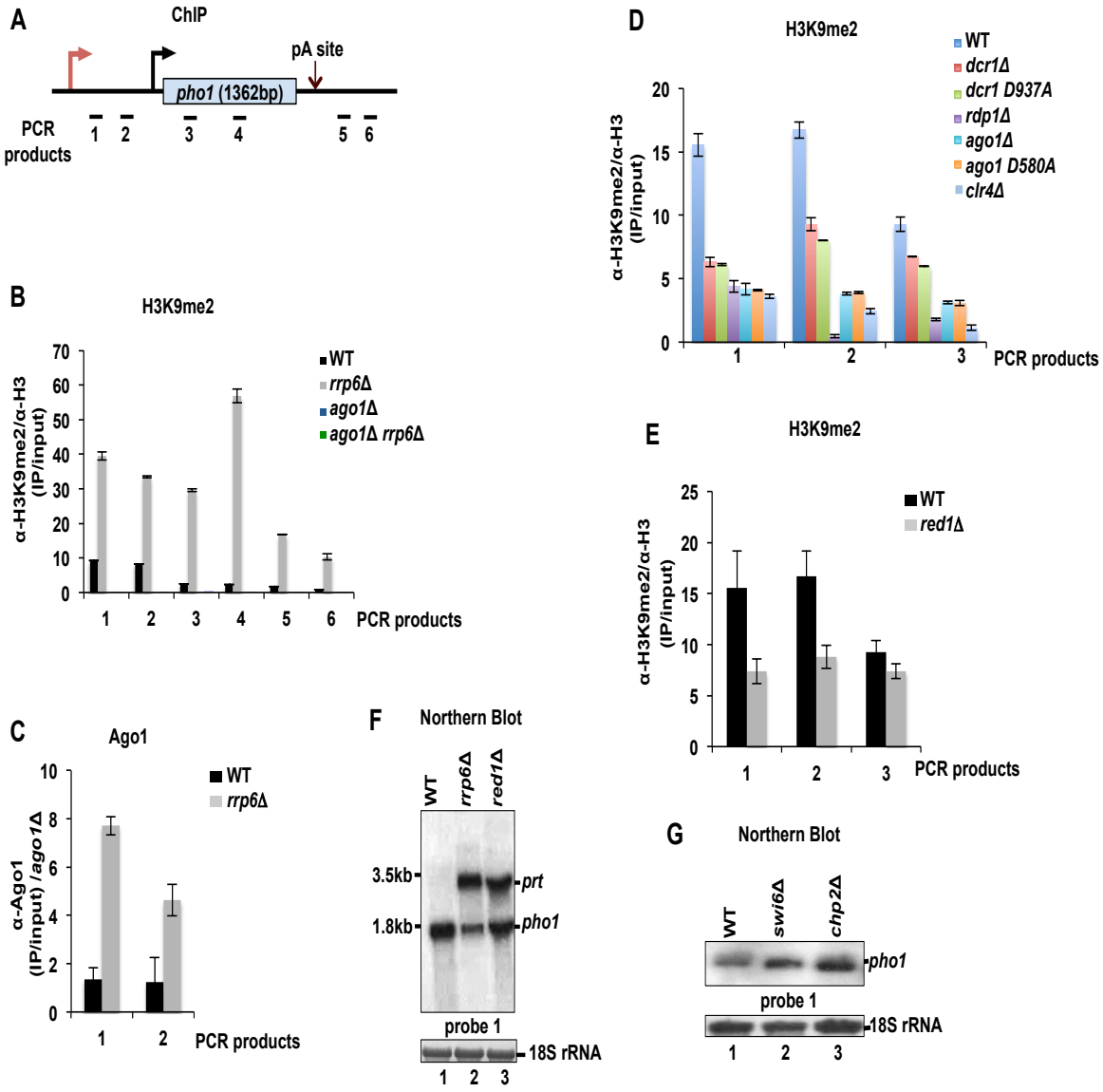


Figure 5—3. Components of RITS, Red1 and HP1 proteins are required for establishment of H3k9me2 at *pho1*.

(A) Schematic representation of the *pho1* gene. Black bars show locations of the PCR products. (B-E) ChIP experiments were performed in the strains indicated. Cells were grown in YES for all experiments. Quantification of results from three independent experiments are shown; the error bars indicate standard error. (B) ChIP analysis of H3K9me2 in the strains indicated (C) Ago1 ChIP. (D) H3K9me2 ChIP analysis in indicated strains. (E) H3K9me2 ChIP in Red1 deletion compared to WT cells. (F,G) Northern Blot analysis of indicated strain, using probes against *pho1* gene body.

5.4 *prt* transcription is essential for RNAi recruitment and heterochromatin formation across *pho1*.

I noticed that H3k9me2 marks are specifically assembled at the *pho1* locus in the presence of Pi, which leads to repression of *pho1* expression. Although the primary outcome of heterochromatin is silencing of gene transcription, it has been shown that transcription is required for assembly and also for maintenance of heterochromatin. Thus, it maybe necessary for *prt* ncRNA to be transcribed, to mediate heterochromatin formation in this region. Previously I have described that *prt* is transcribed only in the presence of Pi and is associated with *pho1* repression (Chapter 3). Thus I reasoned that transcription of *prt* could be involved in heterochromatin formation at *pho1* locus. Interestingly, in strains where *prt* is no longer expressed (due to promoter deletion), I find loss of H3K9me2 in WT and *rrp6* Δ strains (Fig. 4-4A and B), while centromeric heterochromatin remained unaffected (data not shown). Moreover, after 12h induction in no Pi media when *prt* is no longer expressed (Fig. 4-2A), H3K9me2 was no longer detected in WT (Fig. 4-1A and B). Additionally, Ago1 is also recruited to *pr*t (Fig. 4-3C) and siRNA clusters are also found in the region of *prt* that does not overlap with *pho1* mRNA by genome wide analysis (Zofall et al., 2012).

I conclude that *prt* transcription is necessary for H3K9me2 at *pho1*, while lack of nc transcription (in the absence Pi) leads to the loss of H3K9me2.

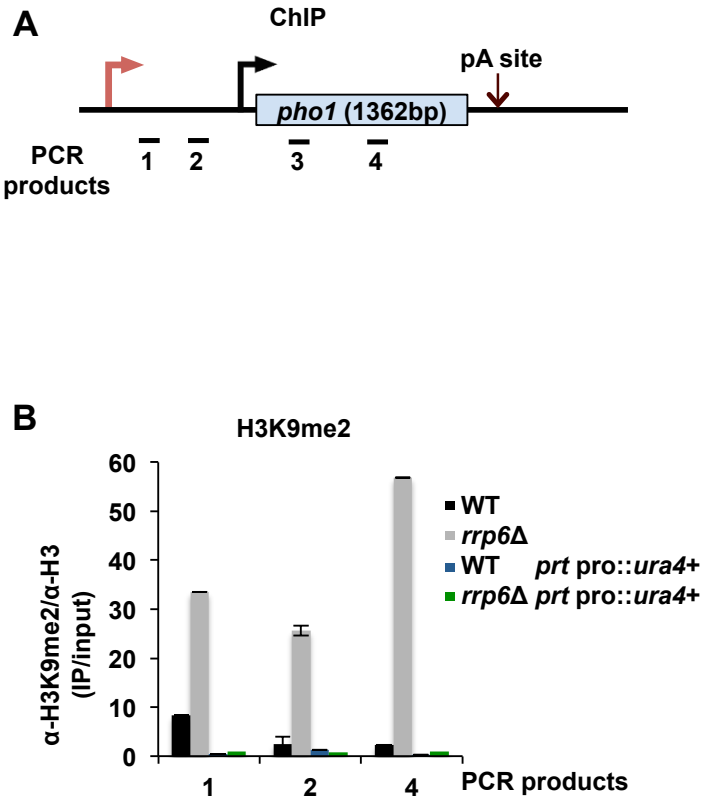


Figure 5—4. *prt* transcription is essential for H3k9me2 at *pho1*.

(A) Schematic representation of the *pho1* gene. Black bars show locations of the PCR products. (B) ChIP experiments were performed in the strains indicated. Cells were grown in YES (+Pi) for all experiments. Quantification of results from three independent experiments are shown; the error bars indicate standard error.

Discussion:

Heterochromatin formation at various chromosomal loci has previously been reported to result in transcriptional silencing of the underlying genes. This mechanism is used for constitutive silencing of regions such as centromeres, telomeres and mating type loci in *S. pombe*. Additionally, facultative heterochromatin, is found to control expression of developmental genes in a cell cycle dependent manner i.e. heterochromatin is formed during mitotic growth and is disassembled during meiotic progression (Tashiro et al., 2013; Zofall et al., 2012). Recently, a more transient heterochromatin was identified in plants called intermediate heterochromatin which responds to environmental stress condition i.e. temperature change (Tittel-Elmer et al., 2010).

In this study, I show that H3K9me2 at the *pho1* locus is dependent on Pi and ncRNA transcription. Interestingly, heterochromatin is formed only in the presence of Pi in the media and lost upon shift to the media with no Pi. Thus, the H3K9me2 is transient and responds to Pi presence in the growth media. Moreover, I demonstrate that the H3K9me2 plays a functional role in delaying induction kinetics of *pho1* mRNA, since upon loss of H3k9me2 in *clr4*Δ, *pho1* is induced faster compared to WT cells. However, steady state *pho1* mRNA levels are not significantly affected by loss of H3K9me2 in Clr4 mutants. This indicates that H3K9me2 may not play a major role in repressing steady state *pho1* mRNA levels. There may be other mechanisms that rely on *prt* transcription, which are important for *pho1* repression, since deleting *prt* promoter leads to full constitutive induction of *pho1*. Also, the fact that H3K9me2 does not affecting gene transcription is in agreement with a recent study that reporting

that the presence of H3k9me2 does not result in transcriptional repression and hence is not functional heterochromatin (Egan et al., 2014). The presence of H3K9me2, ensures slow induction of *pho1* mRNA and hence may be important for regulating induction kinetics. I speculate that H3K9me2 might be a mechanism to assess whether loss of Pi in the extracellular environment is transient or last for a longer length of time before allowing excess Pho1 activity at the cellular membrane.

I find that heterochromatin at the *pho1* locus is dependent on the RITS complex and appears to require HP1 like proteins for its maintenance. However, loss of RITS complex proteins resulted in only partial loss of H3K9me2 compared to that in Clr4 mutants, suggesting other mechanisms are associated with heterochromatin formation at this locus. In agreement with this, I found that RNA binding protein Red1 also contributes to H3k9me2 at *pho1*. Thus the action of Red1 may not be limited to only meiotic genes. Red1 was previously implicated in H3K9me2 at meiotic genes along with Mmi1 (Tashiro et al., 2013; Yamanaka et al., 2013; Zofall et al., 2012). More detailed studies are required to understand whether Red1 acts via the RNAi pathway or recruits Clr4 independently of RNAi factors, since previous studies showed a direct interaction between Red1 and Clr4 (Zofall et al., 2012). How is the RITS complex and Red1 recruited to the *pho1* locus? The role of Mmi1 in H3K9me2 at *pho1* and recruitment of factors responsible for heterochromatin formation will be assessed in Chapter 5 of this thesis.

Additionally, I also find that *prt* expression is essential for H3K9me2 at *pho1*. Interestingly, the de-repression of *pho1* mRNA levels upon loss of *prt* transcription is much higher compared to that when H3K9me2 is abolished. Taken together, these

data suggest that *prt* transcription represses *pho1* mRNA expression in the presence of Pi and uses heterochromatin formation to ensure properly timed induction in the absence of Pi. Results presented in Chapter 5, will address how *prt* mediates recruitment of the factors responsible for H3K9me2.

The role of the exosome at the *pho1* locus still remains a mystery. I find increased H3K9me2 upon loss of Rrp6 and simultaneously lower steady state mRNA levels and delayed mRNA induction kinetics. Previous studies also reported higher small RNA reads and H3K9me2 in *rrp6Δ* (Yamanaka et al., 2013). Whether Rrp6 competes with RNAi factors for binding to *pho1* RNA or does Rrp6 negatively regulates H3K9me2 levels by degrading siRNAs remains an open question. Since *prt* transcription is essential for H3K9me2 and loss of Rrp6 stabilizes *prt*, it is possible that the increase in *prt* levels results in higher amounts of siRNAs and thus an increase in H3K9me2. Further studies are required to dissect the role of exosome in heterochromatin at this locus.

Chapter 5

Role of Mmi1 and the exosome complex in regulating expression of *prt* RNA

Introduction:

Transcription termination and 3′ -end formation of Pol II transcribed RNAs are tightly linked mechanisms (Birse, Lee, Hansen, & Proudfoot, 1997; Buratowski, 2005). In the case of mRNA, 3′-end formation occurs co-transcriptionally and involves cleavage at the poly (A) site and polyadenylation of the nascent transcript synthesized by Pol II. Although the transcribed mRNA is cleaved and released from the DNA template, Pol II continues to transcribe past the poly (A) site. Transcription termination is important to prevent the Pol II from transcribing into neighbouring genes and replenish the Pol II pool in the cell for the next round of transcription. The 5'-3' exonuclease, Dhp1 (Xrn2 in mammals, Rat1 in *S. cerevisiae*) is recruited after the pre mRNA is cleaved, to the exposed 5' end of the nascent transcript. Rat1 degrades the Pol II-associated transcript after poly (A) site cleavage. Thus releasing Pol II from the DNA template via a torpedo like action leading to transcription termination (M Kim et al., 2004; West et al., 2004). So far, the regions of transcription termination are poorly defined and in higher eukaryotes are known to extend up to 2 kb beyond the poly (A) signal (Proudfoot, 1989). The poly (A) site for mRNA in higher eukaryotes is found to be AAUAAA. Mutation of the poly (A) site

abolishes cleavage and subsequent recruitment of Rat1. This results in Pol II transcribing beyond the normal termination site, producing read-through transcripts, which are degraded by the RNA surveillance machinery including the exosome complex.

In *S. pombe*, poly (A) signals have been defined for three genes, namely *ura4, nmt1* and *nmt2* (Birse et al., 1997; Hansen et al., 1998). A recent bioinformatics analysis of transcriptome data showed that the poly (A) sites functional in higher eukaryotes might also be used in *S. pombe* (Schlackow et al., 2013).

In *S. cerevisiae,* another mechanism of transcription termination has been described mainly for short ncRNAs called CUTs, the Nrd1-Nab3-Sen1 dependent termination (Arigo et al., 2006; Colin et al., 2011; Kim et al., 2006; Thiebaut et al., 2006). Nrd1 and Nab3 require (A/U) GUAAA and UCUUG sequences respectively for binding (Carroll et al. 2004; Porrua et al. 2012). Nrd1 and Nab3 binding sites aid recognition of short RNAs and together with Sen1, mediate transcription termination. Nrd1 has been shown to bind specifically to Ser5 phosphorylated polymerase which ensures early termination of Nrd1 dependent transcripts (Vasiljeva et al., 2008). Interestingly, Nrd1 binding also facilitates degradation by the exosome complex (Steinmetz et al., 2001; Thiebaut et al., 2006; Vasiljeva and Buratowski, 2006; Vasiljeva et al., 2008). Thus, via Nrd1 binding, transcription termination is coupled to decay by the exosome complex. The degradation of the CUTs via recruiting the exosome complex is essential to prevent spurious transcripts from accumulating in the cell.

Similarly, in *S. pombe,* a RNA-binding protein, Mmi1, plays a crucial role in selectively eliminating meiosis-specific transcripts in mitotic cells (Chen et al., 2011; Harigaya et al., 2006; St-André et al., 2010; Yamamoto, 2010). Meiotic transcripts targeted by Mmi1 are removed from cells via nuclear exosome- mediated degradation. The canonical poly (A) polymerase, Pla1, and the *S. pombe* homolog of poly (A)-binding protein nuclear 1 (PABPN1), Pab2, play a pivotal role in Mmi1-mediated elimination of meiotic transcripts (Chen et al., 2011; St-André et al., 2010; Yamanaka et al., 2010). The zinc finger protein, Red1, is also known to be involved in the Mmi1/DSR system, although its molecular function remains unknown (Sugiyama and Sugioka-Sugiyama, 2011).

Mmi1 also induces heterochromatin formation at a subset of its target genes (Hiriart et al., 2012; Tashiro et al., 2013; Zofall et al., 2012). Mmi1 was shown to interact with the RITS complex and loss of DSR elements or Mmi1 resulted in loss of H3K9me. Indeed, Red1 was found to interact directly with Clr4 and may mediate H3k9me2 independent of RITS via the NURS complex (Egan et al., 2014; Tashiro et al., 2013; Zofall et al., 2012).

Interestingly, I have found (as described in this chapter) that, in the case of *prt* ncRNA, the nuclear exosome is co-transcriptionally recruited for its transcription termination followed by degradation. Also, Mmi1 and Red1 mediate recognition by the exosome complex for proper termination and consecutive degradation of *prt*.

Indeed, My initial data suggests that Mmi1 and exosome also mediate transcription termination of meiotic RNAs, which are the other substrates of Mmi1. Also, similar to meiotic genes, Mmi1 and Red1 are also involved in heterochromatin formation at the *pho1* locus.

Furthermore, I show that the RITS complex and Clr4 which mediate heterochromatin formation via *prt* transcription may be recruited by Mmi1 and Red1 similar to the scenario described at meiotic loci.

Results:

6.1 Role of the exosome in transcription termination at the *pho1* locus

To test whether transcription is affected upon Rrp6 deletion, I analysed the Pol II levels in an Rrp6 deletion strain compared to WT (Fig. 5-1 A, B and C). Pol II occupancy was detected across *prt* in both WT and exosome mutants suggesting that *prt* is transcribed in the WT (Fig. 5-1A, B and C, lanes 1 and 2). Interestingly, the Pol II profile at *pho1* showed lower levels of Pol II across the *pho1* ORF in *rrp6*Δ cells compared to WT, which corresponds with the lower steady state mRNA levels and higher H3K9me2 levels observed in the *rrp6*Δ strain (Fig. 4-1A, B and Fig. 4-2A). Surprisingly, Pol II levels in *rrp6*Δ did not drop past the poly (A) site as seen in the WT (Fig. 5-1A, B and C, lanes 5 and 6) indicative of a transcription termination defect. Pol II levels were unchanged at *adh1* (Fig. 5-1D, E and F).

Although the Pol II levels across *prt* were lower in the *rrp6*Δ strain than the WT (Fig. 5-1A, B and C, lanes 1 and 2), the steady state *prt* RNA levels were much higher in *rrp6*Δ (Fig. 3-1B). This suggests that *prt* is transcribed in both WT and the exosome mutants, but is degraded by the exosome complex in the WT.

Thus, the exosome complex may be required for transcription termination at the *pho1* locus. It is possible that the termination defect along with the increased H3K9me2 (Fig. 4-1A and B) observed may also contribute to the decrease in mRNA levels seen in the *rrp6*Δ.

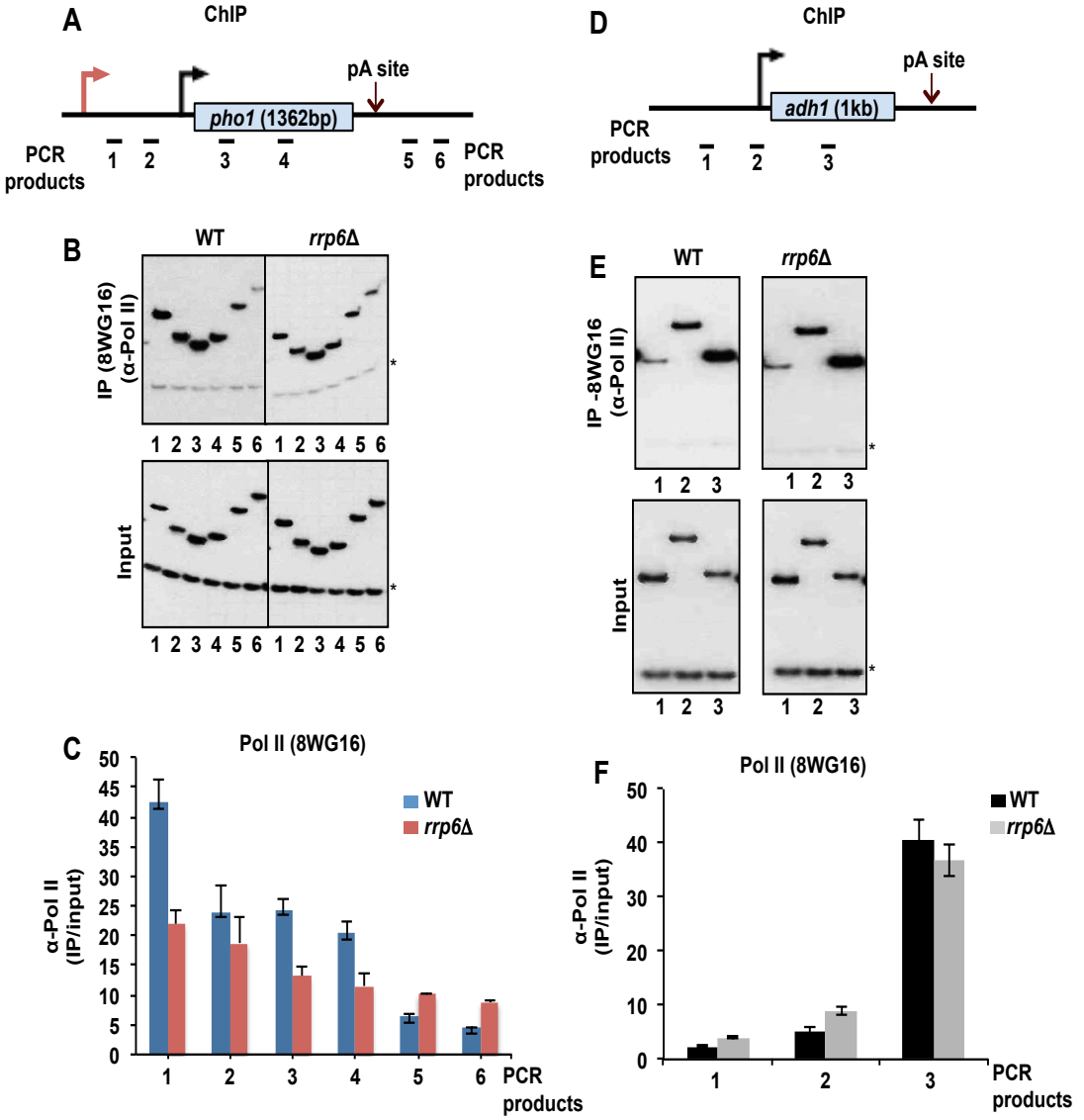


Figure 6—1. Rrp6 affects Pol II levels across *pho1* and is required for transcription termination.

(A, D) Schematic representations of the *pho1* and *adh1* gene respectively. Black bars show locations of the PCR products. (B, E) ChIP analysis of Pol II distribution across *pho1* and *adh1* locus respectively in strains grown in YES media. Labelled PCR products were resolved on polyacrylamide gels and detected by autoradiography. Star indicates position of the PCR product from a non-transcribed region. (C, F) Quantification from four independent experiments; the error bars indicate standard error.

6.2 Exosome is involved in transcription termination of *prt* RNA

Next, I wanted to determine which transcript (*prt* or *pho1*) relies on the exosome for transcription termination.

To test whether Rrp6 is specifically involved in termination of ncRNA, TRO (Transcription Run-On) analysis was performed in *rrp6*Δ in the presence and absence of Pi. As shown in Fig. 3-1A and B, in the presence of Pi both transcripts are produced while, in the absence of Pi, transcription of the *prt* RNA is abolished. This observation is also reflected in the TRO data (Fig 5-2 A and B, compare probe 1). Remarkably, transcription past the poly (A) site was found only in the presence of Pi and not in its absence (Fig 5-2 A and B, compare probes 5 and 6). Taken together this data suggests that Rrp6 is likely to regulate transcription termination of *prt* ncRNA.

To conclusively determine the requirement of Rrp6 of proper termination of *prt*, I used the strains with either the *prt* or the *pho1* mRNA promoter deleted in the WT or *rrp6*Δ background (Fig. 3-3A). Using RT-PCR to analyse levels of read-through transcripts, I found that upon deletion of *pho1* mRNA promoter the read-through levels in the *rrp6*Δ strain were not affected (Fig. 5-2C and D; compare lanes 4 and 5). In contrast, deletion of *prt* promoter resulted in loss of the read-through RNA (Fig. 5-2D, lane 6). These results clearly demonstrate that exosome complex plays a role in transcription termination of *prt* and not the *pho1* mRNA.

Thus, I find transcription termination coupled to decay for *prt* ncRNA in *S. pombe*, which is reminiscent of the Nrd1 dependent mechanism for CUTs in *S.*

cerevisiae. However, unlike CUTs, in the case of *prt,* the exosome complex itself is required for transcription termination. Importantly, this data indicates that the exosome may act co-transcriptionally. Previous genome-wide Dam-ID data showed recruitment of the exosome to chromatin (Woolcock et al., 2012). Dam-ID relies on the abundance of methylation sites for the Dam Methyltransferase in the genomic region of interest. In the case of the pho1 locus due to very few and widely spaced methylation sequences, this technique fails to provide sufficient resolution to identify the region of the *pho1* gene responsible for recruiting the exosome.

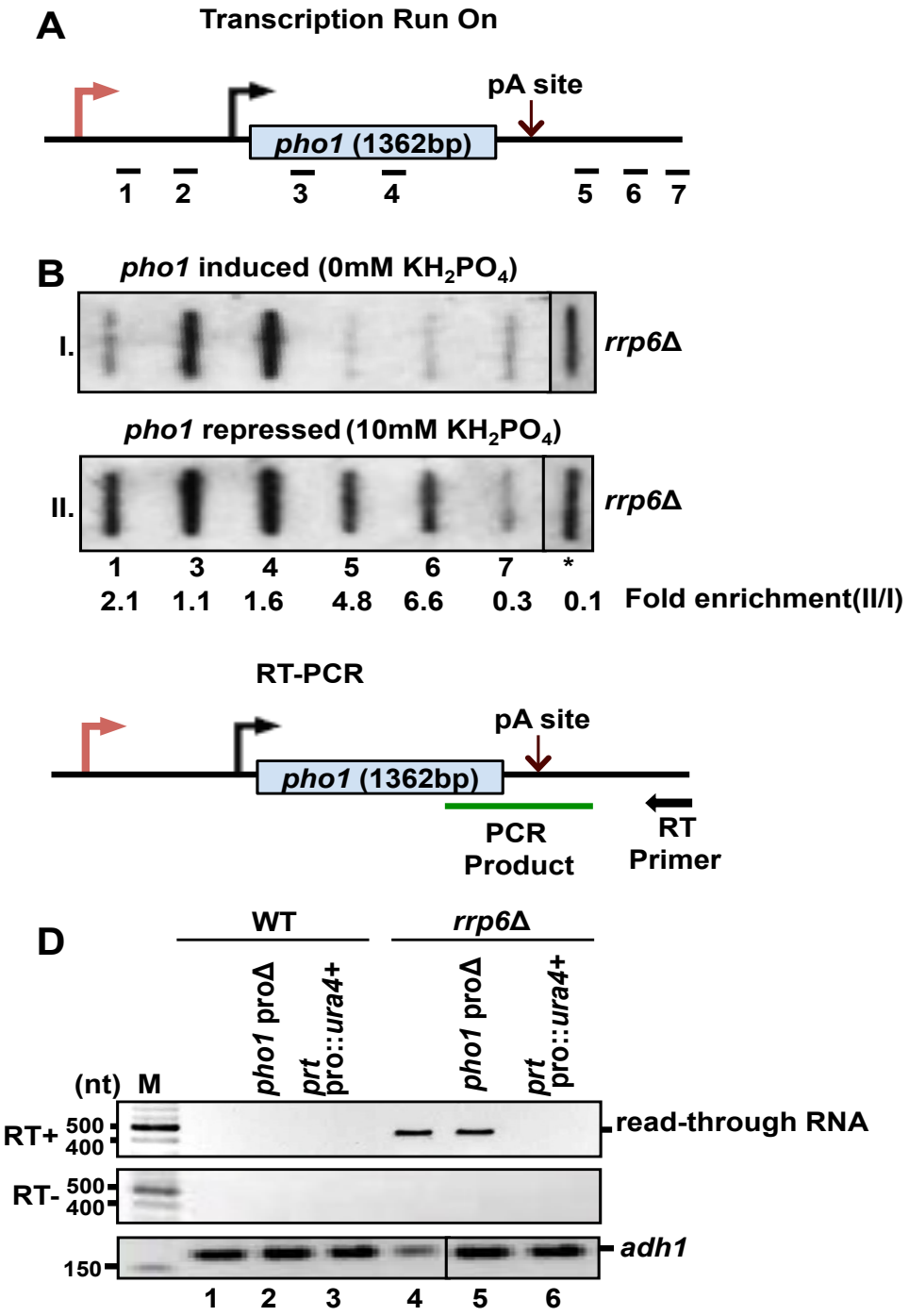


Figure 6—2. Exosome is involved in transcription termination of *prt* RNA.

(A) Schematic diagram showing position of probes (black bars) across *pho1* locus used for TRO assays. (B) TRO was performed on *rrp6* Δ cells grown in EMMG medium with 0 mM or 10 mM KH_2PO_4 for 4h. (C) Schematic diagram of the primer used for Reverse transcription and the PCR product amplified from the cDNA generated by RT reaction. (D). Indicated strains were grown in YES media and RNA was extracted. RT-PCR analysis of the read-through levels in indicated strains. Primer 1 downstream of the 3'UTR was used for reverse transcription and the cDNA generated was amplified to obtain the read-through levels. PCR products were run on a 1% Agarose gel.

6.3 Termination by the exosome complex is additive to Dhp1 (Rat1) function

To determine whether components of the canonical termination pathway (eg. Dhp1) also co-operate with the exosome for transcription termination, I analysed read-through transcription levels in single and double mutants of Dhp1 and Rrp6. To detect read through RNA, RT-PCR using a reverse primer after the poly (A) site to generate cDNA was performed, which was then amplified by PCR with the primers shown in the schematic (Fig. 5-3A). A temperature sensitive mutant of *dhp1* at non-permissive temperature, showed detectable levels of read-through transcription across *pho1* (Fig. 5-3B, lanes 3 and 4). Read-through transcripts from *pho1* were also detected in the *pfs2-11* mutant (Fig. 5-3F, lanes 7 and 8). However, no read-through RNA was detected in WT (Fig. 5-3B lane 2). Since RT-PCR detects steady state RNA I used a technique called Transcriptional Run On (TRO) analysis, which detects nascent Pol II transcription and hence is not affected by post-transcriptional degradation and steady-state abundance of transcripts. In the *dhp1* mutant (Fig. 5-3 C and D), TRO revealed that nascent RNA is transcribed beyond the poly (A) site, confirming the phenotype of the mutant.

To examine whether the exosome is involved in the Dhp1 dependent pathway of transcription termination I generated double mutants (*rrp6Δ dhp1*) and compared read-through levels with those in each of the single mutants and the WT. Interestingly, an additive accumulation of read-through RNAs was observed in the double mutant as compared to each of the single mutants after RNAseH digestion (Fig. 5-3 E and Fig. 5-3F, compare lanes 2 and 4 to 6). Although these results suggest that exosome may play a role in transcription termination independent of the Dhp1

pathway, further analysis is required to determine the contribution of each of these proteins to termination.

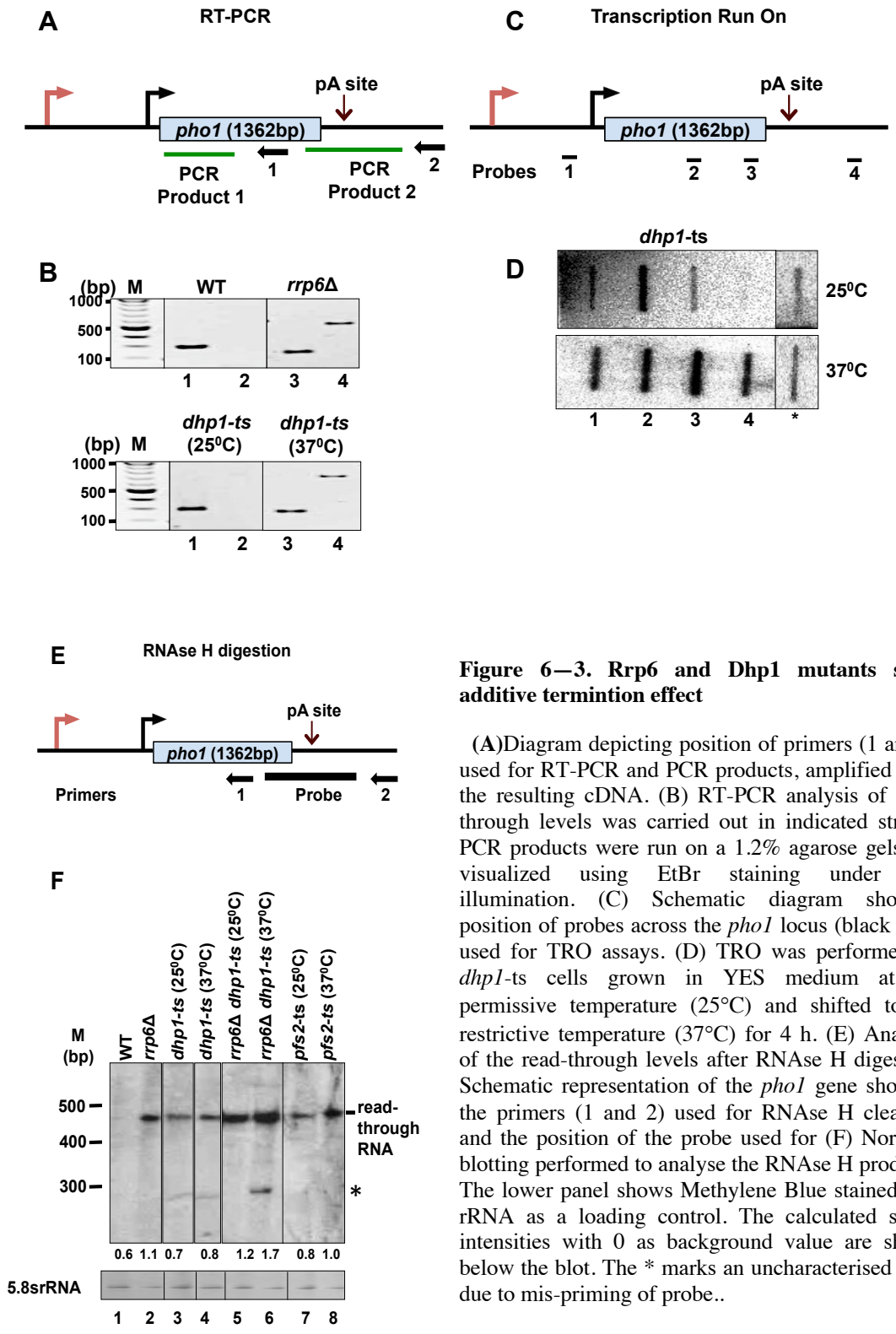


Figure 6—3. Rrp6 and Dhp1 mutants show additive termintion effect

(A) Diagram depicting position of primers (1 and 2) used for RT-PCR and PCR products, amplified from the resulting cDNA. (B) RT-PCR analysis of read-through levels was carried out in indicated strains. PCR products were run on a 1.2% agarose gels and visualized using EtBr staining under UV illumination. (C) Schematic diagram showing position of probes across the *pho1* locus (black bars) used for TRO assays. (D) TRO was performed on *dhp1*-ts cells grown in YES medium at the permissive temperature (25°C) and shifted to the restrictive temperature (37°C) for 4 h. (E) Analysis of the read-through levels after RNAse H digestion. Schematic representation of the *pho1* gene showing the primers (1 and 2) used for RNAse H cleavage and the position of the probe used for (F) Northern blotting performed to analyse the RNAse H products. The lower panel shows Methylene Blue stained 5.8s rRNA as a loading control. The calculated signal intensities with 0 as background value are shown below the blot. The * marks an uncharacterised band due to mis-priming of probe..

6.4 Transcription termination defect is not linked to heterochromatin at the *pho1* locus.

To address whether the termination defect was linked to H3K9me2 at the *pho1* locus found in *rrp6*Δ strain, I used two approaches. First, levels of H3k9me2 in mutants defective in 3'end formation (*dhp1*, *pfs2*-11) were analysed. No change in H3K9me2 levels was observed in these mutants compared to the WT strain (Fig. 5-4 A and B). Second, to address whether H3K9me2 contributes to transcription termination, I examined the read through levels by RT-PCR in *rrp6*Δ compared to double mutants of Rrp6 and the RNAi components essential for H3K9me2 (*ago1*Δ *rrp6*Δ, *clr4*Δ *rrp6*Δ, *dcr1*Δ *rrp6*Δ) (Fig. 5-4C and D). I found no change in the read-through levels in the double mutants compared to each of the single mutant.

Thus, I find no obvious connection between transcriptional termination and heterochromatin formation at the *pho1* locus.

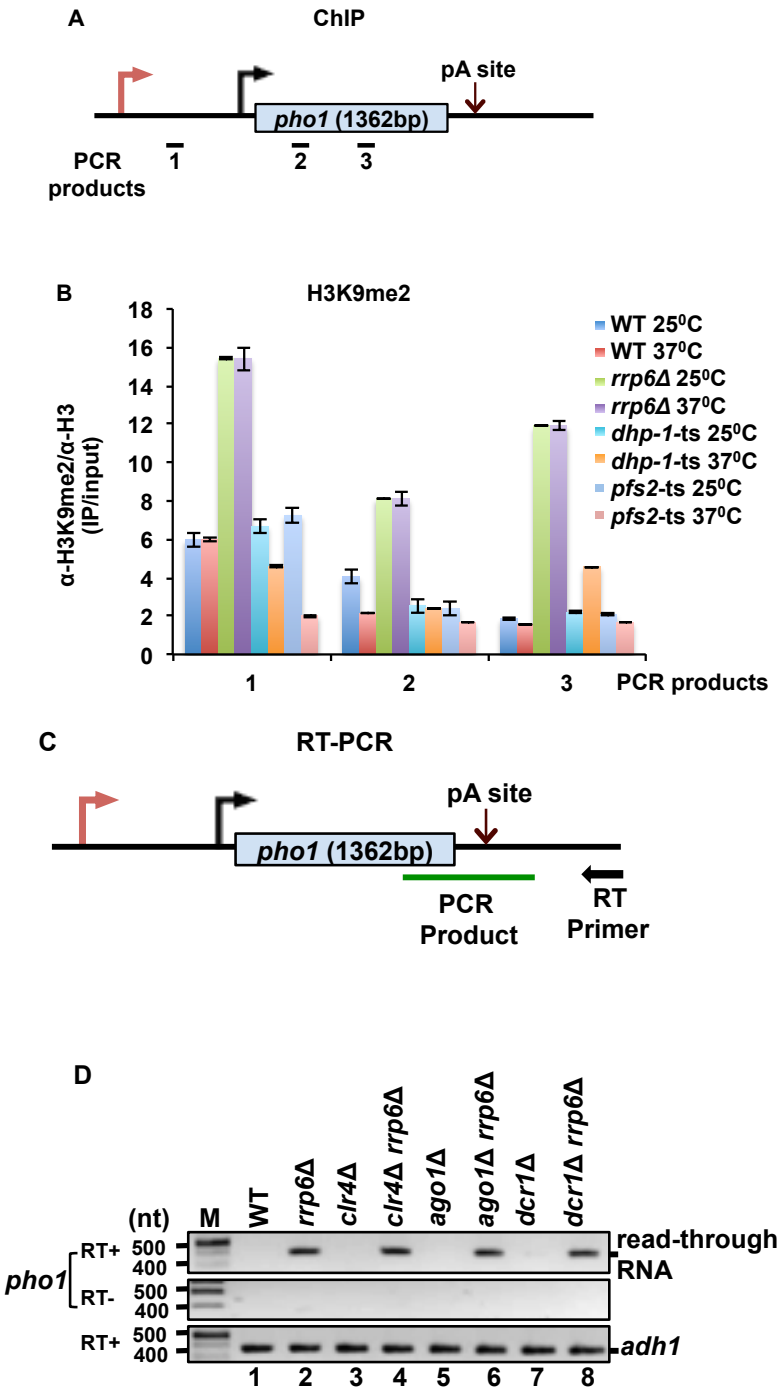


Figure 6—4. Transcription termination defect is not linked to H3K9me2 at the *pho1* locus.

(A) Schematic diagram of the *pho1* gene. Black bars show locations of the PCR products generated in the ChIP experiments below. Strains indicated were grown in YES medium. (B) ChIP for H3K9me2 (C) Schematic diagram of the *pho1* gene depicting primers used for RT and the PCR product amplified from the cDNA generated for detecting read-through products. (D) RT-PCR analysis of read-through levels in indicated strains. PCR products were run on a 1% agarose gel.

6.5 RNA binding protein, Mmi1, recruits the exosome to *prt* RNA

Next, I decided to address how is the exosome specifically targeted to the ncRNA already during transcription to mediate transcription termination and the subsequent decay. Substrate specificity of the exosome has been a long-standing question in this field of research and so far several RNA binding proteins (Nrd1, Nab3 and TRAMP complex is *S. cerevisiae* and Pab2, Red1 and Mmi1 in *S. pombe*) have been shown to recruit the exosome to its substrates. I speculated that the region of *prt* which does not overlap with *pho1* mRNA could harbour the signals to recruit the exosome. I searched the primary sequence for previously identified sequence elements known to recruit the exosome via RNA binding protein, Mmi1. Indeed, I found a cluster of three core hexanucleotide motif U (U/C/G) AAC (Yamashita et al., 2012) known as DSR elements (Determinant of Selective Removal) which recruits Mmi1 and the exosome specifically to meiotic transcripts. These DSR motifs were present in the 1.2 kb region of *prt* which does not overlap with *pho1* mRNA (Fig. 4A). This was surprising, since the *pho1* locus has not been shown to be involved in the meiotic program in *S. pombe*. Although, three additional motifs were found in the 3'UTR region that is shared by both RNAs these were weaker motifs than the stronger core DSR elements present only in *prt*.

My next step, was to determine whether Mmi1 binds to *prt* and whether the DSR elements are functional in recruiting Mmi1 and thereby Rrp6 to *prt* RNA. To do so, Ms. Sina Wittmann (D.Phil student, Vasilieva Lab) performed Mmi1 pull-down followed by RNA extraction from a strain expressing HTP (6X His-TEV-Protein A) -tagged Mmi1 and a non-tagged control (Fig. 5-5A and B). RT-PCR analysis was then used to detect RNAs associated with Mmi1 (Fig. 5-5C). The

meiotic RNA, *sme2* was enriched in the pull-down from the Mmi1-TAP strain, but not in the control sample (Fig. 5-5C, compare lanes 1 and 2). To analyse whether *prt* was bound to Mmi1, we used primers specifically recognizing the *prt* RNA and not *pho1* mRNA and indeed we found *prt* enriched in the Mmi1 pull-down compared to the control sample (Fig. 5-5C, compare lanes 1 and 2).

Since Mmi1 is recruited via DSR elements, I generated a strain deleted for the DSR elements from *prt* and we found loss of *prt* binding to Mmi1 (Fig. 5-5C, compare lanes 1, 2 and 3). The binding of *sme2* was not affected upon deletion of DSR elements from *prt*. These results clearly demonstrate that Mmi1 binds to *prt* directly via the DSR elements. No signal was detected in –RT control (Fig. 5-5C, lanes 4 to 6). The *rps15-2* a highly expressed gene with no obvious DSR elements was found to bind non-specifically to the beads independently of Mmi1 (Fig. 5-5C).

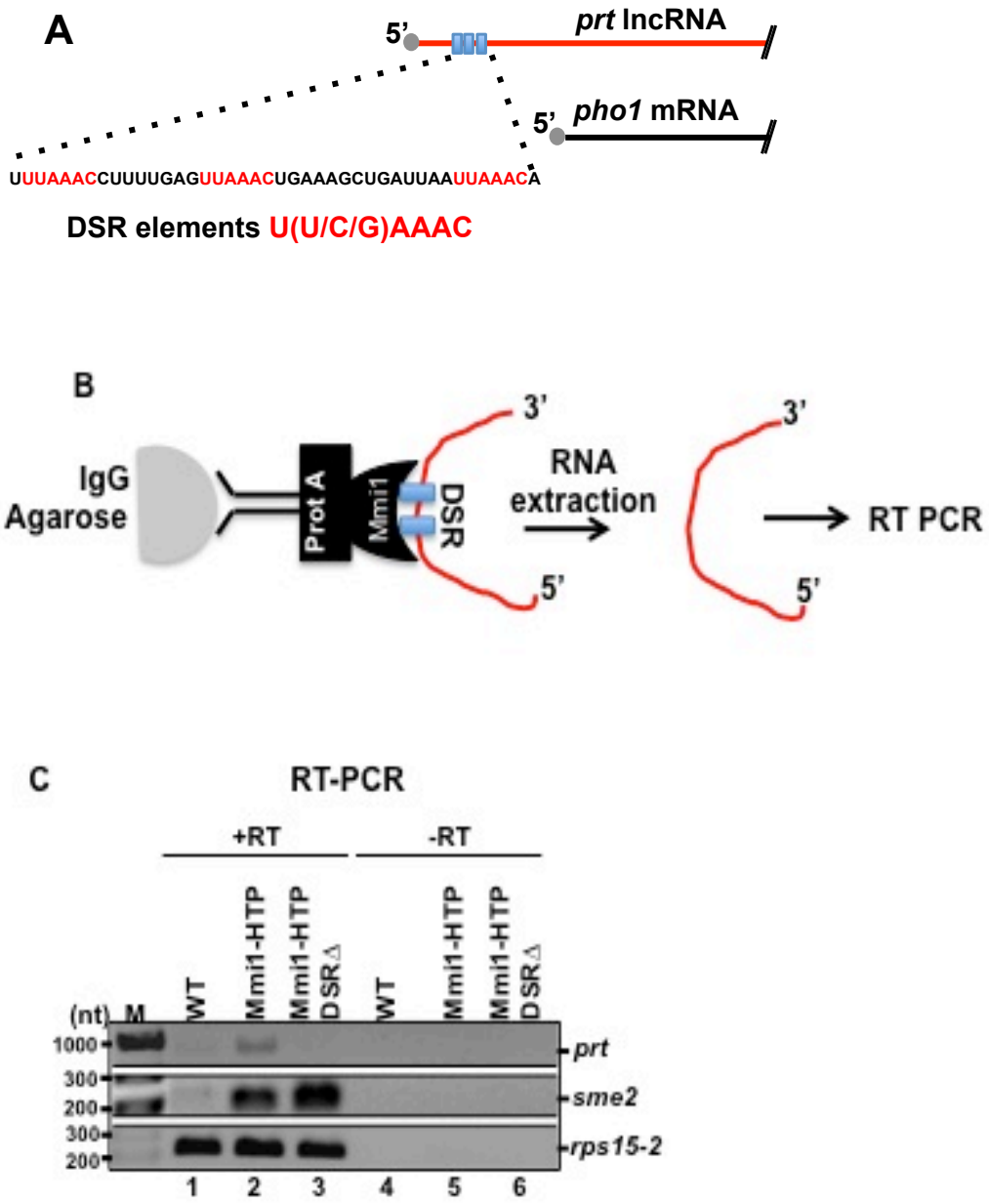


Figure 6—5. Mmi1 binds to *prt* lncRNA

(A) Schematic diagrams highlighting three putative DSRs (in red) in *prt*. (B) Schematic depicting the protocol for detecting RNAs bound to Mmi1. (C) Mmi1 binds to the *prt* RNA. The RNA co-precipitated with Mmi1 was analysed using RT-PCR. Specific primers for *prt* lncRNA and *sme2* (positive control) were used and the PCR products were visualized on a 1% agarose gel.

6.6 Mmi1 and Red1 are involved in mediating transcription termination coupled decay of *prt* via Rrp6.

Next, I wanted to determine what roles of the exosome at the *prt* RNA are mediated via Mmi1- transcription termination, ncRNA degradation, heterochromatin formation or all of the above.

My data shows that the absence of Rrp6 results in defective transcription termination of *prt* (Fig. 5-1). To determine whether loss of Mmi1 also affects transcription termination at the *pho1* locus, I performed RT-PCR analysis.

Indeed, I found accumulation of read-through RNA in an *mmi1*Δ strain compared to its parental strain (Fig. 5-6 A and B, compare lanes 2 and 4). To determine whether Pol II occupancy is also affected in Mmi1 mutants, I performed Pol II ChIP across *pho1* in this strain. Similar to that in *rrp6*Δ, I found that Pol II continues to transcribe past the poly (A) site in *mmi1*Δ (Fig. 5-6C and D). Interestingly, increased Pol II levels past the poly (A) site were found also in a DSR Δ strain, reinforcing the role of Mmi1 in recruiting Rrp6 to mediate transcription termination at the *pho1* locus (Fig. 5-6E and F).

To examine whether Mmi1 binding also mediates the degradation of *prt* via Rrp6. I analysed steady state RNA levels in *mmi1*Δ cells by Northern blot. Interestingly, I found *prt* RNA stabilized in *mmi1*Δ compared to the parental strain. However, I noticed that the levels of *prt* were not comparable to that in *rrp6*Δ (Fig. 5-6G, lanes 2 and 4). This suggested that additional proteins might also be involved in

mediating degradation of *prt* via Rrp6. Another protein that acts in conjunction with Mmi1 is the RNA binding protein Red1 (Sugiyama and Sugioka-Sugiyama, 2011). Indeed, *prt* is stabilized in *red1*Δ at comparable to that in *rrp6*Δ (Fig. 4-3F). However, in contrast to Rrp6 and Mmi1, Red1 is not required for transcription termination at the *pho1* locus (Fig. 5-6H compare lanes 2,3 and 4).

Thus, I find that the transcription termination of *prt* RNA and its subsequent degradation requires the exosome complex, which is recruited via Mmi1 and Red1. Further studies would be required to assess the exact contribution of each of these proteins in regulating expression of RNAs transcribed from the *pho1* locus.

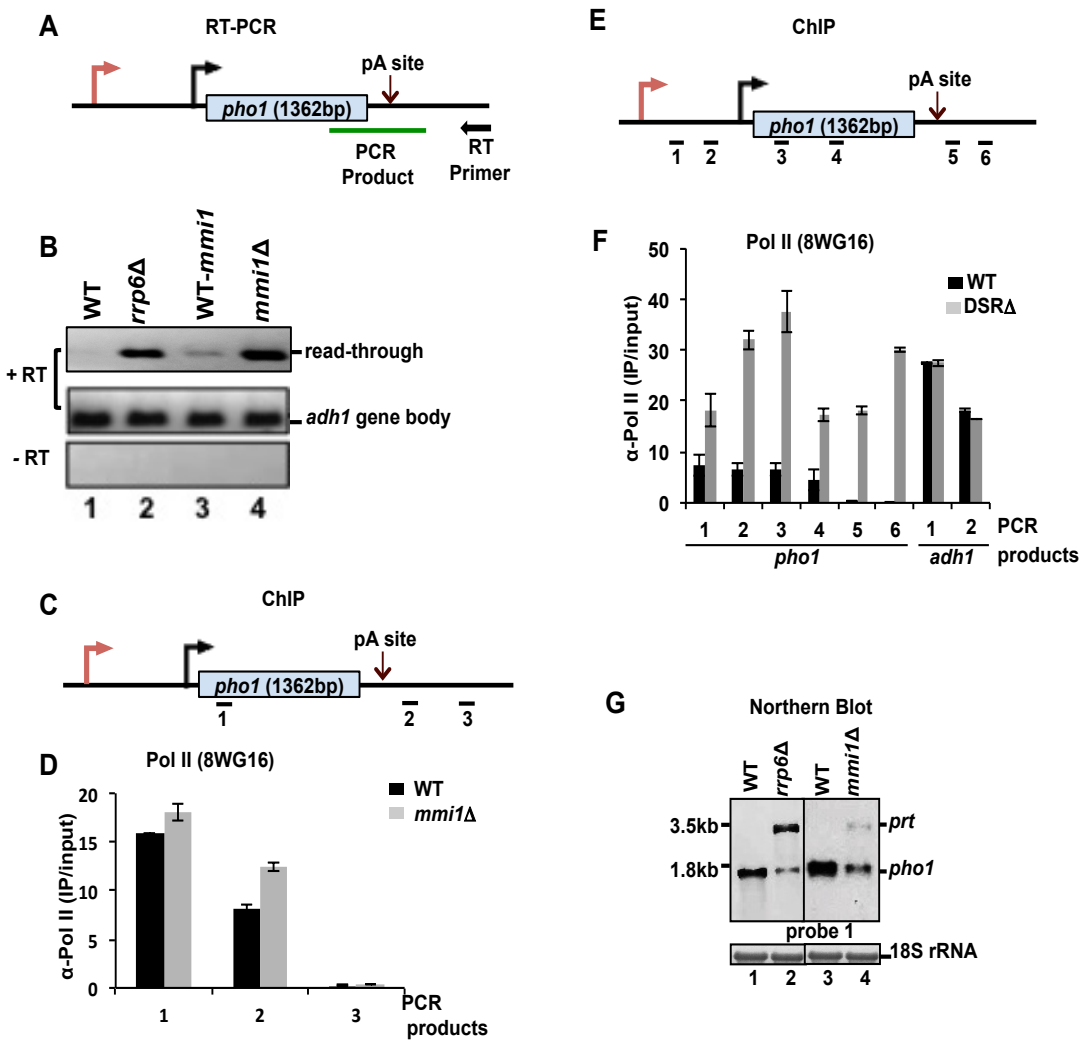


Figure 6—6. Mmi1 but not Red1 contributes to transcription termination and *pho1* locus.

(A) Schematic diagram depicting primers used for RT-PCR. (B) RT-PCR in mutants of Mmi1 and Rrp6, to detect read-through transcripts. (C) Schematic of the *pho1* loci analyzed by ChIP. Black bars indicate the location of the products amplified by qPCR. All strains were grown in YES media. (D) ChIP analysis for Pol II enrichment was carried out in WT and *mmi1* Δ strains. (E) Schematic of the *pho1* loci analyzed by ChIP. Black bars indicate the location of the products amplified by qPCR. All strains were grown in YES media. (F) ChIP analysis for Pol II enrichment was carried out in WT and DSR Δ strains. (G) Northern blot analysis of *pho1* RNA in *rrp6* Δ, *mmi1* Δ and parental strains grown in YES media. (H) Analysis of RNA levels by RT-PCR in mutants of Mmi1 and Rrp6, to detect read-through transcripts.

6.7 Mmi1 and Red1 are also required for *prt* mediated heterochromatin formation

Recent studies showed that, Mmi1 along with Red1 not only regulates mRNA levels by degrading meiotic transcripts during mitosis, but it also mediates facultative heterochromatin formation via recruitment of the RITS complex and Clr4 at meiotic loci (Hiriart et al., 2012; Tashiro et al., 2013; Yamanaka et al., 2013; Zofall et al., 2012). I wondered whether loss of Mmi1 would cause an increase in H3K9me2 similar to that in *rrp6*Δ (Fig. 4-1A and B) or a loss thereof as seen previously at meiotic genes. Surprisingly, I found a reduction in H3K9me2 marks in *mmi1*Δ (Fig. 5-7A and B). This is in contrast to the increased levels of H3K9me2 in *rrp6*Δ mutant (Fig. 4-1B). This suggests that Mmi1 is required for H3K9me2 at *pho1* and might explain increased Pol II levels seen in *DSR*Δ strain and the *mmi1*Δ strains (Fig. 5-6C-F). Furthermore, consistent with the role of Mmi1 in RITS recruitment, reduction in Ago1 levels across *pho1* locus was observed in the absence of Mmi1 (Fig. 5-7C and D).

I conclude that Mmi1 targeting is not restricted to meiotic transcripts as previously suggested. Similar to that shown for other genes (Yamanaka et al., 2013), at the *pho1* locus also, Mmi1 and Red1 are both essential for H3K9me2 at this locus. In contrast, loss of Rrp6 actually results in increased H3K9me2; the reason for this is unknown.

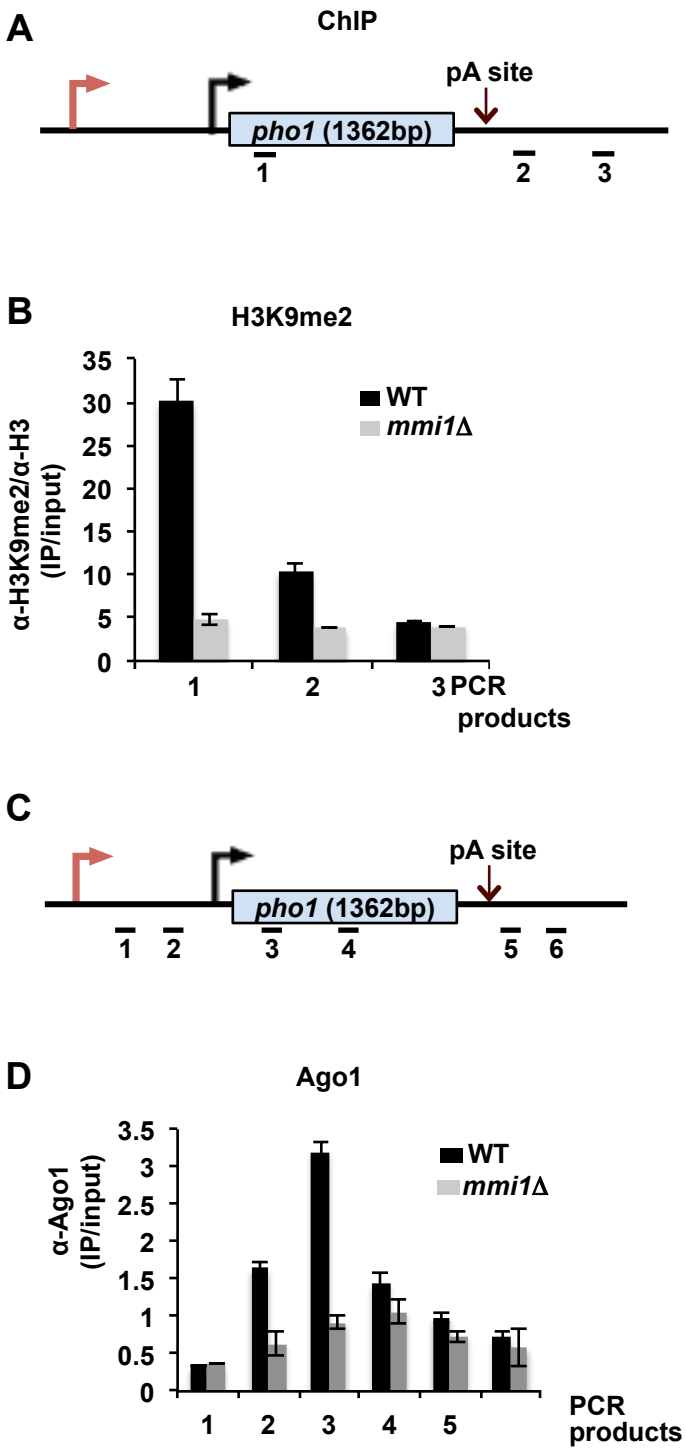


Figure 6—7. Mmi1 contributes to silencing *pho1* locus.

(A) Schematic of the *pho1* loci analyzed by ChIP. Black bars indicate the location of the products amplified by qPCR. All strains were grown in YES media. (B) ChIP analysis for H3K9me2 in WT and *mmi1* Δ strains. (C) (A) Schematic of the *pho1* loci analysed by ChIP. Black bars indicate the location of the products amplified by qPCR. All strains were grown in YES media. (D) ChIP analysis for Ago1 recruitment in WT and *mmi1* Δ strains.

6.8 *prt* RNA is likely to act in *cis* to repress *pho1*

ncRNAs have been shown to act in *cis* and in *trans* to regulate gene expression. I next asked whether *prt* can act in *trans* to mediate repression of *pho1*. To address this, Ms. Wittmann expressed *prt* with and without DSR elements from a plasmid. To ensure that only *prt* RNA is produced from the plasmid, the *pho1* promoter was deleted (Fig. 5-8A). These plasmids were then transformed into strains which had the endogenous *prt* promoter deleted to ensure no interference from transcription of *prt* in *cis*. Northern blot was performed to analyse RNA levels in these strains compared to the WT (Fig. 5-8B). As expected, the overall levels of *pho1* mRNA increased upon deletion of endogenous *prt* promoter. Indeed, we found that expressing *prt* in *trans* could not mimic the effect of *prt* expressed in *cis* since there was no decrease in *pho1* mRNA levels in these strains as seen in the WT strain. Interestingly, we also found that plasmid driven *prt* RNA can be stabilized when DSR elements are not present. Thus, reinforcing the role of DSR in exosome mediated decay of *prt*.

Therefore, unlike the roles of ncRNAs in heterochromatin formation at centromeric loci where ncRNA can act in *cis* and in *trans*, we find that *prt* can mediate repression of *pho1* mRNA only when transcribed in *cis*.

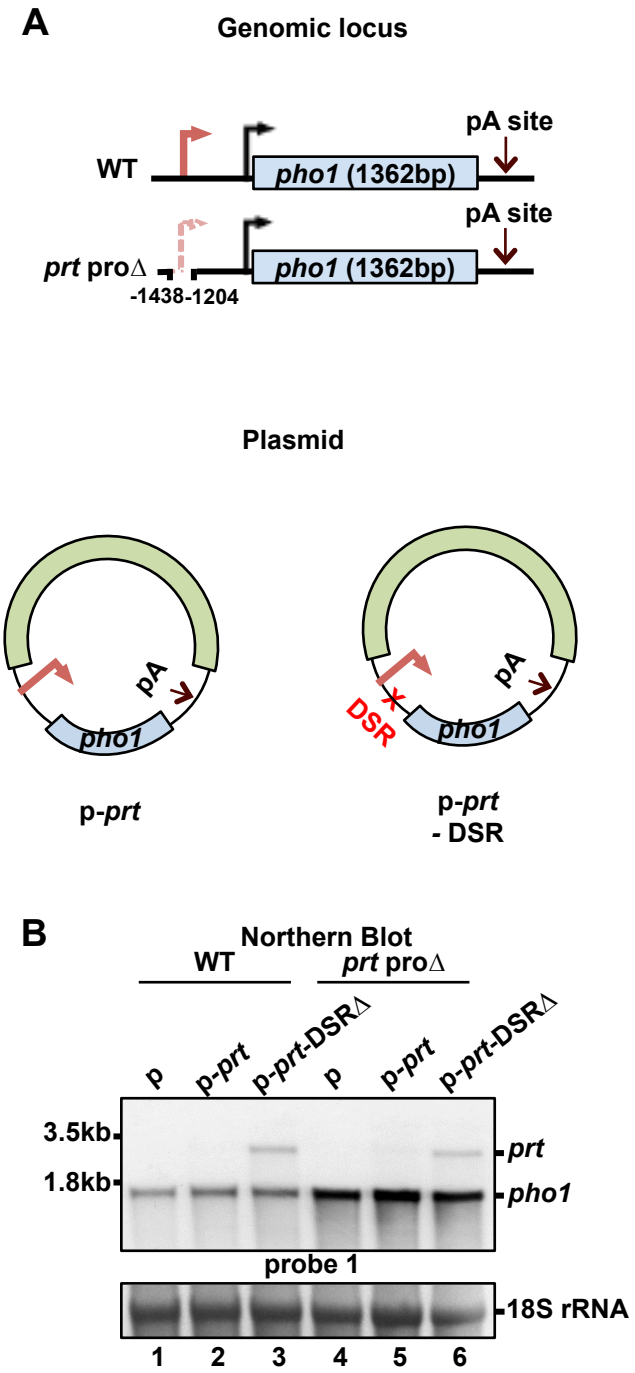


Figure 6—8. *prt* ncRNA acts in *cis* to mediate repression of *pho1* mRNA.

(A) Schematic diagram showing the strains and the plasmids used to express the *prt* RNA in *trans*. The parent strain (WT) and a strain with *prt1* promoter deletion (*prt* Δ) were transformed with the empty parent plasmid (p), a plasmid expressing the *prt* RNA after deletion of the mRNA promoter (p-*prt*) or the *prt* RNA after deletion of both the mRNA promoter and the DSR elements (p-*prt*-DSR Δ) each under control of the endogenous promoter. (B) Analysis of *pho1* levels in strains expressing *prt* RNA +/- DSR elements by Northern blot. Total RNA was extracted from cells grown in EMMG –leu

6.9 Mmi1 and exosome are also involved in termination of meiotic RNAs bound by Mmi1

Next I wanted to check whether Mmi1 and Rrp6 also function in regulating transcription termination of meiotic genes similar to what I have described for *prt*. Interestingly, Pol II ChIP analysis by Dr. Cornelia Kilchert (Post- doctoral researcher, Vasilieva lab) showed an increase in Pol II occupancy downstream of two meiotic genes *sme2* and *mug180* genes in *rrp6*Δ, *mmi1*Δ mutants compared to their parental strains (Fig. 5-9A, B and C). This is consistent with defective termination seen at *pho1* gene (Fig. 5-1).

We reasoned that, if recruitment of Rrp6 for transcription termination of *prt* and meiotic genes is mainly via Mmi1, during meiosis, when Mmi1 is sequestered away from meiotic genes, a transcription termination defect might occur. To test this hypothesis, Dr. Kilchert analysed Pol II occupancy across the candidate genes during a meiotic time course. The increase in RNA levels of *mug180* after 2 and 4h of induction confirmed the induction of meiosis (Fig. 5-9D and E). Indeed, after 6 and 8h of induction increased levels of Pol II occupancy was observed down-steam of *mug180* gene (Fig. 5-9 D and F).

These results suggest that sequestering Mmi1 during meiosis might lead to transcription termination of genes targeted by Mmi1, since Rrp6 is no longer recruited to mediate transcription termination of these genes. Although the biological significance of defective transcription termination in meiosis is unclear, this might be a mechanism used by cells to fine-tune levels of RNAs during meiosis.

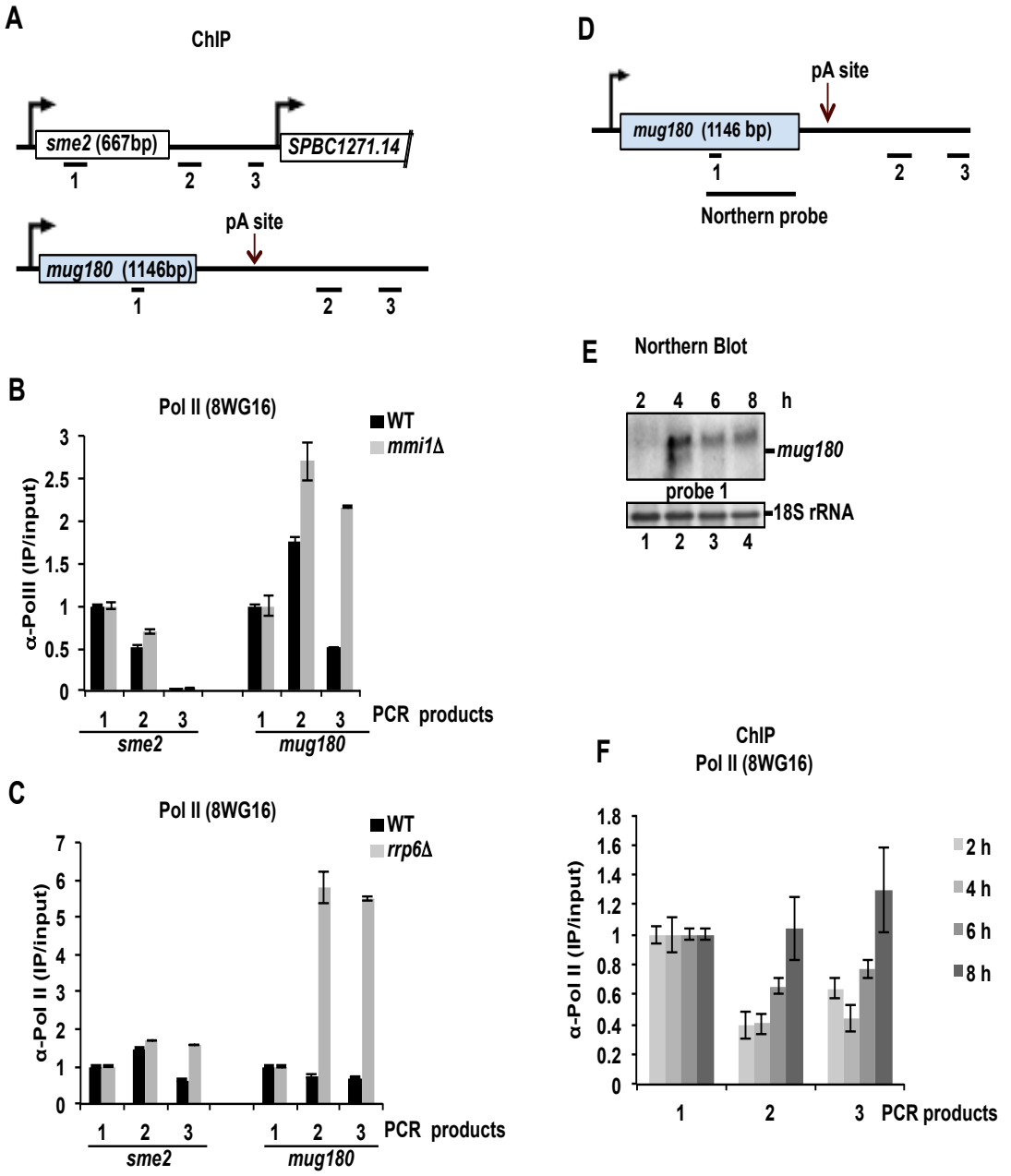


Figure 6—9. Loss of Mmi1 and Rrp6 results in a termination defect at meiotic genes

(A) Schematic diagram of the *mug180* and *sme2* gene. Black bars show locations of the PCR products generated in the ChIP experiments and probes used for Northern blot. (B) ChIP to detect Pol II levels in WT and *mmi1* Δ strains grown in YES medium. (C) ChIP to detect Pol II levels in WT and *rrp6* Δ strains grown in YES medium. (D) Schematic diagram depicting position of the probe used for Northen blot and PCR products for ChIP. (E) A meiotic time course was performed after induction of meiosis in G1-arrested *pat1-114* diploid cells by temperature shift. Samples were removed for chromatin preparation and RNA extraction 2, 4, 6 and 8 h after induction. Northern blot for *mug180* RNA during the meiotic time course. *mug180* is induced after 4h. (F) ChIP analysis of Pol II occupancy over the *mug180* locus during the meiotic time course. IP signals were normalized to the signal obtained over the ORF.

Discussion:

Although *prt* and *pho1* mRNA share the same 3' end and both these transcripts are polyadenylated, the exosome affects termination of only *prt* and not the *pho1* mRNA. I find that specificity is achieved via RNA binding protein Mmi1 that specifically recognizes the *prt* RNA. Mmi1 was previously found to bind meiotic RNAs transcribed during mitosis via the presence of DSR elements (Chen et al., 2011; Yamashita et al., 2012). Indeed, I find that DSR elements are present in *prt* and we show Mmi1 binds to *prt* via the DSR elements. Mmi1 also regulates transcription termination and degradation of *prt*. This implies coupling between 3' end formation and degradation of unstable ncRNAs. This is reminiscent of transcription termination coupled decay shown previously for CUTs in *S. cerevisiae*. However, unlike CUTs, the exosome complex itself is required for transcription termination of *prt*. Interestingly, we also find the other targets of Mmi1 protein (meiotic genes) rely on Mmi1 and exosome for transcription termination of the nascent transcripts. This suggests a more general role of exosome and Mmi1 in transcription termination.

Previous studies on Mmi1 also demonstrated its role in recruiting Clr4 and RITS complex for heterochromatin formation at meiotic genes during mitosis (Hiriart et al., 2012; Tashiro et al., 2013; Zofall et al., 2012). This process also involves Red1. Mmi1 and Red1 are essential for H3K9me2 at the *pho1* locus. Interestingly, as shown previously (Yamanaka et al., 2013), I also detected increased H3K9me2 across *pho1* in Rrp6 mutants. In *S. pombe*, transcription termination defect in genes positioned in a convergent orientation can also give rise to dsRNA and lead to heterochromatin formation. However, in the case of *prt* RNA, I found that the transcription termination

defects and H3K9me2 formed are not linked. These results demonstrate a complex mode of regulation of *pho1* gene expression. Multiple protein complexes provide many layers of regulation. The need for the Mmi1 machinery for regulation clearly suggests that Pi levels may be important for proper meiosis. Also, the need for exosome for transcription termination coupled to decay might imply that *prt* RNA needs to be efficiently degraded. It would be interesting to study overexpression of the ncRNA on the cell growth. Further studies aimed at understanding localization pattern of this ncRNA may give a better understanding of its function. I also show that Mmi1 and Red1 have different roles to play at the *pho1* locus. Hence this gene may serve as a perfect model system to separately analyse roles of the RNA binding proteins Mmi1 and Red1 in RNA degradation, transcription termination and heterochromatin formation.

Chapter 6

GENERAL DISCUSSION

The process of transcription controls gene expression. During differentiation and development as well as in response to stress, cells need to undergo widespread changes in gene expression to adapt and propagate (Kim et al. 2012; Weiner et al. 2012). Interestingly, these genome wide transcriptome changes include differential expression of a number of ncRNAs (Wilhelm et al., 2008). Indeed, ncRNAs may also mediate onset of gene expression changes (Yang et al., 2011). It is becoming apparent that ncRNAs might represent a new set of regulators that have so far been unknown (Berretta and Morillon, 2009). The functions of ncRNAs include modulating chromatin, chromosomes and nuclear architecture, mediating differential recruitment or sequestering of activating or repressing protein complexes etc (Kaikkonen et al., 2011). Alternately, ncRNA can result in chromatin remodelling or histone modifications. With more in-depth studies, we are constantly faced with new ways in which ncRNAs can regulate gene expression, challenging the extent of our current knowledge of RNA (Cech and Steitz, 2014). Considerable focus therefore lies in understanding the role and scope of gene regulation by ncRNA species. So far, studies indicate that no two lncRNAs function in the same manner, which makes in-depth study of individual ncRNAs exciting and necessary.

NcRNAs are most often targeted for rapid degradation (Belostotsky, 2009; Marquardt et al., 2011). Thus, compromising RNA decay pathways is a good strategy to detect unstable transcripts. A number of nc transcripts are produced in an overlapping manner to a coding gene or may arise from intergenic regions and also by alternative splicing of the gene. Inspite of the advances in techniques to map the transcriptome, we haven't still identified all the nc transcripts in the cell. Besides, ncRNAs do not have common distinguishing features making it difficult to classify transcripts (Dinger et al., 2008). In depth analysis of individual loci still remains crucial for identifying transcripts that might not have been identified in genome-wide screens.

Interestingly, upon compromising the exosome complex in *S. pombe*, I identified a novel lncRNA transcribed from the *pho1* locus, which I term ***prt*** (*pho1* repressing transcript). I mapped the *prt* transcript and studied in detail its transcription, regulation and function at the *pho1* locus (Shah et al., 2014).

7.1 *prt* lncRNA transcription overlaps the *pho1* gene.

Pi homeostasis is key to normal cell function and development. A number of enzymatic activities and signalling cascades are regulated by the presence of optimum intracellular Pi concentrations. A dedicated pathway called phosphate-responsive signalling (PHO) pathway, is found to be present in most organisms from yeast to higher eukaryotes that is involved in scavenging extracellular Pi when the internal reserves of Pi in the cell are constrained (Henry et al., 2011; Wykoff and O'Shea, 2001; Yoshida et al., 1989).

The basic components of the *S. pombe,* PHO pathway is the acid phosphatase, Pho1 (SPBP4G3.02, orthologous to Sc*PHO5*) and the Pi transporters (products of the genes SPBC8E4.01c (orthologous to ScPHO84), and SPBC1271.09) (high affinity transporters to enforce active uptake of Pi), transcription factor Pho7, and repressor, Csk1 (Carter-O'Connell et al., 2012; Henry et al., 2011). Interestingly, unlike *S.cerevisiae*, the Pho7 transcription factor binds to the *pho1* promoter even in the presence of Pi. Thus, there is a basal level of *pho1* transcription at all times. It is imperative that repressive mechanisms are in place to inhibit full induction of the *pho1* gene when Pi levels in the cell are sufficient. One of the repressors of *pho1* is the kinase; Csk1. How Csk1 represses pho1 expression is still unknown. It is believed that Csk1 interacts at a site between -1kb and -2kb upstream of the *pho1* promoter. Indeed, deletion of this region, called the Pi sensing element results in loss of Pi sensitivity and thus constitutive expression of *pho1*. Moreover, the levels of Pho1 are dramatically increased upon loss of the Pi sensing element. Thus, indicating the presence of a repressive element in this region (Carter-O'Connell et al., 2012).

7.1.1 Three transcripts were mapped at the *pho1* locus in exosome mutants

Unexpectedly, I find that the Pi sensing element transcribes a long RNA (*prt*). Transcription of this lncRNA from the region of the Pi sensing element indicated that this RNA maybe the unknown feature of the Pi-sensing element. Interestingly, the *prt* completely overlaps with the *pho1* mRNA and is cleaved and polyadenylated at the same poly (A) site as the mRNA. Importantly, in the presence of *prt* transcript, the levels of the mRNA are reduced (Fig. 6-1). Therefore, at least at the RNA levels I

found an inverse correlation between expressions of the two transcripts. But the presence of the long transcript only in the exosome mutant raised the question whether it is transcribed in the WT as well.

7.1.2 Long RNA from the *pho1* locus is a substrate for exosome-mediated decay

Two scenarios can explain the presence of the long RNA only in the exosome mutant. Either *prt* is transcribed upon compromising the exosome complex or it is degraded post-transcriptionally by the exosome complex and hence visualized only in mutants (similar to CUTs (Thiebaut et al., 2006). Indeed, *prt* is a substrate for exosome mediated nuclear decay (Fig. 6-1). However, further analysis is required to assess the localization and involvement of other decay pathways in regulating the long RNA.

The nuclear exosome is responsible for quality control of aberrant mRNAs or ncRNAs by targeting them for degradation (Schmid and Jensen 2012; Schmidt and Butler 2013). Since both the transcripts overlap and have the same 3' end, this raises the questions- why is only the long RNA recognized by the exosome? What specific features contribute to exosome recruitment? Although both transcripts encompass the ORF, is there a difference between the long and short transcript?

7.1.3 Long RNA does not contribute to Pho1 protein activity

Both the transcripts mapped at the *pho1* locus contain the ORF. However, *prt* does not contribute to Pho1 protein activity as against the short transcript. Also, I

found many stop codons in the long RNA, which would inhibit synthesis of peptides. Although no obvious ORFs are found in the lncRNA it is possible that, small peptides may be synthesized (for example upstream ORF's (Barbosa et al., 2013)). Detailed ribosome profiling analysis would be required to determine whether this transcript could encode for a protein.

7.2 LncRNA plays a repressive role at the *pho1* locus and mediates Pi responsiveness of the gene

Interestingly, nc transcription occurs only in the presence of Pi and the transcription is blocked upon shifting the cells to no Pi media. Therefore, this transcript might be one of the repressors for *pho1* expression in presence of Pi. How nc transcription is achieved in the presence of Pi and repressed in its absence. Is still an open question and requires further study. Indeed, loss of nc transcription alleviated *pho1* repression in the presence of Pi and resulted in constitutive induction of *pho1* mRNA and protein. Thus, *prt* plays an important role in repression of *pho1* (Fig. 6-1). Based on this data, I have been able to assign a role to the previously uncharacterised Pi sensing element.

Interestingly, transcription of *prt* in response to nutrients is very similar to the scenario described for the *SRG1* (*SER3* repressing gene) CUT, which regulates the *SER3* gene. In the presence of serine, *SER3* expression is repressed via transcription of an upstream ncRNA, *SRG1*. The ncRNA is rapidly degraded by the exosome complex. Moreover, loss of transcription of *SRG1* results in de-repression of *SER3* expression via loss of transcriptional interference. The *prt* mediated repression of *pho1* might also be a consequence of transcription interference due to *prt*

transcription. However, two main differences between *SRG1* and *prt* are that, *prt* overlaps the entire *pho1* gene unlike *SRG1*, which overlaps with the first few nt of *SER3*. Secondly, the levels of SER3 are not affected in exosome mutants unlike in the case of *pho1*.

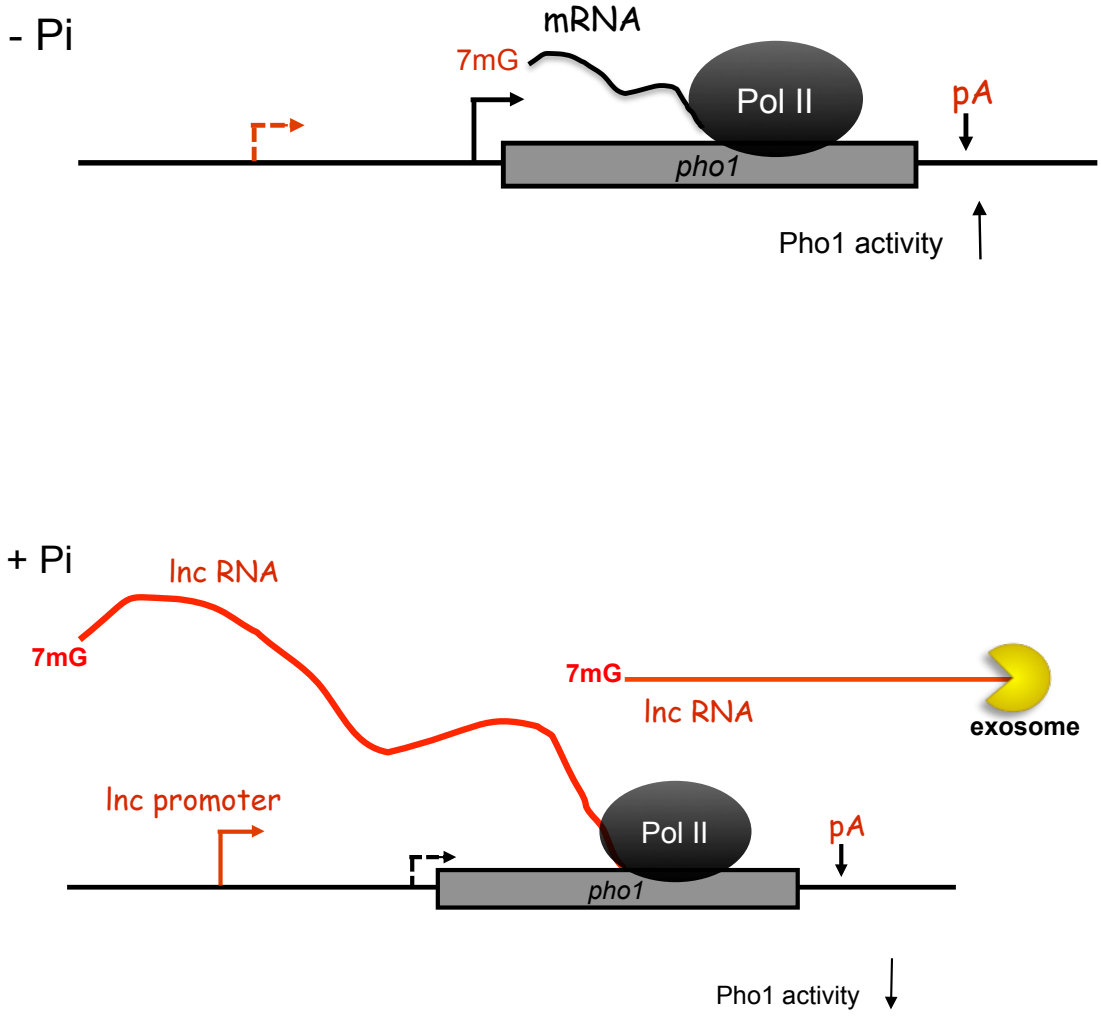


Figure 7—1. Nc transcription results in Pi dependent repression of *pho1*

In the absence of Pi, *pho1* gene expression is induced. However, in the presence of Pi, upstream transcription of an overlapping lncRNA (*prt*) results in repression of *pho1* gene. This lncRNA, is degraded by the exosome complex.

7.3 Transient heterochromatin is responsible for slow induction of *pho1* gene expression

Since, Pi levels regulate *pho1* expression, I decided to follow the induction of *pho1* in WT and exosome mutants over a time course of 24h. The induction profile is much slower in the exosome mutants. RNA analysis revealed that *prt* is not detected in exosome mutants after induction of *pho1*. Thus, loss of *prt* transcription alone cannot account for the slow induction profile. There must be another layer of repressive elements that are slowly lost upon induction thereby delaying the kinetics. This observation points towards an underlying chromatin structure that needs to be remodelled to allow transcription.

Chromatin architecture is altered by changing the 'histone code', which includes compound patterns of methylation at various residues. One of the repressive histone marks is methylation of Lysine 9 on histone H3 (H3K9me, H3K9me2 and H3K9me3). In *S. pombe*, Clr4 is the sole methytransferase responsible for H3K9 methylation. Indeed, genome wide H3k9me2 maps showed the presence of H3K9me2 at the *pho1* locus. Also, a high read of small RNAs was also found. However, the H3k9me2 and small RNAs (hallmarks of heterochromatin) were found only in exosome mutants in the genome-wide screens and hence could be an indirect effect. Thus, the functionality of these marks at the *pho1* locus was not understood.

Surprisingly, I found H3K9me2 in the WT cells as well as the exosome mutants. Although the levels of H3K9me2 were low in the WT they were much above the background levels. Furthermore, these marks were responsive to Pi and were lost upon shifting cells to no Pi media. Additionally, in a Clr4 mutant and a double mutant

of Clr4 and Rrp6, I found faster induction of the *pho1* gene, suggesting that transient heterochromatin state might help in delaying activation of *pho1* gene. However, the levels of mRNA were not derepressed in a Clr4 mutant, as is the case for transcripts from constitutive heterochromatic regions. It was recently shown that, H3K9me2 at meiotic genes does not result in transcriptional repression at meiotic loci, similar to what I find at the *pho1* locus. Conversely, as with *pho1*, it is possible that at meiotic genes, the H3K9me2 is responsible for slowing down their onset of expression by regulating timing. Further studies are required to test this hypothesis.

Heterochromatin at the *pho1* locus is the first example of transient heterochromatin in yeast. In other organisms, intermediate/transient heterochromatin is found in selective regions of the genome or over single genes and possesses considerable transcriptional activity. This process is thought to be a mechanism to fine-tune gene expression in *Arabidopsis* plants wherein a global release of transient heterochromatin in response to environmental stress (temperature) has been described (Tittel-Elmer et al., 2010). Although this scenario resembles heterochromatin at *pho1* locus, a striking difference to plants is the dependence on lncRNA transcription and the RNAi pathway (described later) in fission yeast.

As described in this thesis, I find that transient heterochromatin at the *pho1* locus plays a critical role not in repressing transcription but regulating its timely expression. The need for this delay in activation kinetics may be to allow cells to sense whether the decrease in Pi levels is only a short fluctuation or will persist for a considerable time before embarking on transcription of the entire PHO cascade. Also, it might give the cells enough time to utilize their intracellular Pi reserves in storage

granules before acquiring Pi from external sources.

RNAi pathway contributes to H3k9me2 at *pho1* locus

The RNAi machinery is essential for heterochromatin formation in *S. pombe*. RNAi is mediated by long and short ncRNA (Buhler et al., 2006; Grewal and Moazed, 2003; Motamedi et al., 2004).

At the *pho1* locus, Dcr1, Ago1, Rdp1, Clr4 were all found to be important for heterochromatin formation, since loss of any of these proteins and also catalytic mutants of Dcr1 and Ago1 resulted in partial loss of H3K9me. Also, Chp2 and Swi6 mutants showed slight de-repression of *pho1* mRNA levels. Notably, the loss of H3k9me2 in RNAi mutants was not complete as that in Clr4 mutants, suggesting RNAi independent mechanisms might also be responsible for H3k9me2 at this locus (Fig. 6-2). Interestingly, it was recently shown that Dcr1 could also recruit Clr4 independently of the RITS complex (Yu et al., 2014). Further studies are required to understand the contribution of all factors to H3k9me2 at this locus.

7.4 *prt* transcription mediates heterochromatin formation at the *pho1* locus.

Interestingly, unlike constitutive heterochromatin where ncRNAs mediate H3k9me2 both in *cis* and *trans* (Bühler et al., 2007; Colmenares et al., 2007), at the *pho1* locus, nc transcription in *cis* is required for heterochromatin formation. Thus, the nc transcription itself may be important for heterochromatin formation (Fig. 6-2).

NcRNA mediated chromatin modifications have been previously studied for PHO pathway genes in *S. cerevisiae*. In the case of *PHO84* gene, an antisense ncRNA may act as a scaffold to recruit histone-modifying enzymes to silence expression of the mRNA (eg. HDAC) (Camblong et al., 2007). Similarly, another component of the PHO pathway, *PHO5* gene in *S. cerevisiae*, is positively regulated by a ncRNA that evicts histones from the promoter of *PHO5* upon transcription (Uhler et al., 2007; Wongwisansri and Laybourn, 2005).

It is therefore, interesting that the genes involved in the PHO pathway rely heavily on ncRNA transcription to modulate their expression. Given the importance of Pi for many pathways, the need to precisely maintain Pi levels remains a fascinating area of study that can be addressed using the *pho1* gene.

7.5 The exosome complex is required for proper transcription termination of *prt*

The increase of H3k9me2 in exosome mutant might not explain the decrease of *pho1* transcription and mRNA levels. Since, in Clr4 mutants, where H3k9me2 is lost, *pho1* levels are not de-repressed. However, the defective transcription termination of *prt* in an exosome background might affect recycling of the Pol II at the locus. However, the transcription termination defect is specific for *prt* and hence may not be the only explanation for reduced transcription of *pho1* mRNA.

Even in an exosome mutant background, I found polyadenylated *prt* transcripts, suggesting that role of the exosome in *prt* transcription termination may be a 'fail-safe' mechanism and additive to the canonical transcription termination pathway.

Indeed, fail-safe termination pathways have been described before (Rondón et al., 2009; Sayani and Chanfreau, 2012). But, the role of the exosome complex in any of these pathways has not been reported previously. The role of the exosome in 3'end formation by trimming was described for snRNA and snoRNAs (Kim et al., 2006). A role for the exosome in mediating transcription termination is a unique feature of the *prt* lncRNA. Loss of exosome in *S.cerevisiae,* results in defective Nrd1 recruitment at *PHO84*, causing read-through transcription. Thus, the role of the exosome in transcription termination is unclear and needs more detailed evaluation.

Exosome may act parallel to the Rat1 mediated termination pathway

Our preliminary data suggest that the role of the exosome in transcription termination might be additive to the canonical termination pathway involving Dhp1 exonuclease (Rat1 in *S.cerevisiae*), since, double mutants of Rat1 and Rrp6 showed more read through transcription than either single mutant. To understand the exact contribution of the exosome and the CPA (cleavage and polyadenylation) machinery in terminating *prt* transcription, further analysis is warranted. It is tempting to speculate the endonucleolytic function of Dis3 subunit of the exosome could be responsible for the cleavage at the pA site similar to Ysh1 protein in the canonical pA/ cleavage machinery. However, preliminary results (data not shown) suggest that the endo activity of Dis3 may not play a role in the termination function.

Although more in depth studies are required to uncover the mechanistic details of exosome function in transcription termination, my results strongly suggest that the contribution of the exosome to substrate regulation must be evaluated not only at the

RNA stability level but also at the level of transcription.

This data implies that the exosome mediates transcription termination coupled to decay of *prt* lncRNA (Fig. 6-2). This mechanism has been previously studied for CUTs in *S. cerevisiae* (Colin et al., 2011; Thiebaut et al., 2006). Strikingly, in contrast to CUTs, whose 3'ends are distinct from those of mRNAs, I show that *prt* has the same 3'end as *pho1* mRNA and similar to mRNA is capped and polyadenylated. This suggests that, like mRNAs, lncRNA rely on the pA site for the 3'end formation. This argues against the accepted view that usage of the pA site produces stable mRNA and raises the question of how the fate of this transcript is determined.

7.6 Specificity of *prt* recognition is achieved via binding of Mmi1 and Red1 to *prt*

Previous studies have revealed several RNA binding proteins (Nrd1 and TRAMP complex is *S. cerevisiae* and Pab2, Red1 and Mmi1 in *S. pombe*) that recruit the exosome to its substrates. Interestingly, among these, Mmi1 and Red1 in *S. pombe* can interact with the RNAi machinery via the RITS complex and also by directly recruiting Clr4, for heterochromatin formation at meiotic genes (Hiriart et al., 2012; Holm and Thon, 2012; Tashiro et al., 2013; Yamanaka et al., 2013). Also, the Mmi1-Red1 machinery is responsible for exosome mediated decay of meiotic transcripts expressed untimely during meiosis (St-André et al., 2010; Sugiyama and Sugioka-Sugiyama, 2011; Yamamoto, 2010). But, the role of these proteins outside the context of meiotic genes was unknown.

Surprisingly, I find specific sequences called DSR (Determinants of selective removal) elements present exclusively in *prt*, which serve as a distinguishing feature to recruit Mmi1 (Yamashita et al., 2012). Furthermore, Mmi1 and Red1 mutants show a phenotype similar to Rrp6 mutants. Also, upon loss of both proteins, H3k9me2 levels were affected. The differences between the effects of Red1 and Mmi1 at the *pho1* locus might signify their specific roles. For example, I found Mmi1 via DSR is required for transcription termination whereas Red1 is dispensable for this process. Also, upon loss of Red1, H3k9me2 was dramatically reduced as against loss of Mmi1. Recently, it was shown that Red1 interacts directly with Clr4 (Tashiro et al., 2013) and also that it is essential for heterochromatin formation at the *pho1* locus (Lee et al., 2013). The stabilization of *prt* in Red1 mutants is similar to that in Rrp6 mutants, compared to that in Mmi1 mutants.

Thus, taken together, I find that Mmi1 is in part responsible for recruiting the exosome for transcription termination of *prt*. Conversely, Red1 might mediate degradation of *prt* via Rrp6 and heterochromatin formation at the *pho1* locus. Also, deletion of DSR can stabilize *prt*. However, whether Red1 binds to DSR sequences is unknown.

Thus, we find heterochromatin at the *pho1* locus relies on the ability of *prt* to recruit Red1 and Mmi1 and the RNAi complex (Fig. 6-2). Also, exosome specifically recognizes *prt* as a substrate for transcription termination via the presence of DSR elements and by binding of Mmi1. The *pho1* gene can thus be effectively used to study the roles of Mmi1 and Red1 and also their sequential order of recruitment. Additionally, the use of the Mmi1-Red1-exosome pathway for regulating genes of the

PHO pathway hints towards a potential requirement of optimal phosphate levels during meiosis. A role for *pho1* in meiosis has not been implicated so far.

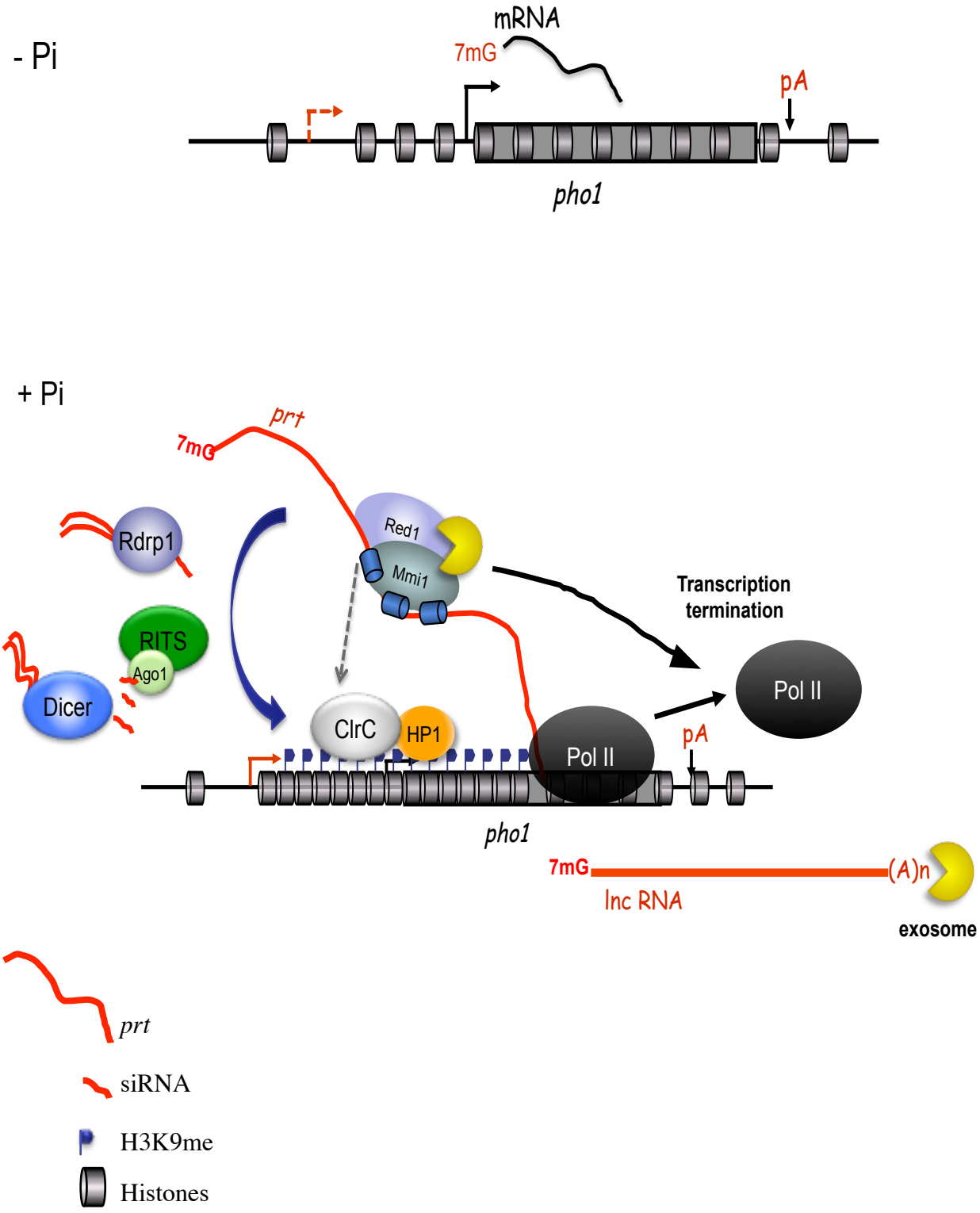


Figure 7—2. ncRNA mediates heterochromatin formation at the *pho1* locus

prt transcription in the presence of Pi, results in H3k9me2 across the *pho1* locus. Binding of Mmi1 and Red1 is required for H3K9me. Also, the RITS complex is involved in this process. Further work is required to understand whether multiple mechanisms are at play and the contribution of each of the pathways in H3k9me2 at this locus.

7.7 Meiotic genes also rely on the exosome and Mmi1 for proper termination.

The presence of DSR sequences mediating transcription termination is not a feature restricted to *prt*. I found other meiotic genes (eg. *sme2, mug180*) that demonstrate defective transcription termination in exosome and Mmi1 mutants. In fact, subjecting cells to a meiotic time course, which results in loss of binding of exosome and Mmi1 to meiotic transcripts, resulted in read-through transcription. This underlines the fact that the role of exosome in mediating transcription termination is generalized and depends on the presence of Mmi1 binding sites. This study provides a good platform for genome-wide analysis of transcription termination defect in exosome mutants. Also, it is imperative to search the genome for additional targets of Mmi1, since; Mmi1 binding does not seem to be restricted to meiotic genes. The termination defect during meiosis might be a mechanism to check for unwarranted excess transcripts during meiosis which are no longer targets of exosome mediated decay unlike in mitosis when a proper control is exerted on their expression.

7.8 The role of the exosome complex at the *pho1* locus

The exosome complex, a major component of RNA surveillance pathways has so far been studied in context with its role in quality control and 3' end formation of RNAs. However, more general themes suggest that the quality control complexes may have a role in co-transcriptional processing of RNA (Kilchert and Vasiljeva, 2013). Indeed, my findings re-inforce the idea that the role of the exosome needs to be addressed at the level of transcriptional regulation (Coy and Vasiljeva, 2010). Additional proteins that may aid these functions of the exosome (eg. in transcription termination) still remain undiscovered. Furthermore, the increase in H3k9me2 and

small RNA reads in an exosome background is not understood (Shah et al., 2014; Yamanaka et al., 2013).

As previously hypothesized, Rrp6 could compete with the RITS complex for binding to Mmi1 (Yamanaka et al., 2013). Thus, loss of Rrp6 would allow channelling of these transcripts into the RITS pathway and lead to an increase in H3K9me. So far, my findings are not sufficient to confirm this hypothesis since in an Mmi1 mutant I lose both Ago1 and Rrp6 recruitment but do not detect enhanced H3K9me. On the other hand, in a Red1 mutant I lose H3K9me, suggesting that direct Clr4 recruitment via Red1 also plays a role in H3k9me2 at the *pho1* locus. Thus, addressing whether Ago1 recruitment at *pho1* is increased upon loss of Rrp6 would be important.

Although mechanisms are in place to check transcription fidelity and ensure that genes do not make spurious dsRNA, it was reported that defects in transcription termination of convergent genes can give rise to dsRNA. These dsRNA can be processed into siRNAs and trigger heterochromatin formation (Gullerova and Proudfoot, 2008; Gullerova et al., 2011). However, the transcription termination defect in an exosome mutant does not account for the increased H3k9me2 observed.

Another hypothesis is that, stabilizing transcripts in an exosome mutant can result in increased silencing. The ncRNAs, which are usually degraded by the exosome, can fold back into extended hairpins. These dsRNAs can generate endogenous siRNAs (endo-siRNAs) via Dcr1 resulting in heterochromatic silencing. Alternately, the siRNAs themselves may be a target for exosome mediated decay, this

would explain the increase in small RNA reads in an exosome mutant (Yamanaka et al., 2013). Furthermore, Cid14, Cid12 (TRAMP complex components which are involved in exosome mediated decay of transcripts) also contribute to efficient gene silencing at constitutive heterochromatic loci (Reyes-Turcu et al., 2011; Zhang et al., 2011). It is also possible that the effects of the exosome at constitutive, facultative and transient heterochromatin are distinct from each other. Thus, more detailed studies are required to specifically focus on the role of the exosome in light of this new data and so understand its contribution to gene expression and silencing.

The sequence dependence for termination of unstable *prt* transcripts mediated via RNA binding protein Mmi1, is reminiscent of the Nrd1-exosome mediated termination of CUTs in *S. cerevisiae* (Arigo et al., 2006; Carroll et al., 2007; Vasiljeva et al., 2008). However, a notable difference is that the exosome was shown to be required only for the degradation of transcripts and did not affect termination per say. Also, termination by Nrd1 occurs close to its binding site. However, in the case of *prt,* the transcription termination does not take place at the Mmi1 binding site. Thus, evaluating the poly (A) site and in connection with exosome mediated termination may help understand what pathways and proteins are likely to play a role.

Thus, I find that transcription termination coupled decay mechanism is conserved between the two yeasts (Fig. 6-3). The dependence of other meiotic transcripts on the exosome for termination points towards a more generalized role for the exosome mediated degradation coupled to transcription termination. Interestingly, the use of the Mmi1 machinery for this purpose indicates that more genes may be involved in proper meiotic progression other than those so far identified. Maybe the

concerted effects of nutrient homeostasis pathways (such as PHO pathway) are required to maintain optimum nutrient levels for proper cell division. Thus the targets of Mmi1-exosome mediated regulation need to be re-assessed. Furthermore, the role of heterochromatin at meiotic loci must also be re-assessed to understand whether the progression of meiosis is delayed or the genes that need to express during late phase meiosis are affected by the presence of heterochromatic marks.

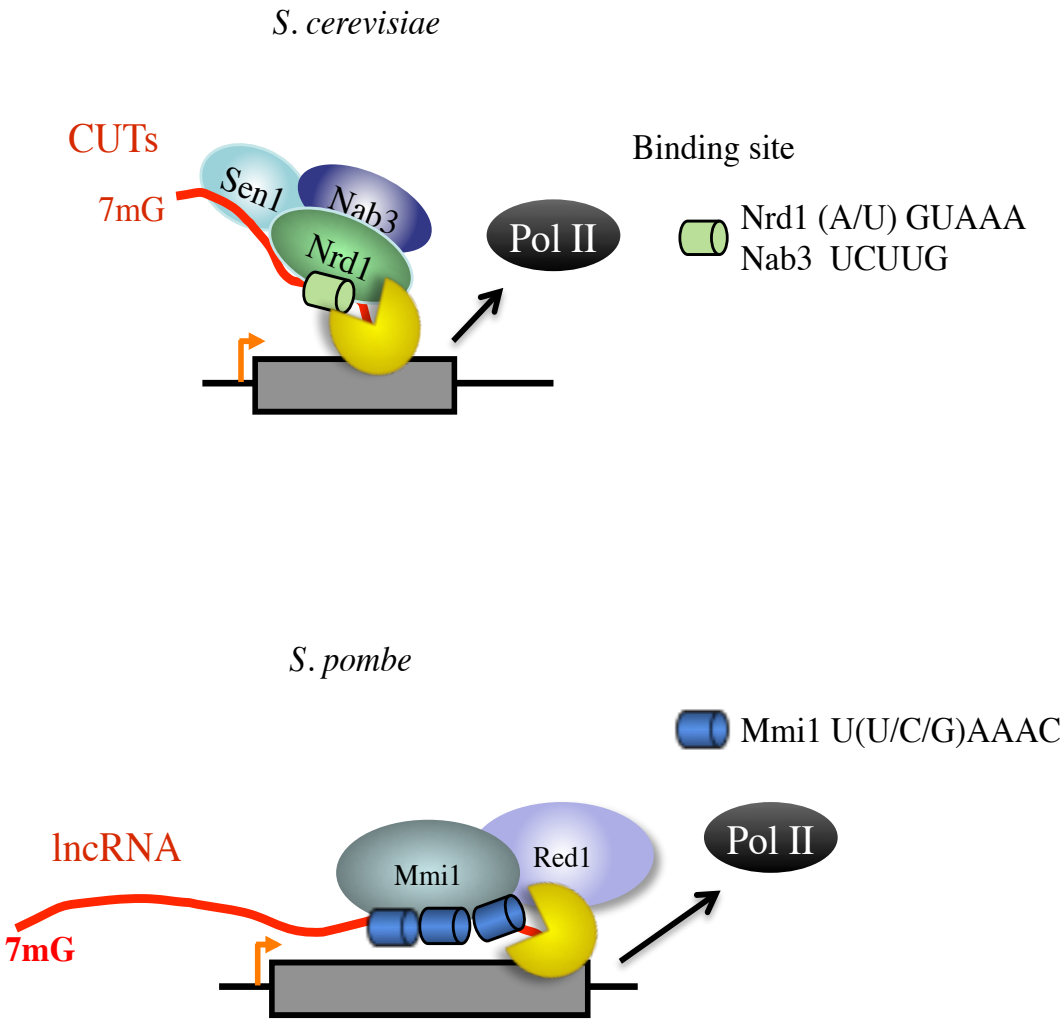


Figure 7—3. Transcription termination coupled to decay.

Based on my findings, I hypothesize that RNA binding proteins such as Nrd1/Nab3 in *S.cerevisiae* and Mmi1/Red1 in *S. pombe*, help to target the exosome complex to specific substrates. The exosome complex unlike *in S. cerevisiae* is involved in transcription termination in *S. pombe* as exemplified at the pho1 locus and meiotic genes.

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