The role of long non-coding RNAs and the RNA exosome in regulation of gene expression

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DOCTORAL DISSERTATION

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Supervisor:
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Sveučilište u Zagrebu

Prehrambeno-biotehnološki fakultet

Ana Novačić

ULOGA DUGIH NEKODIRAJUĆIH MOLEKULA RNA I RNA EGZOSOMA U REGULACIJI EKSPRESIJE GENA

DOKTORSKI RAD

Mentor:
izv. prof. dr. sc. Igor Stuparević

Zagreb, 2022.

Ana Novačić

The role of long non-coding RNAs and the RNA exosome in regulation of gene expression

Supervisor:
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This is an article-based doctoral thesis, known as Scandinavian model, which consists of already published scientific papers accompanied by a chapter with the critical review, which was written in accordance with Article 14 of the Doctoral Studies Regulations at the University of Zagreb (2016).

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The role of long non-coding RNAs and the RNA exosome in regulation of gene expression

Ana Novačić, mag. ing. biotechn.

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Short abstract: The RNA exosome is a conserved protein complex involved in 3'-to-5' degradation and processing of all types of RNA classes in eukaryotic cells. The recently characterized substrates of the RNA exosome complex are long non-coding RNAs (lncRNAs), transcripts that do not code for proteins, but have the potential to regulate gene expression. The main objective of this work was to determine the role of lncRNAs and the RNA exosome complex in the regulation of stress-related genes in yeast. This work demonstrates that the RNA exosome is critical for maintaining yeast cell wall stability by regulating the expression of genes encoding enzymes involved in protein glycosylation, which is essential for supporting the cell wall structure. In maintaining cell wall stability, the RNA exosome functions in parallel with Ssd1, an RNA-binding protein that regulates translation of cell wall-related mRNAs. Furthermore, this work elucidates the molecular mechanisms through which lncRNAs and the RNA exosome regulate the expression of stress-related genes. A regulatory role has been demonstrated for the lncRNA transcribed at the gene locus encoding the protein Psa1, which is involved in the synthesis of the mannosylation precursor. Furthermore, this work demonstrates a negative regulatory role of antisense transcription at the *PHO5* model gene locus at the level of promoter chromatin structure.

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Uloga dugih nekodirajućih molekula RNA i RNA egzosoma u regulaciji ekspresije gena

Ana Novačić, mag. ing. biotechn.

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Kratki sažetak: RNA egzosom je evolucijski očuvani proteinski kompleks uključen u razgradnju i procesiranje svih klasa RNA u smjeru 3'-5' u eukariotskim stanicama. Nedavno karakterizirani supstrati RNA egzosoma su duge nekodirajuće molekule RNA (lncRNA), transkripti koji ne nose uputu za sintezu proteina, ali imaju potencijalne uloge u regulaciji ekspresije gena. Glavni cilj ovog rada bio je odrediti ulogu lncRNA i kompleksa RNA egzosoma u regulaciji ekspresije gena kvasca čija je funkcija povezana sa stresom. Ovaj rad pokazuje da je RNA egzosom ključan za održavanje stabilnosti stanične stijenke kvasca, budući da regulira ekspresiju gena koji kodiraju za enzime uključene u glikozilaciju proteina, koja je bitna za održavanje strukture stanične stijenke. U održavanju stabilnosti stanične stijenke, RNA egzosom funkcionira paralelno sa Ssd1, RNA-vezujućim proteinom koji regulira translaciju mRNA koje nose upute za proteine uključene u staničnu morfogenezu. Nadalje, ovaj rad razjašnjava molekularne mehanizme putem kojih lncRNA i RNA egzosom reguliraju ekspresiju gena povezanih sa stresom. Pokazana je regulatorna uloga lncRNA transkribirane na lokusu gena koji kodira za protein Psa1, koji je uključen u sintezu manozilacijskog prekursora. Dodatno, ovim je radom pokazana negativna regulatorna ulogu *antisense* transkripcije na lokusu modelnog gena *PHO5* na razini strukture kromatina promotorske regije.

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The topic of the doctoral thesis was accepted at the 7th regular session of the Faculty Council of the Faculty of Food Technology and Biotechnology in Zagreb in the academic year 2021/2022 held on April 26th, 2022. The Senate of the University of Zagreb decided to initiate the procedure for obtaining a Doctor of Science degree within the doctoral study at a session held on July 19th, 2022.

My first thanks go to my doctoral thesis supervisor Igor Stuparević. I have grown a lot in the last five years, as a person and as a scientist, and this would not have been possible without the freedom, flexibility and resources granted to me in his lab. It would also not be wrong to say that during this time we shared the experience of starting a research group from scratch, and I am immensely proud of what we have achieved together.

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I have always liked that Stryer's Biochemistry textbook opens with the acknowledgement "To our teachers and our students". I am grateful for what my professors taught me, but my doctoral thesis was much more defined by the bachelor and master students I was lucky to teach and to supervise. One of the most rewarding parts of my work was finding a way for each of them to experience the joy of scientific work and I hope I was successful in doing that.

I am grateful that I have always had many people behind me who supported me unconditionally. That also seems to be necessary to do great science, because you have something to be happy about, even when you are dealing with very challenging situations and scientific problems. So, to Filip, my friends, parents, and grandparents – thank you! ♡

EXTENDED ABSTRACT

The RNA exosome is a conserved multiprotein complex involved in 3'-to-5' degradation and processing of all types of RNA classes in eukaryotic cells. The dysregulation of the RNA exosome therefore leads to broad changes in gene expression patterns and is also implicated in various human diseases. The recently characterized substrates of the RNA exosome complex are long non-coding RNAs (lncRNAs), transcripts longer than 200 nt that do not carry information for protein synthesis but have the potential to regulate gene expression. Even though lncRNAs are generated at many eukaryotic gene loci, their roles are still poorly characterized. The main objective of this work was to determine the role of lncRNAs and the RNA exosome complex in regulation of stress-related genes in the model eukaryotic yeast *Saccharomyces cerevisiae.* In this thesis, the role of the RNA exosome in the yeast stress response was shown to stem from its crucial function in regulating the cell wall structure. This regulatory activity of the exosome requires the exoribonuclease activity of the catalytic subunit Dis3, a non-catalytic function of the second catalytic subunit Rrp6, and several cofactors of the nuclear exosome. RNA exosome mutants therefore show cell wall instability phenotypes such as temperature sensitivity and sensitivity to cell wall stressors. In RNA exosome mutants, the cell wall is destabilized due to dysregulation of genes implicated in protein glycosylation, which is essential for supporting the cell wall structure. The RNA exosome regulates cell wall stability through a pathway parallel with that of Ssd1, an RNA-binding protein that regulates translation of cell-wall related mRNAs. Consequently, inactivation of the RNA exosome results in more severe phenotypes in genetic backgrounds in which Ssd1 is not functional. Furthermore, this work demonstrates lncRNA-mediated regulatory mechanisms for certain yeast gene loci encoding proteins with stress-related functions. A negative regulatory role has been demonstrated for the lncRNA transcribed at the gene locus encoding the protein Psa1, which is involved in synthesis of the mannosylation precursor. Degradation of the corresponding lncRNA by the RNA exosome complex was shown to be important for proper protein glycosylation and therefore cell wall stability under stress conditions. Additionally, this work demonstrates a negative regulatory role of antisense transcription at the *PHO5* model gene locus, which encodes a periplasmic acid phosphatase. The corresponding lncRNA is transcribed through the *PHO5* promoter region, resulting in a more repressive chromatin conformation which is harder to remodel upon gene induction.

Keywords: Gene expression, lncRNAs, RNA exosome, stress conditions, yeast

PROŠIRENI SAŽETAK

RNA egzosom je evolucijski očuvani multiproteinski kompleks uključen u razgradnju i procesiranje svih klasa RNA u smjeru 3'-5' u eukariotskim stanicama. Poremećaji u regulaciji RNA egzosoma stoga dovode do ekstenzivnih promjena u ekspresiji gena i identificirani su kod raznih ljudskih bolesti. Nedavno karakterizirani supstrati RNA egzosoma su duge nekodirajuće RNA molekule (lncRNA), transkripti dulji od 200 nt koji ne nose informaciju za sintezu proteina, ali imaju potencijal regulirati ekspresiju gena. Iako se lncRNA transkribiraju na lokusima mnogih eukariotskih gena, njihove su uloge još uvijek slabo definirane. Glavni cilj ovog rada bio je utvrditi ulogu lncRNA i RNA egzosoma u regulaciji ekspresije gena povezanih sa stresom u modelnom eukariotskom kvascu *Saccharomyces cerevisiae*. U ovom je radu pokazano da uloga RNA egzosoma u kvaščevom odgovoru na stres proizlazi iz njegove ključne funkcije u regulaciji strukture stanične stijenke. Ova regulatorna aktivnost egzosoma zahtijeva aktivnost egzoribonukleaze katalitičke podjedinice Dis3, nekatalitičku funkciju druge katalitičke podjedinice Rrp6 i nekoliko kofaktora nuklearnog egzosoma. Mutanti RNA egzosoma stoga pokazuju fenotipove karakteristične za stanice sa nestabilnom staničnom stijenkom, kao što su temperaturna osjetljivost i osjetljivost na stresore stanične stijenke. U mutantima RNA egzosoma, stanična stijenka je destabilizirana zbog štetnih promjena u regulaciji ekspresije gena koji kodiraju za enzime uključene u glikozilaciju proteina, procesa bitnog za strukturu stanične stijenke. RNA egzosom regulira stabilnost stanične stijenke paralelno sa Ssd1, RNA-vezujućim proteinom koji regulira translaciju mRNA koje nose upute za sintezu proteina uključenih u staničnu morfogenezu. Posljedično, inaktivacija RNA egzosoma rezultira izraženijim fenotipovima u linijama sojeva u kojima Ssd1 nije funkcionalan. Nadalje, ovaj rad razjašnjava regulatorne mehanizme posredovane lncRNA za određene lokuse gena kvasca koji kodiraju za proteine čije su funkcije povezane s odgovorom na stres. Pokazana je negativna regulatorna uloga lncRNA transkribirane na lokusu gena koji kodira za protein Psa1, koji je uključen u sintezu manozilacijskog prekursora. Pokazano je da je razgradnja odgovarajuće lncRNA RNA egzosomom važna za pravilnu glikozilaciju proteina i stoga za stabilnost stanične stijenke u uvjetima stresa. Osim toga, ovaj rad pokazuje negativnu regulatornu ulogu *antisense* transkripcije na lokusu modelnog gena *PHO5*, koji kodira za periplazmatsku kiselu fosfatazu. Odgovarajuća lncRNA transkribira se kroz promotorsku regiju gena *PHO5*, što rezultira represivnom konformacijom kromatina koju je teže remodelirati nakon indukcije gena.

Ključne riječi: Ekspresija gena, lncRNA, RNA egzosom, stresni uvjeti, kvasac

Information about the supervisor – PhD Igor Stuparević, Associate Professor

IGOR STUPAREVIĆ was born in 1978 in Slavonski Brod. He studied at the Faculty of Food Technology and Biotechnology and graduated with a Masters degree in 2004. He received his PhD in Biochemistry from the Faculty of Science, University of Zagreb in 2010, where he studied the role of yeast cell wall proteins in cell wall biogenesis. After defending his PhD thesis, he spent two years at CNRS (Centre national de la recherche scientifique), CBM (Centre de biophysique moléculaire) in Orleans, France, in the group of Rachid A. Rahmouni, where he worked on transcription elongation, mRNP biogenesis and nuclear quality control. From 2013 to 2015, he worked as a postdoctoral fellow at INSERM (Institute national de la santé et de la recherche médicale), U1085-IRSET (Institut de Recherche en Santé, Environnement et Travail) in Rennes, France in the group of Michael Primig. During this time, he continued to work on the regulation of transcription in the context of growth and development, in particular on yeast meiotic progression and the role of non-coding RNAs in the regulation of transcription. In 2015, he returned to the Faculty of Food Technology and Biotechnology at the University of Zagreb as an Assistant Professor, where he studied the interplay of non-coding RNAs and yeast cell wall biogenesis. Since 2021, he has been an Associate professor in the Laboratory of Biochemistry at the Faculty of Food Technology and Biotechnology. He is the main lecturer for the Molecular Biology course, one of the main lecturers for the Biochemistry I and Biochemistry II courses and participates in teaching of Biochemical Analytics and Protein Purification and Characterization courses. I. Stuparević is the project leader of the research project "*In silico* and *in vivo* analysis of yeast *Saccharomyces cerevisiae* cell wall transcriptome and its application in construction of novel biotechnological strains", funded by the Croatian Science Foundation, and the project "Interplay of RNA exosome complex and non-coding RNAs in preserving cellular integrity" funded by the COGITO programme. He has been the supervisor of 15 bachelor's and 7 master's theses. In addition to his own scientific production (17 publications), he has contributed to many other collaborations. Since 2020, he has been the FEBS Ambassador for Education and Treasurer of the Croatian Society of Biochemistry and Molecular Biology. He is also a member of the Croatian Microbiological Society and the Croatian Association of Genetic Engineers.

TABLE OF CONTENTS

1. GENERAL INTRODUCTION

Gene expression is an essential cellular process that is crucial for maintaining cellular homeostasis upon physiological and stress conditions. This process is therefore extensively regulated at many different levels, to ensure proper expression patterns according to cell type, cell cycle, life cycle stages and external conditions. Recently identified regulators of eukaryotic gene expression are long non-coding RNAs (lncRNAs), transcripts longer than 200 nt which do not carry information for protein synthesis. Since eukaryotic genomes are pervasively transcribed, lncRNAs are generated at many gene loci, however, their roles are still mostly uncharacterized (Villa and Porrua 2022). The unicellular eukaryotic yeast *Saccharomyces cerevisiae* is used as a model system for studying lncRNAs, as it does not generate small non-coding RNAs due to the loss of the RNA interference pathway during evolution (Alcid and Tsukiyama 2016), and many gene regulation mechanisms are conserved from yeast to human. lncRNAs are degraded by the RNA exosome, a multiprotein complex which catalyzes 3'-to-5' RNA degradation and processing in the nucleus and the cytosol compartments of all eukaryotic cells (Zinder and Lima 2017). The RNA exosome is therefore a crucial factor in eukaryotic gene regulation, as its dysregulation leads to broad changes in gene expression patterns and is also implicated in various human malignancies and disorders (Morton *et al.* 2020). In yeast, all subunits of the RNA exosome are essential for viability, except for the catalytic subunit Rrp6 which is essential for growth at high temperatures (Mitchell *et al.* 1997, Briggs *et al.* 1998), showing that the exosome is necessary for the stress response in yeast. Main objectives of this dissertation are to determine the role of lncRNAs and the RNA exosome complex in maintaining the cell wall structure of yeast cells under stress conditions and to elucidate the molecular mechanisms by which they regulate expression of certain stress-related genes.

2. THEORETICAL BACKGROUND

2.1. Regulation of gene expression in eukaryotes

In the process of gene expression, eukaryotic protein-coding genes are first transcribed into messenger RNAs (mRNAs) through a process catalyzed by RNA Polymerase II (Pol II) in the nucleus. These mRNAs are co-transcriptionally processed through splicing, 5'-cap and 3'-poly-A tail addition and are bound by regulatory and export factors to form ribonucleoprotein complexes (RNPs). The mature RNPs are exported to the cytosol, where the mRNAs are translated on ribosomes to synthesize proteins. The process of gene expression can be considered complete when the protein is localized in the proper cellular compartment and, if required for its activity, is modified through post-translational modifications. Gene expression is, therefore, a complex process that can be regulated at practically any step. This leads to a variety of transcriptional, post-transcriptional and post-translational mechanisms that control the expression patterns of eukaryotic genes. However, gene expression is most commonly regulated at the first step, *i.e.*, gene transcription, especially its initiation, as it is the most efficient control point (Haberle and Stark 2018). Transcriptional regulation is a complex process controlled by numerous regulatory proteins that work together to activate or repress the expression of different genes depending on environmental signals, cell cycle and life cycle stage. Although there are some species-specific differences, the basic mechanisms of transcriptional regulation are highly conserved in eukaryotes from yeast to humans, making the budding yeast *Saccharomyces cerevisiae* a widely used model organism for studying the principles of gene regulation (Hahn and Young 2011). Some examples of conserved features of eukaryotic gene regulation are (*i*) the assembly of Pol II and general transcription factors (GTFs) at the promoter region to form the pre-initiation complex (PIC), (*ii*) regulation through sequence-specific transcription factors (TFs) and (*iii*) the organization of the genome into a well-defined chromatin structure which can be modified through remodeling and covalent modifications (Venters and Pugh 2009).

2.1.1. Transcriptional initiation

PIC or the so-called closed complex assembly (**Figure 1**) involves the binding of GTFs and Pol II at the core promoter region (~50 base pairs (bp) upstream and ~50 bp downstream of the transcription start site (TSS)) and is the first and often limiting step in the initiation of

gene transcription (Thomas and Chiang 2006). The GTFs function as the basal transcriptional machinery that supports the loading and release of Pol II at the TSS. During PIC assembly, the 14-subunit transcription factor IID (TFIID) recognizes the TATA box motif in core promoters through its TATA box binding protein (TBP) subunit. The vast majority of eukaryotic coding gene promoters are TATA-less, *i.e.*, they lack the consensus TATA box motif (~80% in yeast, [Basehoar *et al.* 2004]), yet TFIID is required for almost all Pol II-mediated gene transcription (Warfield *et al.* 2017, Haberle and Stark 2018). TATA-less promoters are typical of housekeeping genes that are constitutively expressed, whereas TATA-containing promoters are typical for highly regulated genes associated with responses to stress (Basehoar *et al.* 2004). This is followed by sequential binding of TFIIA, TFIIB, the TFIIF-Pol II complex, TFIIE and TFIIH. The last two recruited factors regulate Pol II activity and facilitate promoter clearance. In particular, TFIIH has ATP-dependent helicase activity that is important for DNA strand separation and formation of the open complex. The Kin28/Cdk7 kinase subunit of TFIIH phosphorylates serine (S) residues at the 5th position of the repeated YSPTSPS motif in the C-terminal domain (CTD) of the largest Pol II subunit, a process that mediates the transition from transcriptional initiation to elongation (Valay *et al.* 1995).

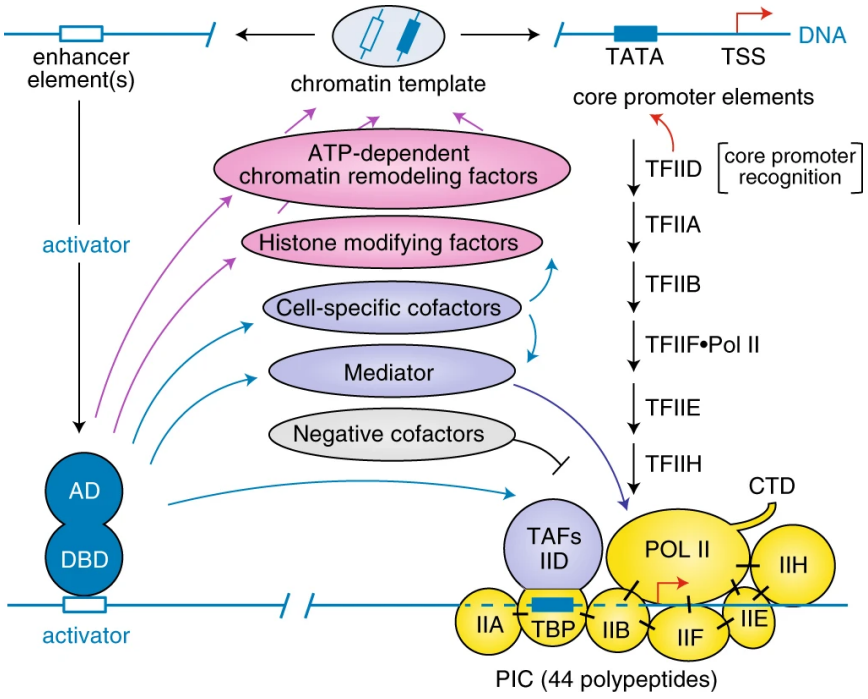

Figure 1. A model for the regulation of eukaryotic PIC assembly. Assembly of Pol II and GTFs (in yellow) at the core promoter region is regulated by regulatory factors and their interactions with other cofactors that mediate chromatin-remodeling and modifying and

protein-protein interactions. TAFs, TBP-associated factors. AD, activation domain. DBD, DNA binding domain. TSS, transcription start site (Roeder 2019).

Transcription is also regulated by sequence-specific TFs, which bind to *cis*-regulatory elements (CREs) called upstream activating sequences (UAS) in yeast or enhancers in metazoans. Specific TFs can target multiple (*e.g.,* Pho4 ~20) or several hundred *(e.g.,* Rap1 ~300) promoters in yeast, whereas in metazoans some TFs target thousands of promoters (Venters and Pugh 2009, Korber and Barbaric 2014, Haberle and Stark 2018). However, unlike GTFs, they are not required for all Pol II-mediated gene transcription. Specific TFs regulate the recruitment of GTFs, Pol II (*via* the multi-subunit Mediator complex), as well as chromatin-remodeling and modifying complexes to gene promoters (Venters and Pugh 2009). Specific transcriptional activators consist of at least one DNA-binding domain (DBD) and at least one activating domain (AD), whereas repressors instead contain a repression domain (RD). The activity and subcellular localization of specific TFs are often regulated through covalent modifications, such as phosphorylation, or targeted proteolysis. While UASs in yeast are typically found several hundred bps upstream of TSS (Harbison *et al.* 2004), enhancers in metazoans can be located several thousand bps away (Haberle and Stark 2018). In addition to the conserved TATA box motif typically present in the core promoter region 30-60 bp from TSS, additional core promoter elements in metazoans include the Initiator (Inr), downstream promoter element (DPE), motif ten element (MTE), and TFIIB recognition element (BRE) (Venters and Pugh 2009).

2.1.2. Transcriptional elongation and termination

During transcriptional elongation, 8-9 nucleotides (nts) of nascent RNA and the template DNA form an RNA-DNA hybrid at the core of the elongating Pol II, which is called a transcription bubble. The elongation step is regulated through proteins which function as elongation factors, such as the Spt4-6 proteins and the PAF complex in yeast (Noe Gonzalez *et al.* 2021). In metazoans, an important regulatory step is the promoter-proximal pausing, a phenomenon in which Pol II pauses at the promoter-proximal region in an early elongation step, awaiting signals for its rapid release and activation. This signal requires the kinase activity of the cyclin-dependent kinase 9 (CDK9) subunit of the Positive Transcription Elongation Factor b (P-TEFb), which phosphorylates the NELF and DSIF complexes, as well as the 2nd serine residue of the Pol II CTD repeat, rendering it elongation-competent (Noe Gonzalez *et al.* 2021). Pol II CTD is critical for regulation of eukaryotic transcription, as it recruits proteins

involved in elongation, termination, mRNA processing and histone modifications. The number of CTD heptapeptide repeats can vary from 26 in yeast to 52 in humans and each heptapeptide carries five potential phosphorylation sites, arguing in favor of a CTD code model in which different phosphorylation patterns recruit different factors (Egloff and Murphy 2008). Phosphorylation of the 5th serine residue marks the transition from initiation to elongation, but also functions as a platform for recruitment of the mRNA capping enzyme, the elongation factor PAF complex and the Nrd1-Nab3-Sen1 (NNS) RNA surveillance complex. The 7-methylguanylate (m^7G) 5' cap protects the transcript from 5'-3' exoribonucleolytic degradation and marks it for export to the cytosol and translation. The NNS complex consists of the RNA-binding proteins Nrd1 and Nab3 and the helicase Sen1, which track along with Pol II to induce early termination of transcripts with Nrd1 binding motifs (*e.g.*, UCUUG). These motifs are primarily present in pervasively transcribed non-coding transcripts and direct them for degradation through the RNA exosome complex and are rarely present in protein-coding mRNAs (Villa and Porrua 2022).

Towards the 3' end of a gene, serine-2 phosphorylation of the Pol II CTD predominates over serine-5 phosphorylation, serving as a mark for recruitment of the 3' end RNA processing machinery. The termination factor Pcf11 of the cleavage and polyadenylation factor (CPF)-cleavage factor (CF) complex is recruited to the CTD to mediate transcriptional termination. The nascent mRNA is cleaved at the poly-A site by the CPF endonuclease subunit Ysh1, which is subsequently polyadenylated by the CPF-associated poly-A polymerase Pap1. The 3' poly-A tail is bound by the poly-A binding protein Pab1, which protects it from 3'-5' exoribonucleolytic degradation and promotes its nuclear export (Porrua and Libri 2015). Rtt103, which is also recruited by the CTD, is involved in termination through interactions with the Rat1/XRN2 exonuclease, which degrades RNA downstream of the mRNA cleavage site (Kim *et al.* 2004).

2.1.3. Chromatin structure

The defining feature of eukaryotic genome organization is the chromatin structure, in which DNA forms a complex with histone proteins. More specifically, 147 bps of DNA are wrapped around a histone octamer (consisting of two copies each of histones H2A, H2B, H3 and H4) to form the nucleosome, the repeating unit of chromatin structure (Luger *et al.* 1997). Nucleosomes serve to condense the genome, but also restrict access to DNA of many DNA-

binding proteins, such as those involved in the processes of transcription, DNA replication, recombination and repair. Importantly for gene expression, DNA regions which interact with histone proteins are not free for interaction with the transcriptional machinery, such as sequence-specific TFs and GTFs, making the chromatin structure generally repressive for transcription. However, the chromatin structure is highly dynamic and is affected by the activity of enzymes that catalyze covalent modifications of histones, termed chromatin modifiers, and enzymes that catalyze ATP hydrolysis to reposition or eject histones from DNA, termed chromatin remodelers. The activity of both classes of complexes can result in specific types of local chromatin architecture, *i.e.*, the composition of nucleosomes and their positioning relative to *cis*-regulatory sites, which is particularly important in the context of promoter regions from which transcription initiates (Cairns 2009).

Gene promoters can be broadly divided into two classes based on their chromatin architecture: open and covered (Cairns 2009) (**Figure 2**). Open promoters are typical for constitutively expressed genes that encode proteins with housekeeping functions, such as maintaining of essential metabolic processes and cell structure. These promoters typically contain a large (~150 bp) nucleosome-depleted region (NDR) upstream of the TSS which contains key *cis*-regulatory elements such as binding sites for transcriptional activators, allowing them free binding access. Furthermore, open promoters are typically TATA-less and usually contain the H2A histone variant H2AZ (Htz1 in yeast) at the +1 or -1 nucleosome. In contrast, closed promoters are typical of highly regulated genes, such as those induced only under specific stress conditions. The TSS and activator binding sites on closed promoters are covered by precisely positioned nucleosomes, impairing the access of specific activators and GTFs to the promoter. Therefore, transcriptional activation of genes regulated by closed promoters depends on the chromatin remodeling process, which results in sliding or ejection of promoter histones to expose the *cis*-regulatory elements. Closed promoters usually contain a TATA box element, which is usually partially covered inside the edge of the proximal nucleosome. Transcriptional activation at closed promoters often requires the Spt-Ada-Gcn5 acetyltransferase (SAGA) complex which catalyzes histone acetylation and deubiquitylation, but also shares a number of subunits with TFIID, namely the TATA box-binding TBP subunit (Hahn and Young 2011). It is important to note that the open and closed promoter architectures are useful models for promoter classification, but that many promoters do not strictly belong to either of these classes, but rather contain a mixture of their properties (Cairns 2009).

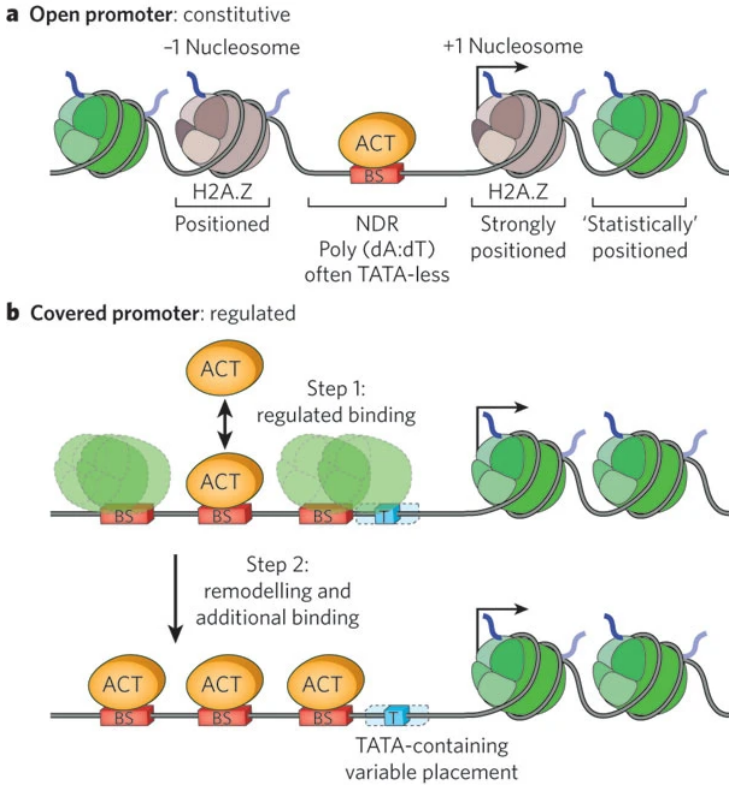

Figure 2. Chromatin architecture of open and closed promoters. Open promoters have a broad NDR (nucleosome-depleted region) that allows free binding of activators (ACT) to their binding sites (BS) **(A)**, whereas in closed promoters the activator binding sites and the TSS are covered by nucleosomes and require chromatin remodeling to become exposed for transcriptional machinery binding **(B)** (Cairns 2009).

2.1.3.1. Chromatin modifications

Histones are dynamically modified through covalent modifications of amino acid residues at their N-terminal tails, which protrude from the core structure of the nucleosome. Common post-translational modifications of histones include acetylation, methylation and ubiquitination of lysine residues and phosphorylation of serine residues, catalyzed by specific chromatin-modifying complexes (Desjarlais and Tummino 2016). The enzymes that mediate histone modifications are sometimes referred to as “writers” and those that remove them as “erasers”. Multiple modifications can occur at the same nucleosome, providing combinatorial complexity and dictating specific remodeling outcomes, a hypothesis known as the histone code (Strahl and Allis 2000). Some chromatin modifications, such as histone acetylation and deacetylation, can directly affect condensation of chromatin structure, by inhibiting or

promoting the formation of electrostatic interactions between histones and DNA. Additionally, some modifications function as docking sites for specific "reader" domains of other regulatory proteins such as chromatin remodelers. For example, bromodomains recognize acetylated histone lysine residues, while chromodomains recognize methylated lysine residues (Clapier and Cairns 2009).

Histone acetylation is the first identified and best studied histone modification. It is catalyzed by histone acetyltransferases (HATs), enzymes which catalyze the acetylation of the ε-amine of histone lysine residues with acetyl-CoA as the acetyl donor. This modification neutralizes the positive charge of the lysine residues and prevents them from forming electrostatic bonds with the negatively charged phosphates of the DNA backbone, resulting in a more relaxed chromatin structure. Eukaryotic HATs are divided into five major families: HAT1, Gcn5/PCAF, MYST, p300/CBP and the fungal-specific Rtt109 (Marmorstein and Zhou 2014). Histone deacetylases (HDACs) catalyze the cleavage of acetyl groups from histone lysine residues and utilize either zinc or NAD+ as cofactors for catalysis. Human HDACs are divided into four families according to their homology to yeast HDACs: classes I (homologous to yeast Rpd3), II (homologous to yeast Hda1), III (also known as sirtuins) and IV (homologous to yeast Hos3) (Park and Kim 2020). In general, histone deacetylation promotes chromatin condensation and transcriptional repression.

2.1.3.2. Chromatin remodeling

Chromatin-remodeling complexes utilize ATP hydrolysis to slide histones along DNA or evict them from DNA. Eukaryotic remodelers share a conserved ATPase domain and are classified into four families based on their unique flanking domains: switch/sucrose non fermentable (SWI/SNF), imitation switch (ISWI), chromodomain helicase DNA-binding (CHD) and inositol requiring 80 (INO80) (Clapier and Cairns 2009). Their ATPase domain uses DNA translocation to disrupt contacts between histones and DNA and drives DNA along the histone surface (Clapier *et al.* 2017). Coordination of their activities ensures proper density and spacing of nucleosomes along the genome and enables regulation of gene transcription at the level of promoter chromatin structure remodeling.

The yeast SWI/SNF remodeler family comprises the SWI/SNF and the RSC complex, with their respective ATPase subunits Snf2 and Sth1 (Clapier and Cairns 2009). Their catalytic

ATPase subunit includes two RecA-like lobes flanking a small conserved insertion, an N-terminal helicase-SANT-associated (HSA) that binds actin or actin-related proteins (Arps), a post-HSA domain, and a C-terminal bromodomain. Additionally, RSC complex subunits Rsc1, Rsc2 and Rsc4 contain tandem bromodomains (Zhang *et al.* 2010). Both yeast complexes contain an Arp protein heterodimer, Arp7/Arp9, which regulates the ATPase subunit activity (Clapier *et al.* 2016). This remodeler family can slide or evict histones, making chromatin more accessible for binding of transcriptional activators or repressors (Clapier *et al.* 2017). Due to their important regulatory roles, alterations in various human SWI/SNF subunits have been linked to several cancers and neurological diseases (Shain and Pollack 2013).

2.1.3.3. The yeast *PHO5* gene as a model for transcriptional regulation through chromatin remodeling

Inorganic phosphate (P_i) is a crucial nutrient because it is incorporated into nucleic acids, phospholipids, and ATP, and is often limiting in the environment of microorganisms such as yeast. The expression of genes encoding proteins involved in phosphate homeostasis, such as genes for phosphate uptake, export, and storage, must be highly coordinated. Such genes encode secreted acid phosphatases that release P_i from environmental substrates (Pho5, Pho3, Pho11 and Pho12), a vacuolar alkaline phosphatase (Pho8), P_i membrane importers with high (Pho84 and Pho89) and low (Pho87 and Pho90) affinity, and others (Austin and Mayer 2020). Some of these genes are constitutively expressed, however ~20 of them are highly regulated, forming the phosphate-responsive (*PHO*) regulon regulated by the sequence-specific transcriptional activator Pho4. Pho4 is constitutively expressed and imported into the nucleus, but upon high intracellular P_i levels it is phosphorylated by the cyclin/cyclin-dependent kinase complex Pho80/Pho85 at five specific serine residues, leading to its nuclear export (Komeili and O'Shea 1999). Upon P_i limitation, the Pho80/Pho85 complex is inactivated by Pho81, leaving the unphosphorylated Pho4 in the nucleus, where it can bind the CACGTG element of the Upstream Activating Sequence phosphate (UASp) in the promoters of PHO genes, leading to their transcriptional activation (Korber and Barbaric 2014).

One of *PHO* regulon genes is the *PHO5* gene, which encodes a periplasmic acid phosphatase and has become a widely used model system for eukaryotic gene regulation at the level of promoter chromatin structure over the last three decades (Korber and Barbaric 2014). The *PHO5* gene became interesting in the 1980s due to its promoter region which is covered by five

nucleosomes in the repressed state but is remodeled into a ~600 bp NDR upon gene induction (Almer *et al.* 1986). This extensive transition of chromatin structure is atypical even for eukaryotic regulated genes, as usually only one nucleosome is remodeled to activate transcription (Shivaswamy *et al.* 2008). To activate *PHO5* gene transcription, the pioneer TF Pho4 binds to the low affinity UASp1 element located in a short NDR between nucleosomes numbered -2 and -3, in cooperation with the transcriptional coactivator Pho2. Chromatin modifiers and remodelers are recruited to the promoter, leading to removal of histones, and exposing a high affinity UASp2 element and two Pho2 binding sites (previously covered by nucleosome -2), and the TATA box/TSS (previously covered by nucleosome -1), allowing full gene induction (Korber and Barbaric 2014). Many chromatin modifiers and remodelers are targeted to the *PHO5* gene promoter to maintain the tightly repressed state or to enable the large chromatin transition required for promoter opening. For example, the H3K4 histone methyltransferase Set1 and the histone deacetylase Rpd3 introduce histone modifications which cause chromatin condensation and maintain tight repression when intracellular P_i is abundant (Wang *et al.* 2011). When intracellular P_i becomes limiting, Pho4 binds to UASp1, and its activation domain recruits the SAGA complex with its catalytic subunit Gcn5 that acetylates promoter histones (Barbaric *et al.* 2003). Hyperacetylated histones make chromatin more accessible and serve as docking sites for bromodomains of remodeling complexes such as SWI/SNF (Syntichaki *et al.* 2000). Five remodelers from all four remodeler families are recruited to enable *PHO5* promoter opening: SWI/SNF, RSC, Chd1, Isw1 and INO80 (Barbaric *et al.* 2007, Musladin *et al.* 2014). Of these, the most abundant and only essential yeast remodeler RSC is responsible for most of the remodeling activity required for this transition (Musladin *et al.* 2014).

2.2. Non-coding RNAs

Advances in deep sequencing technologies over the last two decades revealed that eukaryotic genomes are pervasively transcribed, resulting in production of a variety of non-coding RNAs (ncRNAs). Non-coding RNAs are transcripts which are transcribed by Pol II but do not encode proteins. They are usually considered separately from known functional non-coding transcripts such as ribosomal RNAs (rRNAs) or transfer RNAs (tRNAs) (Villa and Porrua 2022). Many eukaryotic promoters seem to be intrinsically bidirectional, meaning that Poll II can transcribe in the appropriate sense direction to generate protein coding RNAs, but also in the antisense direction to generate divergent ncRNAs (Xu *et al.* 2009). Furthermore,

NDRs at the 3' end of protein-coding genes often contain cryptic promoter regions with permissive chromatin structure, resulting in transcription of non-coding antisense transcripts complementary to mRNAs of corresponding genes (Murray and Mellor 2016). The function of these transcripts is often controversial, but many of them have been implicated in gene regulatory mechanisms (Statello *et al.* 2021). Moreover, even in the case of functional ncRNAs, the regulatory effect could be provided by the ncRNA transcript itself or be due to the act of its transcription through the ORF and/or the promoter region of the corresponding coding gene (Li *et al.* 2021).

2.2.1. Classification of non-coding RNAs

Long non-coding RNAs (lncRNAs) are a highly heterogeneous class of transcripts which are distinguished from small non-coding RNAs (sncRNAs) based on their size (Till *et al.* 2018). While lncRNAs are ≥200 nt in length, sncRNAs are <200 nt and are in general not direct products of transcription but are processed from precursor transcripts by the RNA-induced silencing complex (RISC). RISC is a multi-protein complex containing Dicer, an RNase II endonuclease which cleaves long dsRNAs into 21-23 bp fragments, of which the antisense strand is incorporated into RISC as a guide. When an mRNAs complementary to the guide subsequently binds to it, it gets degraded by the RISC endonuclease Ago2 (Argonaute), through the RNA interference (RNAi) pathway (Wilson and Doudna 2013). A large number of budding yeast species including *Saccharomyces cerevisiae,* have lost the RNAi pathway during evolution and are therefore unable to generate sncRNAs (Drinnenberg *et al.* 2009). In turn, the lncRNA budding yeast transcriptome is exceptionally developed, as its lncRNAs show higher expression levels, lengths, and degree of overlap with coding genes in comparison to yeasts which kept the RNAi pathway (Alcid and Tsukiyama 2016). This makes the budding yeast *Saccharomyces cerevisiae* an ideal model organism for studying the mechanisms of lncRNA-mediated gene regulation.

Transcription of lncRNAs is subject to early termination by the NNS complex and subsequent degradation by the RNA exosome complex, so inactivation of these complexes was necessary to enable detection of these transcripts (Xu *et al.* 2009). Consequently, the classification of lncRNAs in yeast is based on their sensitivity to a particular degradation pathway or the conditions under which they are transcribed (**Figure 3**). Cryptic unstable transcripts (CUTs) are degraded by the Rrp6 catalytic subunit of the RNA exosome complex, whereas Xrn1-sensitive

Unstable Transcripts (XUTs) are degraded by the 5'-3' exoribonuclease Xrn1 (Xu *et al.* 2009, Van Dijk *et al.* 2011). In contrast, lncRNAs which are stable enough to be detected in wild-type cells are referred to as stable unannotated transcripts (SUTs) (Xu *et al.* 2009). Specific lncRNA classes are also related to yeast meiotic development, as meiotic unannotated transcripts (MUTs) show peaks in expression in meiosis, while those that show peaks in expression in respiring or sporulating cells are referred to as rsSUTs (Lardenois *et al.* 2011). It is worth noting that there are large overlaps between these classes, *e.g.*, many SUTs are degraded by the RNA exosome complex albeit less efficiently (Gudipati *et al.* 2012), and the main reason for stabilization of MUT levels in meiosis is the downregulation of Rrp6 levels (Lardenois *et al.* 2011).

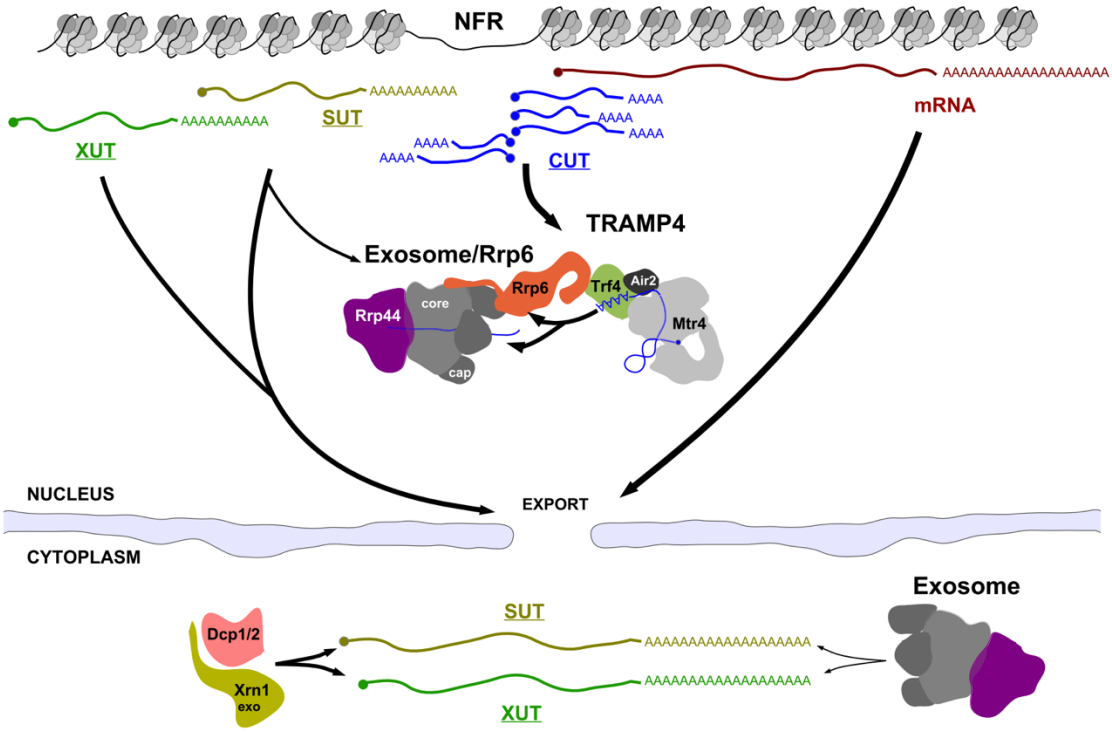

Figure 3. Nuclear and cytoplasmic degradation pathways for yeast lncRNAs. While yeast mRNAs are exported to the cytoplasm for translation, lncRNAs are subjected to nuclear or cytoplasmic degradation. CUTs are degraded almost exclusively by the nuclear RNA exosome, along with a small fraction of SUTs. Most SUTs and XUTs are preferentially exported to the cytoplasm, where they are either decapped by Dcp1/2 and degraded by Xrn1 or, less commonly, degraded by the cytoplasmic RNA exosome (Tudek *et al.* 2015).

2.2.2. Gene regulation through long non-coding RNAs

Even though non-coding transcription is a genome-wide phenomenon, only a small fraction of lncRNAs have been functionally described. As they do not encode proteins, they are mostly implicated in regulation of protein coding genes. They can act *in cis*, to influence expression of the corresponding gene at its gene locus, or *in trans*, to influence expression of the - usually complementary in sequence - gene that is transcribed at another locus. Even *in cis*, lncRNAs can be transcribed in the sense or the antisense direction through the corresponding gene's ORF and/or promoter region, leading to various regulatory outcomes. Generally, lncRNAs act by repressing the expression of the corresponding gene, although some positive regulatory roles of lncRNAs have been described (Li *et al.* 2021).

Several molecular mechanisms have been proposed for gene regulation through lncRNAs *in cis*. In the transcriptional interference model, the transcription from the lncRNA promoter interferes with that of the coding gene promoter, *i.e.*, relies on the assumption that two Pol IIs cannot transcribe the same region simultaneously because collisions may occur. In the promoter interference model, the transcription of the lncRNA through the coding gene promoter displaces transcriptional activators and other components of the transcriptional machinery that need to be assembled for coding gene transcription (Pelechano and Steinmetz 2013). Another chromatin-based model postulates that lncRNA transcription through the coding gene promoter can influence the recruitment of chromatin modifiers and remodelers which render the local chromatin structure more or less accessible for transcriptional activation at the coding gene promoter (Soudet and Stutz 2019). The chromatin-based model also represents an attractive hypothesis for how lncRNAs transcribed *in trans* could be regulating transcription of genes with lncRNA-complementary sequences in their transcription units (Camblong *et al.* 2009). It has also been shown that some lncRNA function at the post-transcriptional level by hybridizing with complementary mRNAs (Sinturel *et al.* 2015, Xie *et al.* 2019). Although yeast does not have a functional RNAi pathway, lncRNA-mRNA pairs could still inhibit nuclear export or translation of the respective mRNAs. Importantly, these mechanisms are not necessarily mutually exclusive, and they have all been shown to control gene regulation at specific yeast gene loci (Li *et al.* 2021).

Although the techniques to detect lncRNAs are now well developed, the development of techniques suitable to decipher their functions is still largely lacking (Liu and Lim 2018).

Functional studies of lncRNAs are complicated by the fact that their transcription units often overlap with those of their corresponding genes, making it difficult to block lncRNA transcription without affecting the transcription of their putative target genes. In addition, lncRNA transcription units tend to have poorly defined core promoters and TSSs, so targeting lncRNA promoters or inserting termination sequences rarely works to inactivate lncRNA transcription. CRISPRi (clustered regularly interspaced short palindromic repeats interference) is a recently developed technique to modulate lncRNA transcription levels without introducing mutations into transcription units (Gilbert *et al.* 2013). It relies on targeting a nuclease dead Cas9 (dCas9) protein to the desired genomic region through a designed guide RNA (gRNA), where the dCas9-gRNA complex provides a transcriptional block for the elongating Pol II. Interestingly, silencing is independent of the DNA strand when the CRISPRi system targets a promoter, whereas targeting a coding sequence only induces silencing of the non-template DNA strand, making this system promising for studying the effects of antisense transcription (Qi *et al.* 2013).

2.2.2.1. lncRNA-regulated yeast genes

One of the first functionally characterized yeast lncRNAs was *SER3* regulatory gene 1 (*SRG1*), which is transcribed in the sense direction through the *SER3* gene promoter region and represses *SER3* transcription (Winston *et al.* 2005). When serine is abundant, *SRG1* transcription is promoted by the activity of the transcriptional activator Cha4 together with the SAGA and SWI/SNF complexes. This leads to Spt6/Spt16 elongation factor-dependent histone deposition behind the elongating Pol II as it transcribes through the *SER3* promoter region, which negatively regulates *SER3* transcription (Hainer *et al.* 2011). Two genes encoding proteins that initiate meiosis, *IME1* and *IME4*, are also regulated *in cis* by lncRNAs. *IME1* expression is subject to negative regulation of the IME regulatory transcript (*IRT1*) which is transcribed in the sense direction through its promoter and recruits the chromatin modifiers Set2, which deposits the repressive histone mark H3K36, and Set3, a histone deacetylase. The resulting repressive chromatin conformation at the *IME1* promoter prevents the recruitment of transcriptional activators and thus gene transcription (Van Werven *et al.* 2012). Transcription of the *IRT1* transcript was demonstrated to be activated by the repressor of meiosis (Rme1) regulator. In contrast, lncRNA *IRT2* is transcribed in the antisense orientation relative to the *IME4* gene when the corresponding coding gene is not expressed, possibly repressing it through a transcriptional interference mechanism (Hongay *et al.* 2006). *GAL* genes are repressed or non-

induced upon growth in glucose or raffinose, respectively, and induced upon growth in galactose, and the *GAL1/10* divergent promoter is an example of a highly regulated chromatinized promoter (Elison *et al.* 2018). Transcription of a lncRNA is initiated under repressive conditions at the 3' end of the *GAL10* gene, resulting in increased Set2-mediated H3K36 histone methylation and Rpd3-mediated histone deacetylation across the *GAL10* coding region (Houseley *et al.* 2008). Transcription of the *cis*-acting lncRNA *pHO* is initiated upstream of the gene encoding the endonuclease HO which is responsible for mating type switching in yeast. Its transcription leads to nucleosome deposition over the *HO* promoter region and displaces the Swi4/Swi6 cell-cycle box binding factor (SBF), thereby repressing HO expression during the re-entry into the cell cycle (Yu *et al.* 2016).

2.2.2.1.1. lncRNA-based regulation of yeast *PHO* genes

The yeast *PHO84* gene is currently the best characterized model gene for lncRNA-mediated gene regulation in yeast. It encodes a high affinity P_i membrane importer and belongs to the *PHO* gene regulon (Korber and Barbaric 2014). *PHO84* expression is regulated by an Rrp6-degraded antisense lncRNA which originates from its gene ORF and is elongated through its promoter region where it recruits the histone deacetylase Hda1/2/3 complex to contribute to its repression (Camblong *et al.* 2007). Single-cell analysis showed anti-correlation of *PHO84* mRNA and antisense RNA levels and clarified that Rrp6 inactivation leads to stabilization of the antisense levels due to increased transcriptional elongation, as early termination by the NNS complex is reduced in these cells (Castelnuovo *et al.* 2013). A subsequent transcriptome-wide study defined a class of 28 genes similar to *PHO84*, which are repressed by antisense transcription through a mechanism dependent on the histone deacetylases Hda1 and Rpd3, as well as the histone methyltransferase Set1 (Castelnuovo *et al.* 2014). Interestingly, there is evidence that the *PHO84* antisense RNA can also act *in trans*, as introduction of an additional copy of the *PHO84* gene results in the co-silencing of both gene copies, and that this mechanism is dependent of Set1, but independent on Hda1 (Camblong *et al.* 2009). In contrast, the *PHO5* gene, which belongs to the same gene regulon as *PHO84*, is one of the rare examples where non-coding antisense transcription has been reported to play a positive gene regulatory role (Uhler *et al.* 2007). This is based on the finding that active transcription is required for the physiological kinetics of *PHO5* promoter chromatin opening and on the fact that a construct in which an *URA3* marker gene was inserted in place of a large portion of the 3' *PHO5* ORF

sequence (to inactivate antisense transcription) exhibits slower chromatin opening kinetics (Uhler *et al.* 2007).

2.3. The RNA exosome complex

The RNA exosome is a highly conserved complex that catalyzes 3'-to-5' processing and degradation of practically all types of RNA substrates in eukaryotic cells (Zinder and Lima 2017). The RNA exosome was first identified by the Tollervey laboratory in 1997 in yeast *Saccharomyces cerevisiae* as the complex required for 3' processing of the 5.8S rRNA (Mitchell *et al.* 1997), which is why most yeast exosome subunits are referred to as Rrp (ribosomal RNA processing) proteins. In contrast, the human exosome subunits are referred to as EXOSC (exosome components) proteins. The RNA exosome has subsequently been found in all studied eukaryotic organisms, and a similar complex was also discovered in Archaea (Lykke-Andersen *et al.* 2009). This complex is found in the nucleus and the cytosol of eukaryotic cells, and the two isoforms differ somewhat in structure and function. The nuclear exosome is responsible for processing rRNAs, small nuclear RNAs (snRNAs) and small nucleolar RNAs (snoRNAs) (Allmang *et al.* 1999). Furthermore, the exosome degrades hypomodified tRNAs and is involved in normal turnover of mature tRNAs and mRNAs (Kadaba *et al.* 2004, Gudipati *et al.* 2012). Both exosome isoforms are involved in RNA surveillance by targeting improperly processed RNAs (Houseley *et al.* 2006). In this context, the cytoplasmic exosome plays a role in many RNA quality control pathways such as nonsense-mediated decay (NMD), non-stop decay (NSD), and the no-go decay (NGD), which target transcripts containing premature termination codons, transcripts lacking termination codons and transcripts with delays in translational elongation, respectively (Łabno *et al.* 2016). Important targets of the nuclear exosome are unstable non-coding RNAs termed CUTs in yeast (Xu *et al.* 2009) and promoter upstream transcripts (PROMPTs) or upstream antisense RNAs (uaRNAs) in human cells (Preker *et al.* 2008, Flynn *et al.* 2011).

The nine structural subunits of the RNA exosome core associate to form a barrel shaped structure comprised of a central RNAse PH-like 6-subunit ring (EXOSC4-9; Rrp41/42/43/45/46/Mtr3) and a S1/KH-like 3-subunit cap (EXOSC1-3; Csl4/Rrp4/Rrp40) (**Figure 4**). The catalytic subunit of the exoribonuclease and endoribonuclease Dis3 (also known as Rrp44 in yeast and DIS3 in humans) associates with the 6-subunit ring at its base, thereby forming the 10-subunit core exosome which is present in both the nucleus and cytosol

(Makino *et al.* 2013). Whereas yeast cells encode only one Dis3 protein, human cells encode two Dis3 enzymes, DIS3 and DIS3L, localized in the nucleus and cytoplasm, respectively (Tomecki *et al.* 2010). All Dis3 variants catalyze processive Mg^{2+}-dependent hydrolytic 3′-to-5′ exoribonuclease activity (Dziembowski *et al.* 2007, Tomecki *et al.* 2010), whereas yeast Dis3 and human DIS3 additionally contain a PIN domain which catalyzes distributive Zn^{2+}/Mn^{2+}-dependent endoribonuclease activity (Schneider *et al.* 2009). The second catalytic Rrp6 binds the 3-subunit cap only in the yeast nuclear isoform of the complex and is enriched in the nucleoplasm and the nucleoli of human cells (Tomecki *et al.* 2010, Makino *et al.* 2013). Rrp6 catalyzes the Mg^{2+}-dependent distributive 3′-to-5′ hydrolytic exoribonuclease activity (Burkard and Butler 2000). In recent years, several structural studies have elucidated how RNA substrates are processed or degraded by the RNA exosome. The classical degradation pathway is that single-stranded RNA substrates are shuttled through the central channel of the ring structure to the Dis3 catalytic subunit, where they are degraded in a processive manner (Makino *et al.* 2013). However, substrates can also be targeted to the Dis3 PIN domain directly through channel-independent mechanisms (Makino *et al.* 2015) or to the second catalytic subunit Rrp6 *via* the cap structure for distributive processing or degradation (Wasmuth *et al.* 2014).

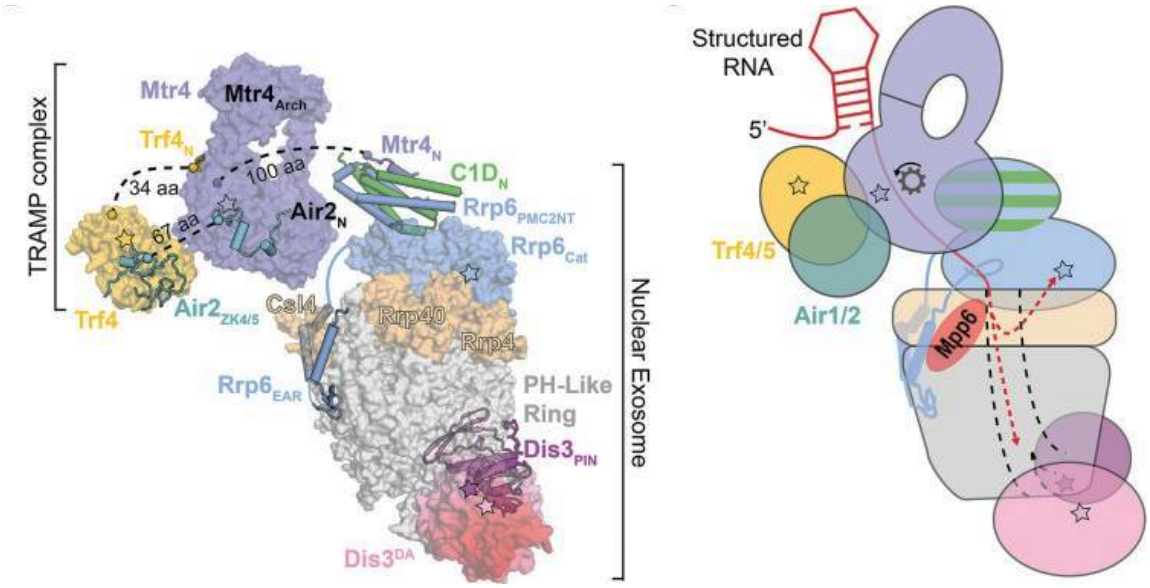

Figure 4. Structures of the nuclear RNA exosome and its cofactors. The RNA exosome interacting with an RNA substrate and its nuclear cofactors, the TRAMP complex, Mpp6 and C1D (Rrp47). The catalytic sites are indicated by stars, the central channel by black dashed lines, and RNA by a red line (Zinder and Lima 2017).

Because of the extensive role of the RNA exosome in regulation of gene expression, this complex is important for viability and development in eukaryotes. Genes encoding all core exosome subunits are essential for viability in yeast, while Rrp6 is required for growth at high temperatures and for progression through meiosis and gametogenesis (Briggs *et al.* 1998, Lardenois *et al.* 2011). In *Drosophila* and mice, all exosome components studied to date have been shown to be essential for viability or embryogenesis (Tomecki *et al.* 2014, Petit *et al.* 2022), while EXOSC10 has been shown to be important for progression through the mitotic cell cycle in cultured human cancer cells (Blomen *et al.* 2015). It is therefore not surprising that mutations in genes encoding RNA exosome subunits cause rare diseases, collectively termed exosomopathies. These disorders are usually due to missense mutations in genes encoding structural exosome subunits, as more extensive mutations are unlikely to be compatible with life. Exosomopathies present with different clinical profiles, but what they have in common is abnormal development or degeneration of the cerebellum, also known as cerebellar hypoplasia or pontocerebellar hypoplasia (Morton *et al.* 2020). Studies in model organisms such as yeast, *Drosophila* and zebrafish have been very informative in modeling of the molecular consequences of RNA exosome mutations found in patients to determine how they affect the stability and function of this complex (Amorim *et al.* 2020). Mutations in genes encoding exosome subunits are also associated with cancer, as the *DIS3* gene, which encodes the exosome catalytic subunit, has been identified in multiple screens as one of the most mutated genes associated with multiple myeloma (Chapman *et al.* 2011, Lohr *et al.* 2014).

2.3.1. Exoribonuclease Rrp6

The exoribonuclease Rrp6 is the nuclear-specific catalytic subunit of the RNA exosome complex, which participates in all nuclear exosome-related processing and degradation pathways. The human EXOSC10 protein was originally discovered as the PM/Scl-100 auto-antigen, as its autoantibodies were first found in patients suffering autoimmune disorders polymyositis, scleroderma, and their overlaps (Gelpi *et al.* 1990). In yeast, 733 amino acid residues of Rrp6 are divided into at least four functional domains. The N-terminal domain of polycystin 2 (PMC2NT) binds its stabilization partner Rrp47 (Costello *et al.* 2011). The central region forms the catalytic module (CAT) comprised of the exoribonuclease (EXO) domain and the helicase and RNase D carboxy terminal (HRDC) domain which regulates the activity of the EXO domain. The DEDD active site of the enzyme is located in the EXO domain, in which negatively charged aspartate and glutamate residues coordinate two metal

ions which deprotonate and activate a water molecule that carries out a nucleophilic attack on the RNA phosphodiester bond (Phillips and Butler 2003). The C-terminal region of Rrp6 comprises the exosome associating region (EAR), which interacts with the RNA exosome (Makino *et al.* 2013, Wasmuth *et al.* 2014) and the highly basic and unstructured Rrp6 C-terminal tail which binds RNA (Wasmuth and Lima 2017). The C-terminal regions also contain a putative bipartite nuclear localization signal (NLS) (Phillips and Butler 2003).

It is now known that the two exosome catalytic subunits, Rrp6 and Dis3, have both shared and distinct RNA substrates (Gudipati *et al.* 2012, Schneider *et al.* 2012). Interestingly, Rrp6 also has a non-catalytic function which contributes to regulation of the RNA exosome and stimulates degradation by Dis3. This function is fulfilled by the unstructured C-terminal tail of Rrp6 known as the lasso, which binds RNA (Wasmuth and Lima 2017). Work with the evolutionarily distant yeast *Schizosaccharomyces pombe* also showed Rrp6 to have some non-catalytic protein-dependent functions by acting as an adaptor for specific RNA substrates that are degraded by the core exosome (Mukherjee *et al.* 2016). Mutants for Rrp6 catalytic activity also show less pronounced temperature sensitivity compared with the *rrp6* deletion mutant, although the underlying cause for this phenotype remains to be determined (Phillips and Butler 2003).

2.3.2. Cofactors of the RNA exosome complex

Exosome cofactors mediate interactions between the RNA exosome and its RNA substrates. Because only single-stranded RNAs are suitable substrates for exosome degradation or processing, association of the RNA exosome with a cofactor that provides RNA helicase activity is often essential for its activation *in vivo* (Weick and Lima 2021). Important activators of the RNA exosome in the nucleus and the cytosol are the Trf4/Trf5-Air1/Air2-Mtr4 polyadenylation (TRAMP) complex and the Superkiller (Ski) complex, respectively, while the nuclear exosome additionally associates with the monomeric cofactors Rrp47 and M-phase phosphoprotein 6 (Mpp6).

The TRAMP complex is a heterotrimeric complex comprised of the essential helicase subunit Mtr4, a poly-A polymerase subunit Trf4 or Trf5 and a zinc finger RNA-binding subunit Air1 or Air2 (ZCCHC7 in humans). TRAMP assists the nuclear exosome in degrading CUTs and processing rRNAs, snRNAs and snoRNAs (LaCava *et al.* 2005, Wyers *et al.* 2005). A model for TRAMP activity postulates that its polyadenylation activity generates 3′ single-stranded

tails that are long enough to be captured by Mtr4 which then catalyzes further unwinding of RNA to generate a single-stranded RNAs long enough to be threaded into the central channel of the RNA exosome (Zinder and Lima 2017). Analysis of yeast TRAMP complex isoforms indicates that Air1 interacts with both Trf4 and Trf5, while Air2 interacts only with Trf4, resulting in three distinct TRAMP isoforms (Delan-Forino *et al.* 2020). Simultaneous *trf4 trf5* deletion is inviable (Castaño *et al.* 1996), while the Air subunits are dispensable for viability (LaCava *et al.* 2005). In metazoans, the TRAMP complex is restricted to the nucleolus, but MTR4 is a part of at least two nucleoplasmic complexes: the nuclear exosome targeting (NEXT) and the poly-A tail exosome targeting (PAXT) complex. The NEXT complex degrades short transcripts lacking mature poly-A tails and besides MTR4 contains the RNA-binding protein RBM7 and the zinc finger protein ZCCHC8 while the PAXT complex targets longer polyadenylated transcripts and besides MTR4 contains the zinc finger protein ZFC3H1 that interacts with RNA-binding proteins such as nuclear poly-A binding protein PABPN (Meola *et al.* 2016).

Rrp47 (also known as Lrp1 in yeast and C1D in humans) is a nuclear exosome cofactor which forms a very stable complex with the N-terminal PMC2NT domain of Rrp6. The interaction between the N-terminal regions of Rrp47 and Rrp6 stabilizes Rrp6 (Feigenbutz *et al.* 2013, Stuparevic *et al.* 2013), but also provides an interface for interaction with Mtr4 helicase of the TRAMP complex (Schuch *et al.* 2014). The monomeric cofactor Mpp6 associates with the exosome cap protein Rrp40 *via* its central region and with Mtr4 *via* its N-terminal region (Wasmuth *et al.* 2017), supporting the roles of both Rrp47 and Mpp6 as adapters for recruitment of the TRAMP complex to the RNA exosome.

The cytoplasmic Ski complex comprises the RNA helicase Ski1, the scaffold protein Ski3, and two copies of the WD40- repeat protein Ski8p (van Hoof *et al.* 2000). Yeast cells also contain the Ski7 protein which bridges the Ski complex with the exosome (Araki *et al.* 2001). The Ski complex assists the cytoplasmic RNA exosome in mRNA turnover and co-translational RNA surveillance decay pathways (Łabno *et al.* 2016). Consistent with its critical role in RNA decay, inactivation of the Ski complex is synthetically lethal with inactivation of the Xrn1-guided 5'-to-3' decay pathway (Halbach *et al.* 2013).

2.4. Regulation of genes related to yeast cell wall integrity through RNA-based mechanisms

Yeast cells are encapsulated by an essential structure absent from mammalian cells, the cell wall. The inner layer of the cell wall is composed mainly of the carbohydrate polymers **β**-1,3-glucan, **β**-1,6-glucan, and chitin, while the outer layer is formed of highly glycosylated mannoproteins that are covalently or non-covalently bound to the carbohydrate components (Klis 1994). The cell wall determines cell shape, provides osmotic resistance, and shields the cell from various environmental stressors. To survive exposure to these stressors, the cell wall needs to be remodeled. This occurs by regulating the expression of genes encoding proteins that serve as structural cell wall components, enzymes that control modifications and cell wall-binding of structural proteins and enzymes that remodel the carbohydrate network (Teparić and Mrša 2013). This condition-specific cell wall stress response must occur rapidly to maintain cell wall integrity under stress and is achieved through a variety of regulatory mechanisms. The best characterized of these mechanisms is the conserved fungal cell wall integrity pathway (CWI) which is controlled by the mitogen-activated protein kinase (MAPK) Slt2. The CWI regulatory cascade is activated through membrane receptors, leading to phosphorylation events that converge in the phosphorylated Slt2, that activates two TFs: Rlm1 (which primarily regulates CWI-responsive genes) and SBF (Swi4/6; which primarily regulates cell cycle genes) (Sanz *et al.* 2022). Other transcriptional responses to cell wall stress require signal transduction through the high osmolarity glycerol pathway (HOG) and the protein kinase A (PKA) pathway (Levin 2005).

Post-transcriptional regulation of genes related to cell wall integrity is much less well studied. RNA-based mechanisms of post-transcriptional regulation are generally accomplished through RNA-binding proteins, which can act as ribonucleases, direct localization of mRNAs to specific cellular compartments or serve as adaptors for other regulatory proteins (Hall and Wallace 2022). For example, the ribonuclease Rnt1, specific for double-stranded RNA (dsRNA), has been shown to degrade mRNAs encoding proteins involved in morphogenesis and the CWI pathway to optimize the cell's stress response (Catala *et al.* 2012). Another well-studied RNA-binding protein which regulates cell wall integrity is the Ssd1 protein, which binds to untranslated regions (UTRs) of mRNAs encoding glucanases and chitinases required for cell separation and directs them for incorporation into processing bodies (P-bodies) and stress granules for storage, thereby preventing their translation (Kurischko *et al.* 2011). Ssd1 is evolutionarily related to RNases of the eukaryotic Dis3L2 family, but its nuclease activity has

been lost and a new RNA-binding site has been acquired in certain fungi (Ballou *et al.* 2020, Bayne *et al.* 2022). Other RNA-binding proteins such as Pub1 and Mrn1 have been shown to bind U-rich elements at 3' UTRs of cell wall-related mRNAs, but their precise functions remain to be elucidated (Hogan *et al.* 2008, Reynaud *et al.* 2021). Yeast cell wall integrity could also be regulated through the family of RNA-binding PUF proteins. Puf1 and Puf2 have been shown to negatively regulate the abundance of *ZEO1* mRNA, which encodes a plasma membrane protein involved in cell wall stress response (Haramati *et al.* 2017). Another PUF protein, Puf5, regulates the CWI pathway by negatively regulating *LRG1* mRNA, which encodes a GTPase activating protein for the small GTPase Rho1 (Viet *et al.* 2018). Finally, recent work showed that inactivation of the CWI pathway aggravates the temperature sensitive phenotype of RNA exosome mutants (Wang *et al.* 2020), hinting at a potential role of the role of this ribonuclease complex in maintenance of yeast cell wall stability.

3. SCIENTIFIC PAPERS

3.1. List of scientific papers

1. **Novačić, A.**, Beauvais, V., Oskomić, M., Štrbac, L., Dantec, A. L., Rahmouni, A. R., & Stuparević, I. (2021). Yeast RNA exosome activity is necessary for maintaining cell wall stability through proper protein glycosylation. *Molecular biology of the cell*, *32*(5), 363-375.

2. **Novačić, A.**, Šupljika, N., Bekavac, N., Žunar, B., & Stuparević, I. (2021). Interplay of the RNA Exosome Complex and RNA-Binding Protein Ssd1 in Maintaining Cell Wall Stability in Yeast. *Microbiology Spectrum*, *9*(1), e00295-21.

3. **Novačić, A.**, Vučenović, I., Primig, M., & Stuparević, I. (2020). Non-coding RNAs as cell wall regulators in *Saccharomyces cerevisiae*. *Critical reviews in microbiology*, *46*(1), 15-25.

4. **Novačić, A.**, Menéndez, D., Ljubas, J., Barbarić, S., Stutz, F., Soudet, J., & Stuparević, I. (2022). Antisense non-coding transcription represses the *PHO5* model gene at the level of promoter chromatin structure. *PLoS Genetics*, *18*(10), e1010432.

Paper 1

Novačić, A., Beauvais, V., Oskomić, M., Štrbac, L., Dantec, A. L., Rahmouni, A. R., & Stuparević, I. (2021). Yeast RNA exosome activity is necessary for maintaining cell wall stability through proper protein glycosylation. *Molecular biology of the cell*, *32*(5), 363-375.

Yeast RNA exosome activity is necessary for maintaining cell wall stability through proper protein glycosylation

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ABSTRACT **Nuclear RNA exosome is the main 3′→5′ RNA degradation and processing complex in eukaryotic cells and its dysregulation therefore impacts gene expression and viability. In this work we show that RNA exosome activity is necessary for maintaining cell wall stability in yeast *Saccharomyces cerevisiae*. While the essential RNA exosome catalytic subunit Dis3 provides exoribonuclease catalytic activity, the second catalytic subunit Rrp6 has a noncatalytic role in this process. RNA exosome cofactors Rrp47 and Air1/2 are also involved. RNA exosome mutants undergo osmoremedial cell lysis at high temperature or at physiological temperature upon treatment with cell wall stressors. Finally, we show that a defect in protein glycosylation is a major reason for cell wall instability of RNA exosome mutants. Genes encoding enzymes that act in the early steps of the protein glycosylation pathway are down-regulated at high temperature in cells lacking Rrp6 protein or Dis3 exoribonuclease activity and overexpression of the essential enzyme Psa1, that catalyzes synthesis of the mannosylation precursor, suppresses temperature sensitivity and aberrant morphology of these cells. Furthermore, this defect is connected to a temperature-dependent increase in accumulation of noncoding RNAs transcribed from loci of relevant glycosylation-related genes.**

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INTRODUCTION

In eukaryotic cells, 3′→5′ RNA degradation and processing is accomplished through activity of the RNA exosome complex (Chlebowski *et al.*, 2013; Zinder and Lima, 2017; Lingaraju *et al.*, 2020). It plays a major part in RNA metabolism in the nucleus and cytoplasm because it targets almost all RNA classes: its roles include RNA surveillance; mRNA turnover; processing and maturation of rRNAs, snRNAs, and snoRNAs; and degradation of noncoding transcripts (Allmang *et al.*, 1999a; Hilleren *et al.*, 2001; Wyers *et al.*, 2005). It is therefore not surprising that dysregulation of RNA exosome activity broadly impacts gene expression (Van Dijk *et al.*, 2007; Lardenois *et al.*, 2011; Gudipati *et al.*, 2012; Schneider *et al.*, 2012; Bresson *et al.*, 2017; Davidson *et al.*, 2019) and is also implicated in various human malignancies and disorders (Fasken *et al.*, 2020). Rare diseases caused by mutations in genes that encode human exosome subunits (EXOSC proteins) have been termed exosomopathies. They usually encompass single amino acid substitutions rather than more substantial mutations, as RNA exosome activity is essential for viability (de Amorim *et al.*, 2020).

The central part of the highly conserved RNA exosome complex is the exosome core (Exo9). It encompasses nine subunits that form a doughnut-shaped channel that has a structural and regulatory role (Wasmuth and Lima, 2012; Wasmuth *et al.*, 2014). Catalytic activity is provided by two additional subunits: Rrp6, which has exonuclease activity, and Dis3/Rrp44, which has exonuclease and endonuclease

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Abbreviations used: CFW, Calcofluor White; ChIP, chromatin immunoprecipitation; CR, Congo Red; CUTs, cryptic unstable transcripts; CWI, cell wall integrity; EAR, exosome interacting region; ncRNAs, noncoding RNAs; RT-qPCR, reverse-transcription quantitative PCR; TSS, transcription start site.

activities (Briggs *et al.*, 1998; Dziembowski *et al.*, 2007; Lebreton *et al.*, 2008). In yeast, Dis3 is found in both the nuclear and the cytoplasmic isoforms of the exosome complex, whereas Rrp6 is only found in the nuclear isoform, where it additionally associates with its stabilization partner Rrp47 to form the 12-subunit complex Exo-12$^{Dis3/Rrp6/Rrp47}$ (Feigenbutz *et al.*, 2013; Makino *et al.*, 2015). Activity of the nuclear RNA exosome is also stimulated by its cofactors Mpp6 and the TRAMP complex, which function to guide substrate specificity and aid RNA degradation (Schilders *et al.*, 2005; Stuparevic *et al.*, 2013; Wasmuth *et al.*, 2017). The three-subunit TRAMP complex provides RNA-binding (Air1 or Air2), RNA-helicase (Mtr4), and poly(A)-polymerase (Trf4 or Trf5) activities, which play a major role in noncoding RNA degradation (LaCava *et al.*, 2005; Wyers *et al.*, 2005).

All core exosome subunits, as well as catalytic subunit Dis3, are essential in yeast (Mitchell *et al.*, 1997). In contrast, deletion of the gene encoding the catalytic subunit Rrp6 is viable; however, it results in slow growth at physiological temperature and temperature sensitivity (Allmang *et al.*, 1999b; Phillips and Butler, 2003). Interestingly, these two phenotypes are not both caused by the lack of Rrp6 catalytic activity, as Rrp6 catalytic mutants also grow slowly at physiological temperature, but are not temperature sensitive (Phillips and Butler, 2003). Because of that, it has long been clear that Rrp6 has a noncatalytic role in maintaining cell viability upon heat stress, but the molecular nature of this predicament has not been explained. Recent work connected RNA degradation to the cell wall integrity (CWI) pathway, which regulates gene expression to ensure cellular integrity upon stress, through a MAPK signaling cascade (Catala *et al.*, 2012; Wang *et al.*, 2020). Involvement of Rrp6 in this process was inferred from the additive cell wall instability phenotype of *rrp6*Δ *mpk1*Δ mutant cells, in which CWI signal transduction is inhibited (Wang *et al.*, 2020). Specifically, a role was proposed for a solitary "moonlighting" function of Rrp6, independent of other exosome subunits and its interactors Rrp47 and Isw1, in maintaining CWI at high temperature (Wang *et al.*, 2020).

In this work, we show that the RNA exosome complex is a major regulator of yeast cell wall stability. Exoribonuclease catalytic activity of the Dis3 subunit is essential for maintaining cellular integrity upon heat stress or treatment with cell wall stressors, together with the second catalytic subunit Rrp6 that has a noncatalytic role in this process. The RNA exosome cofactors Rrp47 and Air1/2 also contribute in a significant way. Cells lacking these proteins or Dis3 exoribonuclease activity are not viable at high temperature because of compromised cell wall stability. Importantly, cell bursting and aberrant cell morphology of RNA exosome mutants are suppressed by osmotic support, as well as by overexpression of the Psa1 enzyme, which enables increased production of GDP-mannose that is incorporated into mannoproteins, indicating that protein glycosylation is a major reason for cell wall instability of RNA exosome mutants. Expression of protein glycosylation-related genes *PSA1*, *DPM1*, and *ALG7* is dysregulated in these mutants at high temperature, presumably through mechanisms that involve accumulation of specific noncoding RNAs transcribed from their gene loci.

RESULTS

RNA exosome mutants undergo osmoremedial cell lysis at high temperature

All subunits of yeast RNA exosome complex are essential for viability except for the nuclear-specific catalytic subunit Rrp6, whose inactivation is lethal only above 37°C (Allmang *et al.*, 1999b; Phillips and Butler, 2003). The reason for temperature sensitivity of the *rrp6*Δ mutant remained unknown, especially because Rrp6 catalytic mutants are not temperature sensitive (Phillips and Butler, 2003), and lack of Rrp6 was not linked to significant RNA processing defects at high temperature (Allmang *et al.*, 2000). To test whether *rrp6*Δ cells are inviable at 37°C due to compromised cellular integrity, we supplemented the growth medium with 1 M sorbitol, which acts as osmotic support. We performed all experiments in the W303-derived BMA41 genetic background in which Rrp6-related phenotypes are most pronounced (Klauer and Van Hoof, 2013; Wasmuth and Lima, 2017). Interestingly, osmotic stabilization of the growth medium completely suppressed its temperature-sensitive phenotype and enabled wild-type level of growth after 3 d at 37°C on both YPD and synthetic YNB mediums (Figure 1A). This was due to osmotic stabilization and not sorbitol itself, because the addition of 1 M sucrose, NaCl, or KCl led to a similar level of suppression (Supplemental Figure S1). Also, this effect was not specific to BMA41 genetic background, as growth at 37°C could also be restored with the less temperature-sensitive *rrp6*Δ haploid BY4741 and diploid JHY222 genetic backgrounds (Figure 1B).

Osmotic instability results in cell lysis, so we grew cells in liquid medium for 3 d at 37°C and measured the activity of alkaline phosphatase released into the medium. Alkaline phosphatase is an intracellular enzyme so its release into the medium implies membrane and cell wall lysis. Cells lacking Rrp6 released almost a fivefold higher amount of alkaline phosphatase than wild-type or Rrp6-Y361A catalytic mutant cells, and cell lysis in all strains was completely suppressed by the addition of 1 M sorbitol (Figure 1C) or upon growth at 30°C regardless of sorbitol addition (unpublished data). As higher activity measured with *rrp6*Δ cells could also be due to a change in expression of alkaline phosphatase, we measured intracellular alkaline phosphatase activity with the same cells and found practically no differences between the strains (Supplemental Figure S2), confirming that the extracellular activity observed for the *rrp6*Δ strain is indicative of cell lysis. We also examined the cells by fluorescent microscopy after Calcofluor White (CFW) staining. CFW stains chitin, which in yeast is localized primarily in bud necks and bud scars, as it forms the primary septum (Klis *et al.*, 2002). It was revealed that morphology of *rrp6*Δ cells without osmotic support at 37°C was also consistent with weakened cellular integrity, as the cells were enlarged, unevenly shaped, and grew in clumps (Figure 1D). Based on the intensive staining of cell septa by CFW, it was clearly visible that two or more *rrp6*Δ cells stuck together at their bud necks, meaning the clumps result from a defect in cell separation after division (Figure 1D).

Deletion of the *DIS3* gene encoding the second exosome catalytic subunit is lethal, but it is possible to generate mutants deficient in Dis3 exo- or endoribonuclease activity (Dziembowski *et al.*, 2007; Lebreton *et al.*, 2008). Exo$^-$ (*dis3-D551N*) mutant displays temperature sensitivity (Drąẓkowska *et al.*, 2013; Milbury *et al.*, 2019), so we wondered whether the cause is similar as for *rrp6*Δ. Indeed, the addition of 1 M sorbitol restored growth and morphology of this mutant at 37°C and suppressed its cell lysis, as measured by the release of alkaline phosphatase (Figure 2, A–C). Furthermore, we tested viability at 37°C of mutants in monomeric cofactors Mpp6 and Rrp47, as well as viable mutants in subunits of the TRAMP complex, which function as coactivators of the nuclear exosome, and found that the temperature sensitivity and the temperature-induced cell lysis of *air1*Δ*air2*Δ and *rrp47*Δ mutants are also suppressed by osmotic stabilization (Figure 2, D and E). Taken together, temperature-sensitive mutants of RNA exosome catalytic subunits *rrp6*Δ and *dis3 exo*$^-$, as well as mutants in exosome cofactors Rrp47 and Air1/2, undergo osmoremedial cell lysis at 37°C, which is a phenotype indicative of a weakened cell wall.

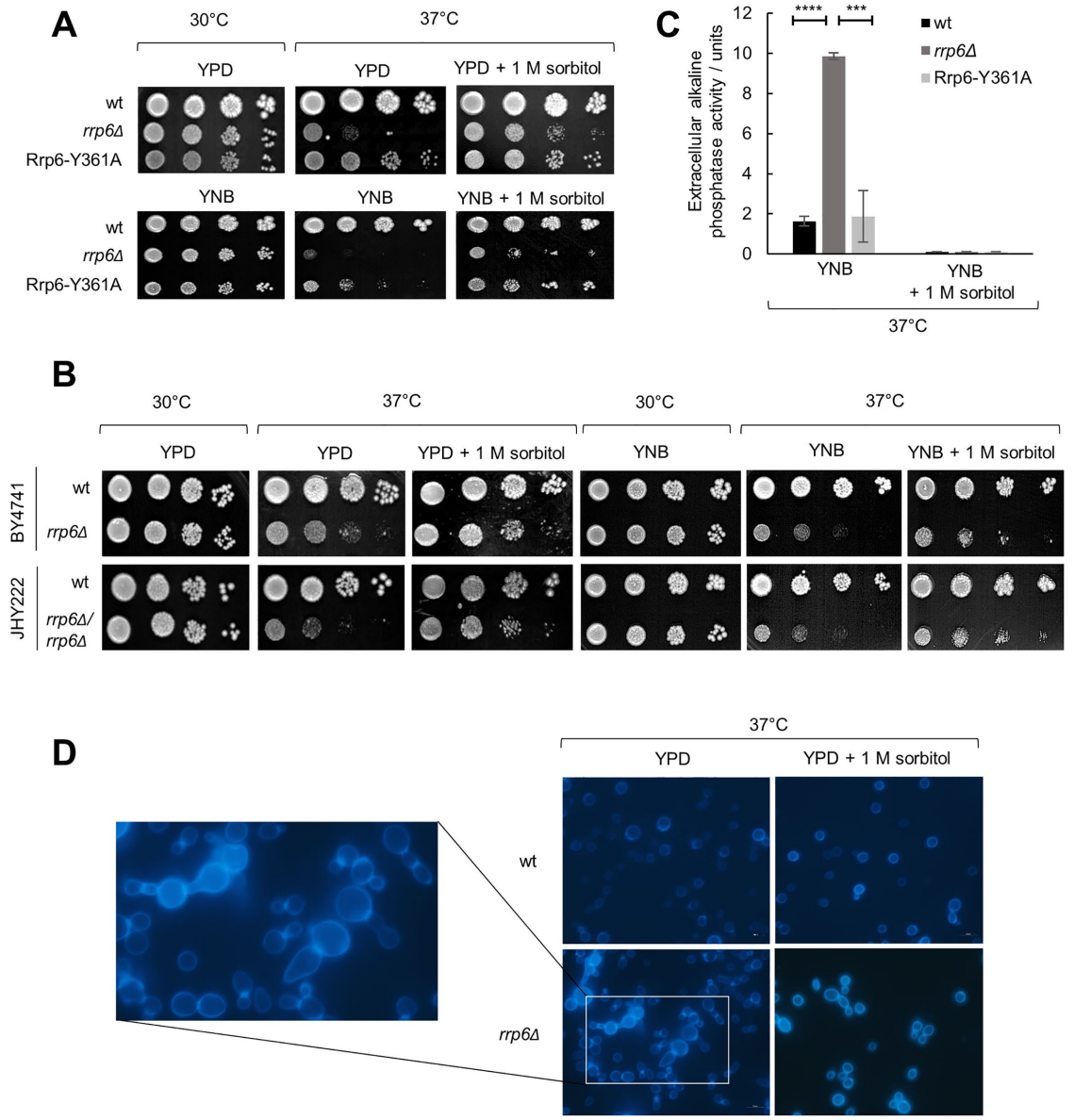

FIGURE 1: Cells lacking Rrp6 display phenotypes indicative of cell wall instability. Strains are BMA41 wild type (wt) and isogenic mutants, unless noted otherwise. Tenfold serial dilutions of cells were spotted on plates and were photographed after 3 d at indicated temperature. (A) Osmotically supporting medium with 1 M sorbitol rescues growth of *rrp6*Δ cells at high temperature. (B) Osmotic support rescues growth of *rrp6*Δ mutants of other genetic backgrounds (haploid BY4741 and diploid JHY222) at high temperature. (C) *rrp6*Δ cells burst at high temperature, unless osmotic support is provided. Strains were grown for 3 d at 37°C and activity of alkaline phosphatase was measured in growth medium. Measurements were performed in duplicate, and reported values represent the means and standard deviations of three independent experiments ($n = 3$). Indicated differences show the significant differences using an unpaired Student's *t* test. Three (***) and four (****) asterisks denote a *p*-value lower than or equal to 0.001 and 0.0001, respectively. (D) Aberrant cellular morphology and cell separation defect of *rrp6*Δ cells at high temperature, visualized by fluorescent microscopy after Calcofluor White staining.

RNA exosome mutants are hypersensitive to cell wall stressors

To investigate whether it is possible to detect cell wall–related phenotypes in RNA exosome mutants at the physiological temperature of 30°C, we examined their growth on media containing known cell wall stressors Congo Red (CR), CFW, caffeine, and SDS. CR and CFW interfere with glucan and chitin assembly, respectively (Roncero and Duran, 1985; Kopecká and Gabriel, 1992); caffeine primarily affects TOR signaling (Kuranda *et al.*, 2006); and SDS is a general cell wall and membrane destabilizer (Popolo *et al.*, 2001). *rrp6*Δ and *dis3 exo*$^{-}$ mutants were hypersensitive to all of these compounds, thereby demonstrating that their cell walls are weaker than those of the corresponding wild-type cells even at the permissive temperature of 30°C when faced with cell wall stressors (Figure 3). Furthermore, their growth was significantly restored by the addition of 1 M sorbitol, which strengthens the argument that the effect is related to cell wall stability (Figure 3).

Regarding mutants in RNA exosome cofactors, for *air1*Δ*air2*Δ and *rrp47*Δ that are temperature sensitive we found that they are also hypersensitive to all tested cell wall stressors, while *mpp6*Δ showed

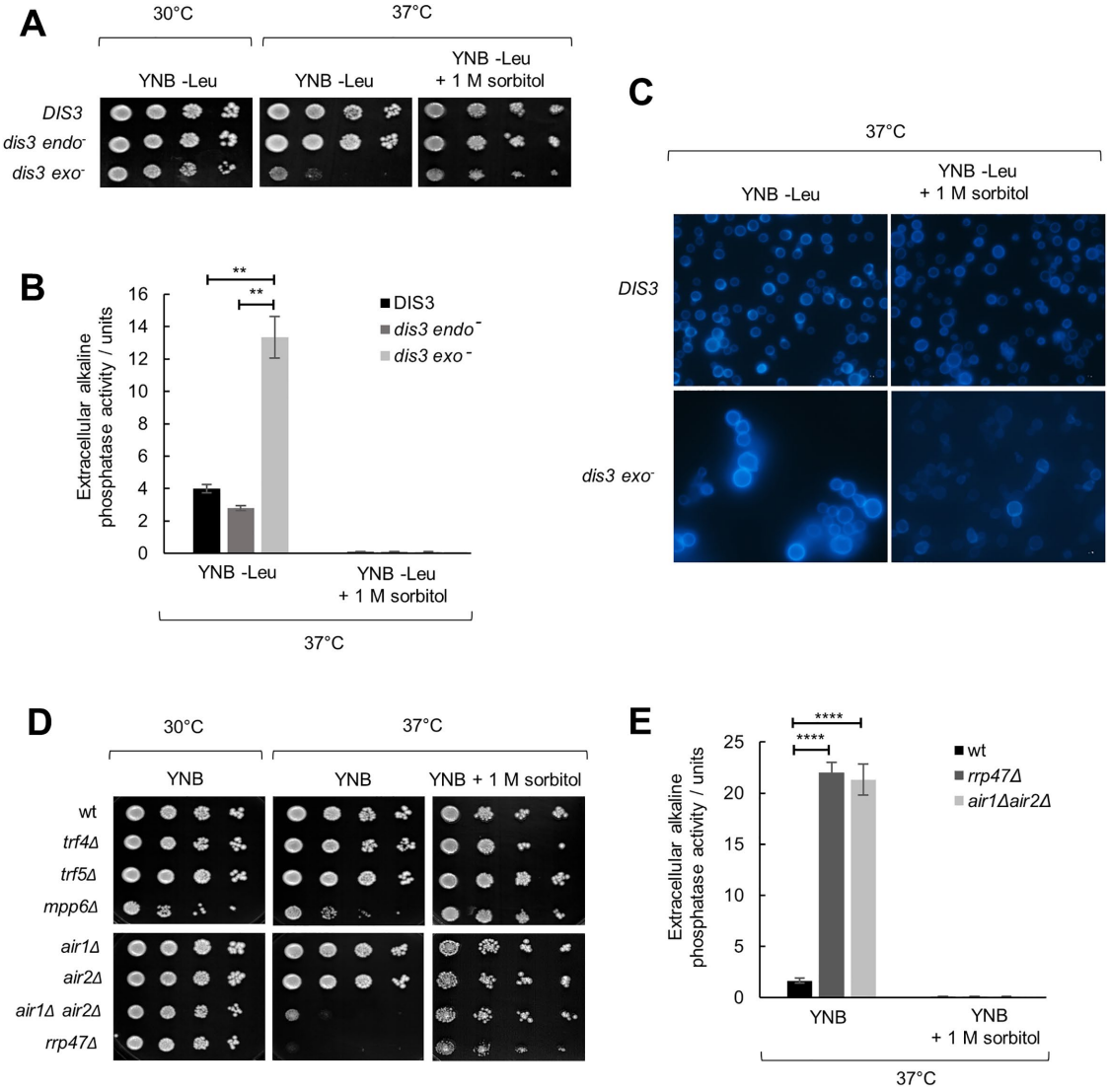

FIGURE 2: Inactivation of Dis3 exonuclease activity and certain exosome cofactors leads to cell wall instability. Strains are W303-derived with genomic copy of *DIS3* gene deleted but bearing a centromeric plasmid that carries the wild-type copy of the *DIS3* gene (*DIS3*) or its alleles with abolished endonuclease (*dis3 endo*⁻, D171N) or exonuclease (*dis3 exo*⁻, D551N) activity. Tenfold serial dilutions of cells were spotted on plates and were photographed after 3 d at indicated temperature. (A) Osmotically supporting medium with 1 M sorbitol rescues growth of *dis3 exo*⁻ at high temperature. (B) *dis3 exo*⁻ cells burst at high temperature, unless osmotic support is provided. Strains were grown for 3 d at 37°C and activity of alkaline phosphatase was measured in growth medium. Measurements were performed in duplicate, and reported values represent the means and standard deviations of three independent experiments ($n = 3$). Indicated differences show the significant differences using an unpaired Student's *t* test. Two (**) asterisks denote a *p*-value lower than or equal to 0.01. (C) Aberrant cellular morphology and cell separation defect of *dis3 exo*⁻ cells at high temperature, visualized by fluorescent microscopy after Calcofluor White staining. (D) Strains are BMA41 wild-type (wt) and isogenic mutants. Mutants in exosome cofactors *rrp47Δ* and *air1Δair2Δ* also show osmoremedial temperature sensitivity. (E) Strains were grown for 3 d at 37°C and activity of alkaline phosphatase was measured in growth medium. Measurements were performed in duplicate, and reported values represent the means and standard deviations of three independent experiments ($n = 3$). Indicated differences show the significant differences using an unpaired Student's *t* test. Four (****) asterisks denote a *p*-value lower than or equal to 0.0001.

specific sensitivity to caffeine (Supplemental Figure S3). The fact that single mutants in the TRAMP RNA-binding subunits *air1Δ* and *air2Δ* did not show cell wall–related phenotypes, but their combined inactivation in the double *air1Δair2Δ* mutant did, indicates their functional redundancy. Inactivation of either Trf4 or Trf5 TRAMP poly(A)-polymerase also did not lead to any cell wall–related phenotypes, while in this case it was not possible to explore whether it is due to functional redundancy because the double mutant is not viable.

Genes involved in protein glycosylation are dysregulated in RNA exosome mutants at high temperature and aiding this process suppresses their temperature sensitivity

The Rrp6-containing RNA exosome is located in the nucleus of the yeast cells, which precludes any direct link to the cell periphery. Instead, given the ubiquitous role of the RNA exosome in gene expression, its role in maintaining cell wall stability upon stress should be visible at the level of mRNAs encoding proteins that are

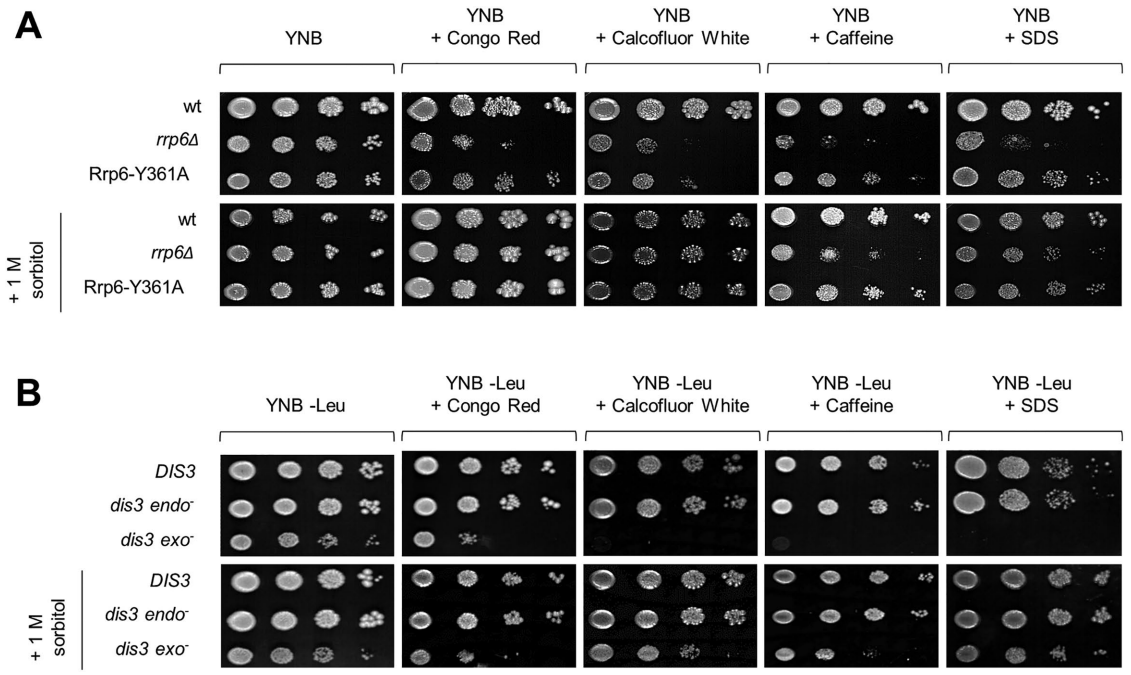

FIGURE 3: Cells lacking Rrp6 protein or Dis3 exoribonuclease activity are hypersensitive to cell wall stressors. Strains are described in Figures 1 and 2. Tenfold serial dilutions of cells were spotted on plates and were photographed after 3 d at 30°C. Concentrations of compounds used: Congo Red 10 µg/ml, Calcofluor White 20 µg/ml, caffeine 6 mM, SDS 0.0075%.

important for cell wall biosynthesis and remodeling. To this aim, we made use of the recently published genome-wide RNA-sequencing analysis that included the datasets of *rrp6*Δ mutant before and after a 45-min heat shock at 42°C (Wang *et al*. 2020). We inspected gene expression profiles of ~180 genes involved in cell wall biogenesis (Orlean, 2012) and visualized them as *rrp6*Δ/wt mRNA ratios on a $\log_2$ scale (Figure 4, A and B, and Supplemental Figure S4). Heat shock–dependent down-regulation in *rrp6*Δ cells as compared with wild-type cells could be seen for a number of genes, such as *GPI12*, encoding an essential protein involved in GPI anchor assembly, and *YPS3*, encoding an aspartic protease (Supplemental Figure S4). However, cell wall–related gene subcategories that encompassed genes with most prominent transcript down-regulation in *rrp6*Δ cells compared with wild-type cells at high temperature were the *precursor supply* gene category, which includes enzymes involved in the synthesis of sugar nucleotides and dolichol phosphate sugars that are precursors for cell wall components, and the *N- and O-glycosylation* category (Figure 4, A and B). In the *precursor supply* gene category, we noticed a strong heat shock–dependent down-regulation of *PSA1* and *DPM1* genes in *rrp6*Δ cells as compared to wild-type cells (Figure 4A). These genes are involved in the synthesis of GDP-mannose and its binding to the dolichol carrier, respectively (Figure 4C). Mannose is exclusively bound to cell wall proteins through N- or O-linked glycosylation in the endoplasmic reticulum and Golgi (Klis *et al*., 2002). Inspection of the *N- and O-glycosylation* category revealed that *ALG7*, which catalyzes the initial step in synthesis of the oligosaccharide precursor for N-glycosylation (Figure 4C), also showed heat shock–dependent down-regulation in *rrp6*Δ cells compared to wild-type cells (Figure 4B). Even though a large number of genes in this category seemed to be up-regulated in *rrp6*Δ relative to wild-type cells, we hypothesized that protein glycosylation in this mutant should nevertheless be affected, because precursor synthesis and the very early steps in the glycosylation pathway are severely impaired. To experimentally verify whether protein glycosylation is affected in RNA exosome mutants, we analyzed the degree of glycosylation of periplasmic invertase, normally a heavily N-glycosylated protein, by following its electrophoretic mobility with subsequent in-gel activity staining. Periplasmic invertase is easily inducible and is secreted even upon glycosylation defects so it provides a simple readout of the glycosylation status of the cell (Esmon *et al*., 1987; Belcarz *et al*., 2002). Positively, we noticed the appearance of a nonglycosylated form of invertase in periplasmic extracts of *rrp6*Δ cells after staining the gel for invertase activity (Figure 4D). This form was also present in periplasmic extracts of other RNA exosome mutants whose cell wall is destabilized: *rrp47*Δ, *air1*Δ*air2*Δ, and *dis3 exo*⁻, and was mostly absent from periplasmic extracts of wild-type and *dis3 endo*⁻ cells (Supplemental Figure S5). Because protein mannosylation is essential for cell viability and its impairment leads to cell wall defects (Janik *et al*., 2012), this analysis opened the possibility that a general defect in protein glycosylation may be the cause of cell wall instability and therefore temperature sensitivity of RNA exosome mutant cells.

Quantification of *PSA1*, *DPM1*, and *ALG7* mRNAs by reverse-transcription quantitative PCR (RT-qPCR) showed that their levels are lower in *rrp6*Δ, *air1*Δ*air2*Δ, and *dis3 exo*⁻ cells than in corresponding wild-type and *dis3 endo*⁻ cells at high temperature (3 h at 37°C; Figure 5A), in line with their down-regulation observed with *rrp6*Δ cells as compared with wild-type cells upon 45 min of heat shock at 42°C (Figure 4, A and B). For some of these genes, down-regulation in certain RNA exosome mutant cells in comparison to wild-type cells could be observed already at 30°C (Figure 5A), which could explain why glycosylation defects can be detected already at this temperature (Figure 4D and Supplemental Figure S5), even though the effect is not strong enough to cause a detectable phenotype. Out of these three genes, *PSA1* acts most upstream in the protein glycosylation pathway, as it encodes the enzyme GDP-mannose pyrophosphorylase, which synthesizes the activated form of mannose

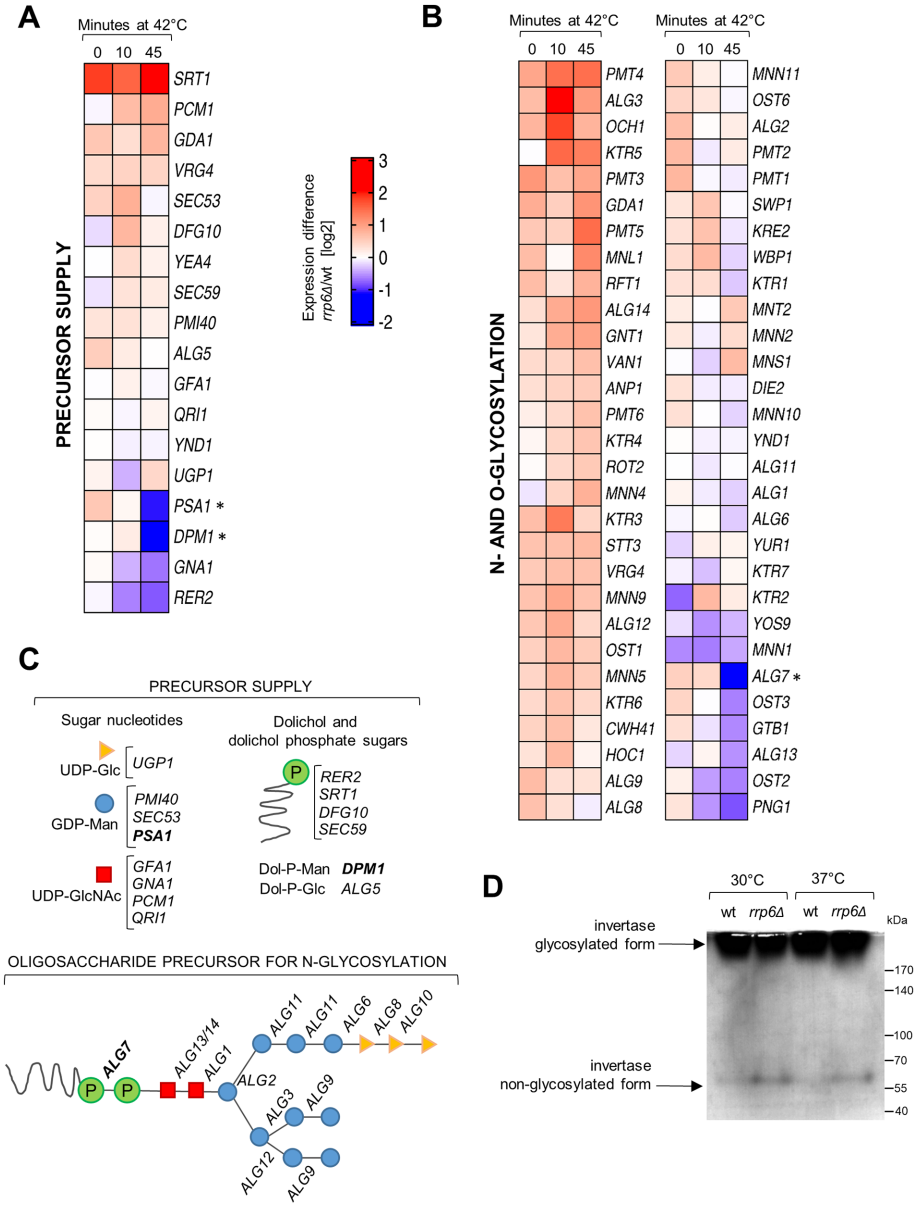

FIGURE 4: Protein glycosylation is dysregulated in cells lacking Rrp6. (A) RNA-seq heat map showing the expression difference of mRNAs encoding genes important for *precursor synthesis* of cell wall components, visualized as *rrp6*Δ/wt mRNA ratio on a $\log_2$ scale. Data is from Wang *et al.* (2020). (B) Same as for A, but for the *N- and O-glycosylation* gene category. (C) Scheme of the genes involved in the synthesis of sugar nucleotides, dolichol and dolichol phosphate sugars, that act as cell wall precursors (above), and in the synthesis of the oligosaccharide precursor for N-glycosylation (below). Genes that are down-regulated in *rrp6*Δ cells upon 45 min at 42°C are marked in bold. (D) Activity staining of invertase from periplasmic extracts. Extracts of *rrp6*Δ cells contain an additional nonglycosylated form of periplasmic invertase, revealing that protein glycosylation is affected in this mutant.

that is incorporated into N- and O-linked glycoproteins (Hashimoto *et al.*, 1997). Psa1 is essential, but partial loss of function of this enzyme or its down-regulation result in phenotypes such as sensitivity to hyposmolarity, cell leakage, and cell separation defects (Zhang *et al.*, 1999; Tomlin *et al.*, 2000; Warit *et al.*, 2000), that are reminiscent of those noticed with the *rrp6*Δ mutant at 37°C. To explore whether the decrease in the *PSA1* mRNA level is reflected by a decrease in the Psa1 protein level in *rrp6*Δ mutant, we C-terminally tagged Psa1 with a Myc tag at its genomic locus and quantified the protein by Western blotting. The Psa1 protein level corresponded well with the mRNA level and confirmed a decrease in the Psa1 level in *rrp6*Δ cells after incubation at 37°C (Figure 5B). Therefore, a low expression level of the essential Psa1 enzyme, or a more general and additive defect in protein glycosylation, could be the reason for the lethality of *rrp6*Δ and other RNA exosome mutant cells at 37°C. To test this hypothesis, we overexpressed Psa1 in these cells from a 2μ plasmid under regulation of its own promoter. Interestingly, overexpression of Psa1 restored the viability of *rrp6*Δ, *rrp47*Δ, and *air1*Δ*air2*Δ mutants at 37°C to a similar degree as osmotic stabilization (Figure 5C), confirming that protein glycosylation was limiting for growth of these cells at 37°C. Overexpression of *PSA1* also partially suppressed the temperature sensitivity of *dis3 exo*⁻ cells (Figure 5C), while in this case the incomplete suppression could be due to the necessity to stably replicate two plasmids (the centromeric plasmid carrying the *DIS3-D551N* allele and the 2μ plasmid carrying the *PSA1* gene) in order to survive. Additionally, overexpression of Psa1 completely suppressed aberrancies in the cell morphology of *rrp6*Δ cells, such as the enlargement of cells and the defect in cell separation (Figure 5D). Taken together, these results demonstrate a role for the RNA exosome in enabling proper protein mannosylation that is needed to preserve cell viability upon temperature-induced stress.

The Rrp6-containing RNA exosome is responsible for the degradation of a class of noncoding RNA transcripts termed CUTs (cryptic unstable transcripts), and inactivation of Rrp6 therefore results in increased CUTs accumulation (Xu *et al.*, 2009). Intriguingly, CUT488 is transcribed in the sense direction through the *PSA1* gene promoter and the 3′ end of this transcript overlaps with the *PSA1* transcription start site (Figure 6A). Quantification of CUT488 by RT-qPCR showed its stabilization in *rrp6*Δ and *dis3 exo*⁻ cells compared with wild-type cells and revealed an additional increase in its level at 37°C (Figure 6B). Because promoter sense transcripts have previously been shown to have gene regulatory roles in yeast (Hainer *et al.*, 2011; Van Werven *et al.*, 2012; Yu *et al.*, 2016), this represents a possible mechanism for *PSA1* down-regulation in *rrp6*Δ and *dis3 exo*⁻ cells. We also found that recruitment of RNA polymerase II to the *PSA1* gene promoter was not significantly changed in these mutant cells compared with wild-type cells at physiological temperature (Supplemental Figure S6). However, at high temperature the occupancy of RNA polymerase II at the *PSA1* gene promoter was drastically decreased in *rrp6*Δ and *dis3 exo*⁻ mutants as compared with wild-type cells or the Rrp6-Y361A catalytic mutant cells (Figure 6C). This was not due to a

general effect on gene transcription in these mutant cells at high temperature, as this effect was not present when probing for RNA polymerase II occupancy at the promoter of the *TAF10* gene (Figure 6D), which is constitutively expressed and does not show any noncoding transcription at its locus. This is conceivably in line with a regulatory mechanism in which accumulation of the normally unstable noncoding RNAs in *rrp6*Δ and *dis3 exo*⁻ cells out-titrates the NNS termination system, thereby promoting read-through of CUT488 into the *PSA1* promoter region, which was recently shown to be a transcriptome-wide phenomenon (Moreau *et al*., 2019; Villa *et al*., 2020). Read-through of CUT488 could limit transcription factor and/or RNA polymerase II recruitment to the *PSA1* promoter region and negatively influence transcription of the *PSA1* gene. We also found that the gene loci of the two other down-regulated glycosylation-related genes, *DPM1* and *ALG7*, show transcription of noncoding antisense transcripts at their genomic loci, which are stabilized in *rrp6*Δ cells at high temperature (Supplemental Figure S7). The antisense transcript at the *DPM1* locus was previously mapped as CUT923, while the antisense transcript at the *ALG7* locus was not mapped but can be seen upon inspection of whole-transcriptome tiling array datasets (Xu *et al*., 2009). Taken together, a possible mechanism for dysregulation of glycosylation-related genes in RNA exosome mutants involves a temperature-dependent increase in accumulation of noncoding transcripts transcribed from their genomic loci.

DISCUSSION

In this work, we demonstrate that the activity of RNA exosome is necessary for maintaining cell wall stability in yeast *Saccharomyces cerevisiae*. RNA exosome mutants undergo osmoremedial cell lysis and show numerous cell wall–related phenotypes that are exacerbated at high temperature. Importantly, this explains that aberrancies in cell wall structure are the reason for temperature sensitivity of these mutants. The essential RNA exosome catalytic subunit Dis3 provides exoribonuclease catalytic activity, while the second catalytic subunit Rrp6 has a noncatalytic role in this process. Besides RNA exosome catalytic subunits, exosome cofactors Rrp47 and Air1/2 are also involved. We show a role for these proteins in maintaining cellular integrity upon heat stress, but also upon treatment with cell wall stressors at physiological temperature, clearly showing that their role is not specific to temperature but to conditions of cell wall stress. Importantly, we provide mechanistic insight into cell wall instability of RNA exosome mutants, as we highlight differential expression of protein glycosylation genes as the factor that disrupts their CWI. Specifically, down-regulation of genes encoding proteins that act in the early steps of the protein mannosylation pathway (*PSA1*, *DPM1*, and *ALG7*) in RNA exosome mutant cells compared with wild-type cells leads to aberrant morphology and temperature sensitivity of these mutants. In addition, artificially aiding protein glycosylation through overexpression of Psa1 suppresses their temperature-sensitive phenotypes, which were previously shown to be due to cell wall instability.

Our results partially contrast with a study that was published during the preparation of this article, which highlighted the role of RNA exosome catalytic subunit Rrp6 in promoting cell survival during heat stress, but argued against involvement of other RNA exosome subunits and cofactors (Wang *et al*., 2020). They proposed that Rrp6 alone has a highly specialized "moonlighting" function in this process, that is independent of all of its currently known interactors, including its stabilization partner Rrp47 (Wang *et al*., 2020). Our results clearly show the importance of the essential RNA exosome catalytic subunit Dis3 in this process, as the catalytically inactive *dis3 exo*⁻ (*dis3-D551N*) mutant displays practically identical cell wall aberrancies as *rrp6*Δ mutant (Figures 1–3), which is also the case for mutants in exosome cofactors Rrp47 and Air1/2 (discussed below). This challenges the idea of a highly specialized Rrp6 function in maintaining CWI and is important to delineate, especially as exosome-independent roles of Rrp6 are a highly debated topic in the field (Callahan and Butler, 2008). Furthermore, the potential role of Rrp6 in the CWI pathway was inferred from the additive cell wall instability phenotype of the double mutant *rrp6*Δ *mpk1*Δ, in which a major CWI signaling component was inactivated (Wang *et al*., 2020). Additivity is suggestive of parallel and redundant functions, and this interpretation was previously applied to the equally severe phenotype of the *rnt1*Δ *mpk1*Δ mutant, which harbors deletion of the dsRNA-specific ribonuclease Rnt1 (Catala *et al*., 2012). It is, however, clear that Rrp6 has a noncatalytic role in maintaining cellular integrity upon heat stress, as previously implied by the fact that all tested Rrp6 catalytic mutants grow normally at high temperature (Phillips and Butler, 2003). The most straightforward explanation, which fits well with our results, could lie in the well-documented role of Rrp6 in allosterically stimulating the activity of RNA exosome through its C-terminal domain, a process which is independent of Rrp6 catalytic activity (Makino *et al*., 2015; Wasmuth and Lima, 2017). In line with this, the deletion of only the Rrp6 EAR (exosome-interacting region) domain leads to temperature sensitivity, which pinpoints it as the region of Rrp6 that is necessary for stress resistance (Wasmuth and Lima, 2017).

Besides mutants in the RNA exosome catalytic subunits, we show that for mutants in RNA exosome cofactors temperature sensitivity is also associated with cell wall instability (Figure 2, D and E, and Supplemental Figure S3). Inactivation of the *RRP47* gene, encoding the obligate stabilization partner of Rrp6, results in osmoremedial temperature sensitivity and hypersensitivity to cell wall stressors. Because Rrp47 is critical for Rrp6 protein stability (Feigenbutz *et al*., 2013; Stuparevic *et al*., 2013), this result confirms the necessity of Rrp6 protein presence for maintaining cellular integrity upon heat stress. Also, simultaneous inactivation of two homologous genes that encode the TRAMP complex subunits Air1 and Air2 results in cell wall–related phenotypes, in contrast to their individual inactivation. Because Air1 and Air2 function as RNA-binding subunits in different isoforms of the TRAMP complex, this indicates that these isoforms have fully redundant roles in ensuring cellular integrity, which is interesting considering that these isoforms were previously shown to have some nonoverlapping roles based on differential substrate specificity (Schmidt *et al*., 2012; Stuparevic *et al*., 2013), somehow similar to what has been recently shown for Trf4 and Trf5 (Delan-Forino *et al*., 2020). Finally, cell wall instability is an elegant explanation for the observation that *rrp6*Δ phenotype, that is, its temperature sensitivity, is most pronounced in W303 and its derived genetic backgrounds, as wild type of this strain was shown to have an already more destabilized cell wall compared with wild types of other backgrounds (Trachtulcová *et al*., 2003; Schroeder and Ikui, 2019).

Yeast cell wall is the outermost part of the cell, which determines its shape and provides physical and osmotic protection. It is a polysaccharide network built out of glucan and chitin to which cell wall proteins are bound (Klis *et al*., 2002). Cell wall proteins function as structural components of the cell wall or enzymes that modify cell wall composition and are often heavily mannosylated through N- or O-linked glycosylation. This modification is vital for yeast, as well as for humans, because it ensures proper protein activity, stability, and localization (Lehle *et al*., 2006). The essential cytoplasmic enzyme GDP-mannose pyrophosphorylase Psa1 catalyzes the production of

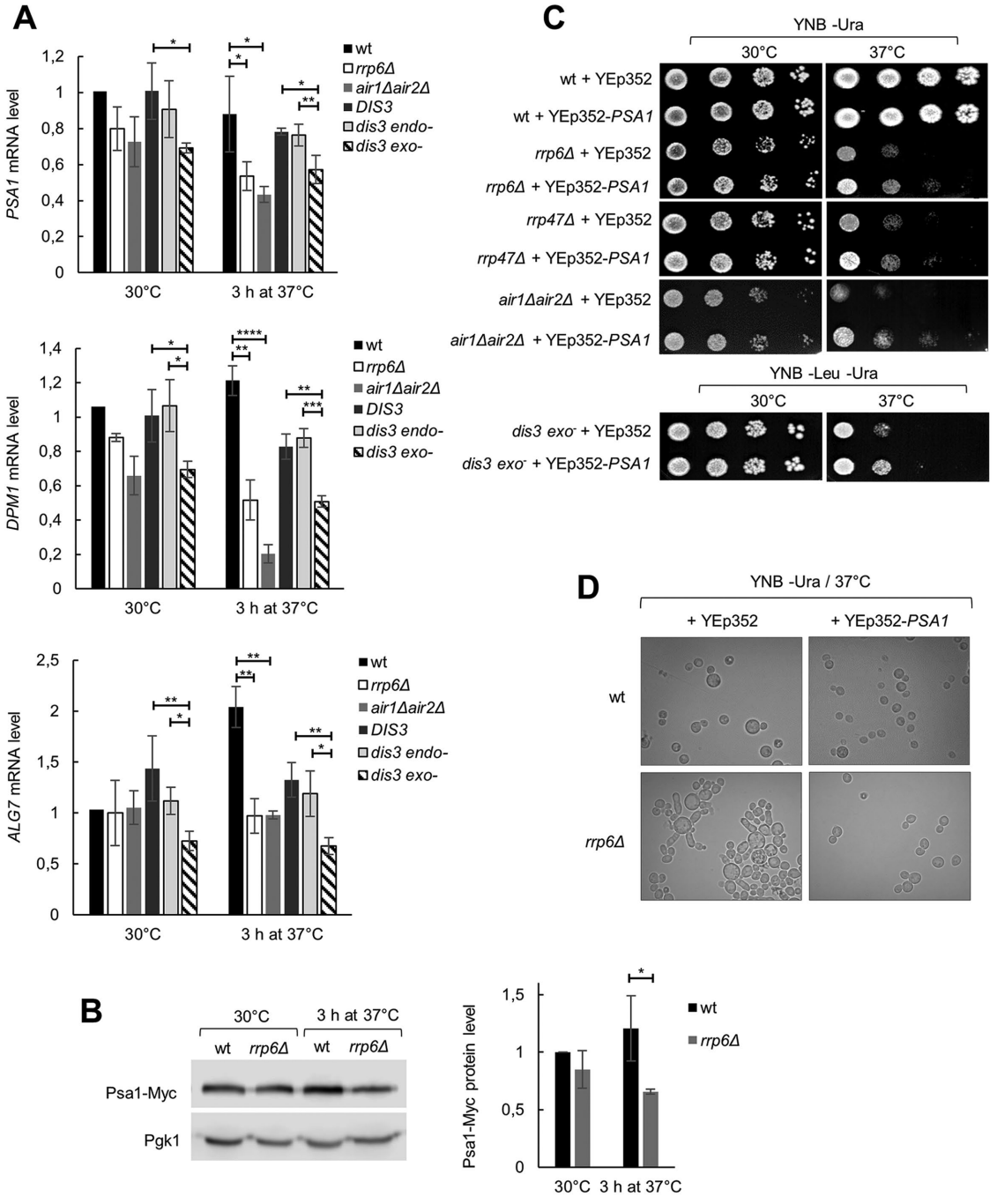

FIGURE 5: Overexpression of *PSA1* rescues temperature sensitivity of RNA exosome mutants. The strains are described in Figures 1 and 2. (A) Levels of *PSA1*, *DPM1*, and *ALG7* mRNAs are lower in *rrp6*Δ, *air1*Δ*air2*Δ, and *dis3 exo*⁻ cells than in the corresponding wild-type cells at high temperature. RT-qPCR values are normalized to *PMA1* mRNA and expressed relative to transcript abundance in wild-type cells at 30°C, which is set as 1. Reported values represent the means and standard deviations of three independent experiments ($n = 3$). Indicated differences show the significant differences using an unpaired Student's *t* test. One (*), two (**), three (***), and four (****) asterisks denote a *p*-value lower than or equal to 0.05, 0.01, 0.001, and 0.0001, respectively. (B) Psa1 protein level is lower in *rrp6*Δ than in wild-type cells at high temperature. Myc-tagged Psa1 was quantified by Western blotting. Values are normalized to Pgk1 and expressed relative to protein abundance in wild-type cells at 30°C, which is set as 1. Reported values represent the means and standard deviations of three independent experiments ($n = 3$). Indicated differences show the significant differences using an unpaired Student's *t* test. One (*) asterisk denotes a *p*-value lower than or equal to 0.05. (C) Overexpression of Psa1 from a multicopy plasmid (YEp352-*PSA1*) fully rescues temperature sensitivity of *rrp6*Δ, *rrp47*Δ, and *air1*Δ*air2*Δ cells and partially of *dis3 exo*⁻ cells. Tenfold serial dilutions of cells were spotted on plates and were photographed after 3 d at indicated temperature. Control cells were transformed with the empty vector (YEp352). (D) Overexpression of Psa1 from a multicopy plasmid (YEp352-*PSA1*) rescues aberrant phenotype of *rrp6*Δ cells at high temperature.

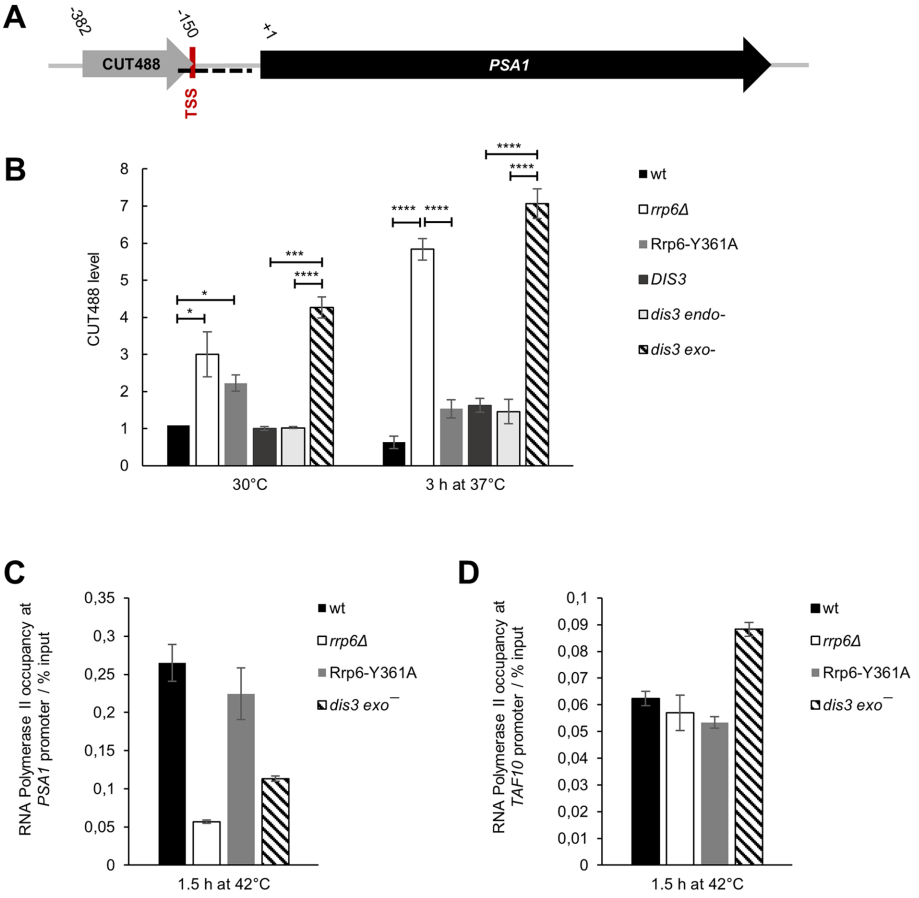

FIGURE 6: Noncoding transcript CUT488 accumulates in cells lacking Rrp6 or Dis3 exoribonuclease activity at high temperature. Strains are described in Figures 1 and 2. (A) Scheme of *PSA1* locus, showing sense transcription of a noncoding transcript CUT488 at its promoter region. Transcription start site (TSS) of *PSA1* is located at position −149 relative to the start of the ORF. The region used for ChIP is marked as a dashed black line. (B) Level of CUT488 RNA is higher in *rrp6*Δ and *dis3 exo*⁻ cells than in corresponding wild-type and Rrp6-Y361A or *dis3 endo*⁻ cells, and that difference is even greater at high temperature. RT-qPCR values are normalized to *PMA1* mRNA and expressed relative to transcript abundance in wild-type cells at 30°C, which is set as 1. Reported values represent the means and standard deviations of three independent experiments ($n = 3$). Indicated differences show the significant differences using an unpaired Student's *t* test. One (*), three (***), and four (****) asterisks denote a *p*-value lower than or equal to 0.05, 0.001, and 0.0001, respectively. (C) Recruitment of RNA polymerase II to *PSA1* gene promoter is decreased in *rrp6*Δ and *dis3 exo*⁻ cells compared with wild-type and Rrp6-Y361A cells at high temperature. Quantification was performed by ChIP of RNA polymerase II using specific antibodies 8WG16. Immunoprecipitated samples (output) were normalized to input following quantification by qPCR. Reported values represent the means and range of two independent experiments ($n = 2$). (D) The decrease of RNA polymerase II occupancy over the PSA1 promoter observed for *rrp6*Δ and *dis3 exo*⁻ cells was not due to a general effect on transcription in these cells because this difference was not present for *TAF10* gene promoter. Quantification was performed as in C.

GDP-mannose, which is the activated form of mannose that gets incorporated into glycoproteins (Hashimoto *et al.*, 1997). Its partial loss of function or down-regulation leads to multiple strong cell wall–related phenotypes such as sorbitol-dependence, cell rupture, and cell separation defects (Zhang *et al.*, 1999; Tomlin *et al.*, 2000; Warit *et al.*, 2000). Importantly, we found that transcription of the *PSA1* gene is down-regulated in RNA exosome mutant cells at high temperature, leading to a lower Psa1 protein level and potentially resulting in *psa1* phenotypes (Figures 4A and 5, A and B). This is strongly supported by rescuing the temperature-sensitive growth and aberrant morphology of these mutants through overexpression of Psa1 (Figure 5, C and D). Because of the previously mentioned strong effects of *PSA1* down-regulation, it is plausible that about a 50% down-regulation of the Psa1 protein level observed in *rrp6*Δ compared with wild-type cells at high temperature can push the protein's enzymatic activity below a physiologically critical level. However, we cannot exclude the possibility that dysregulating expression of genes encoding other proteins involved in the early steps of the glycosylation pathway, such as *DPM1* and *ALG7* (Figures 4, A and B, and 5A) plays a significant contribution in cell wall phenotypes of RNA exosome mutants and that Psa1 overexpression rescues them by generating more precursor supply (Janik *et al.*, 2003). We also hypothesize that a possible reason for transcriptional down-regulation of the *PSA1* gene in RNA exosome mutant cells compared with wild-type cells is the temperature-induced increased accumulation of CUT488, a noncoding transcript transcribed through the *PSA1* gene promoter, which is accompanied by a decrease in RNA polymerase II recruitment at this promoter (Figure 6). At the *PHO84* gene, which is regarded as a model gene for transcriptional regulation through noncoding RNA transcription, loss of Rrp6 was shown to lead to higher production of the antisense transcript due to the decreased recruitment of the NNS complex that normally terminates its transcription (Castelnuovo *et al.*, 2013). Recent transcriptome studies from ours and the D. Libri laboratory showed that out-titration of the NNS complex, accomplished either by perturbation of mRNP biogenesis or inactivation of the RNA exosome, leads to termination defects at ncRNA-producing targets (Moreau *et al.*, 2019; Villa *et al.*, 2020). In line with this mechanism, out-titration of the NNS complex in *rrp6*Δ and *dis3 exo*⁻ cells, because of the more prominent accumulation of ncRNAs in these mutant cells at high temperature, could lead to transcriptional read-through of CUT488 through the *PSA1* gene promoter and negatively influence *PSA1* gene transcription. Notable examples of loci regulated by noncoding promoter transcription in yeast include *SER3*, *HO*, and *IME1* genes, which are all negatively regulated by transcription of a sense transcript at their promoter regions (Winston *et al.*, 2005; Hainer *et al.*, 2011; Van Werven *et al.*, 2012; Yu *et al.*, 2016). While these are nonessential genes expressed specifically under a certain physiological or life/cell cycle condition, *PSA1* is essential and constitutively expressed. However, it is strongly cell cycle regulated, peaking at the START phase of the cell cycle (Benton *et al.*, 1996), so the possibility of a cell cycle–based regulation of its transcription through noncoding RNA transcription could be an exciting subject for future

investigation. Of course, such broad dysregulation of cell wall stability in *rrp6*Δ cells is most probably due to effects on expression of multiple cell wall–related genes and could involve regulatory roles of noncoding transcription (Novačić *et al.*, 2020), as well as the CWI pathway (Wang *et al.*, 2020). In line with this, we also noticed increased accumulation of noncoding antisense transcripts that are transcribed at *DPM1* and *ALG7* gene loci in *rrp6*Δ cells at high temperature (Supplemental Figure S7).

Another interesting point is that the increase in accumulation of CUT488 at high temperature is independent of Rrp6 catalytic activity but is dependent on the presence of Rrp6 protein and the exoribonuclease activity of Dis3 (Figure 6B). This implies that this CUT is degraded primarily by Dis3 at high temperature and that Rrp6 provides a noncatalytic function in this process, probably that of an equivalent of an RNA exosome cofactor (as discussed in the second paragraph of the *Discussion*). Allosteric stimulation of Dis3 activity by Rrp6 probably happens by direct RNA binding, as well as the widening of the RNA exosome channel through which RNAs need to be threaded to reach the active site of Dis3 (Kilchert *et al.*, 2016). Transcripts termed as CUTs were originally identified as ones that accumulate in *rrp6*Δ deletion mutant (Xu *et al.*, 2009) and comparison of the transcriptome of this mutant with that of Dis3 catalytic mutants revealed some unique and some specific roles of the two catalytic subunits (Gudipati *et al.*, 2012); however, the transcriptome of the Rrp6 catalytic mutant was studied only with *Schizosaccharomyces pombe* cells (Mukherjee *et al.*, 2016). The study with *S. pombe* revealed that some RNA targets of Rrp6 depended mainly on its structural role, such as RNAs of early meiotic and iron metabolism genes (Mukherjee *et al.*, 2016). Noncatalytic roles of Rrp6 have not yet been explored transcriptome-wide in *S. cerevisiae* but could be an interesting subject to study, especially with relation to unique cellular states, such as meiosis or conditions of heat shock.

The cell wall structure is absent from mammalian cells; however, protein glycosylation is conserved and essential for viability from yeast to human. The importance of protein glycosylation is underscored by the congenital disorders of glycosylation syndrome, which encompasses multisystemic diseases in children that result from defects in various steps along glycan modification pathways (Chang *et al.*, 2018). While final sugar composition and branching differs between yeast and human, the earliest steps in the glycosylation pathway, precursor synthesis and initial N-glycosylation reactions, are highly conserved (Lehle *et al.*, 2006). Our work in yeast clearly shows that one of the molecular consequences of RNA exosome inactivation is impairment of protein glycosylation at these early steps. Given the high conservation of both the RNA exosome complex and the glycosylation pathway, as well as the association of both with human diseases, this study opens the possibility for future investigation with human cells.

SUMMARY

RNA exosome activity, accomplished through Dis3 exonuclease activity and a noncatalytic function of Rrp6, is necessary for maintaining cell wall stability in yeast *Saccharomyces cerevisiae*.

A defect in protein glycosylation is a major reason for cell wall instability of RNA exosome mutants.

Genes encoding proteins involved in the early steps of protein glycosylation are dysregulated in RNA exosome mutants through mechanisms that involve increased accumulation of noncoding RNAs at high temperature.

MATERIALS AND METHODS

Request a protocol through *Bio-protocol*.

Strains, media, plasmids, and strain construction

Yeast strains and primers used in this study are listed in Supplemental Tables S1 and S2, respectively. Experiments were performed with the BMA41 (W303-derived) strain background, unless noted otherwise. Yeast strains were grown in YPD (containing per liter 20 g peptone, 10 g yeast extract, 20 g glucose, 0.1 g adenine) or YNB medium (containing per liter 6.7 g yeast nitrogen base without amino acids, 2 g drop-out mix as in Musladin *et al.*, 2014, 20 g glucose) supplemented with the required amino acids and uracil (80 mg/l each). Plasmid YEp352-*PSA1* is a high copy vector that carries the *PSA1/MPG1* gene (Janik *et al.*, 2003).

Psa1 was tagged at its genomic locus with a C-terminal 9xMyc tag. The tagging cassette was PCR amplified from plasmid pYM20 (Janke *et al.*, 2004) using the primer pair PSA1Ctag_fwd/ PSA1Ctag_rev and transformed into BMA41 wild-type and *rrp6*Δ strain by a standard lithium acetate procedure. Transformants were selected on Hygromycin B (0.3 mg/ml, Roche) plates and the presence of the tag was confirmed by Western blotting. The *RRP6* gene was deleted in BY4741 strain using a disruption cassette generated by PCR with primers RRP6-Kan1 and RRP6-Kan2 (Mosrin-Huaman *et al.*, 2009).

Phenotypic assays

Sensitivities to CR, CFW, caffeine, and SDS were tested by spotting assays. Exponential phase cultures were adjusted to an OD_{600} of 1 and four 10-fold serial dilutions of that sample were spotted onto plates supplemented with indicated amounts of each compound. Plates were incubated at 30°C or 37°C for 3 d and photographed using a UVIDOC HD6 camera (Uvitec, Cambridge).

Alkaline phosphatase activity assays

Activity of alkaline phosphatase released into the medium was measured as in Molina *et al.* (1998) with slight modifications. Supernatant (500 µl) from liquid culture was mixed with equal volume of 20 mM *p*-nitrophenylphosphate in Tris-HCl buffer, pH 8.8 and assayed for alkaline phosphatase activity. The reaction was performed at 30°C, stopped by the addition of 500 µl of 1 M NaOH, and absorbance of liberated *p*-nitrophenol was measured at 420 nm using a Helios Gamma spectrophotometer (Thermo Fisher). Enzyme activity was normalized to OD_{600} of the culture and the assay time in minutes and was expressed in arbitrary units: A_{420}*10,000/[OD_{600}*(*t*/min)*(V_{sample}/V_{total})]. Intracellular activity of alkaline phosphatase was measured exactly as described in Münsterkötter *et al.* (2000).

Fluorescence microscopy

Cells were stained with CFW stain (Sigma) and observed with an Olympus BX51 fluorescence microscope. The fluorescence from CFW was filtered with a DAPI filter.

RNA-seq data processing and computational analysis

Raw data from Wang *et al.* (2020) were downloaded via GEO (accession number GSE140504). Alignment and reads abundance estimation were conducted as described in the original publication. In short, Hisat2 was used to align reads against *S. cerevisiae* reference genome (taken from SGD, release R64-1-1); read abundance for mRNAs was estimated with HTseq-count (with the option –s reverse). Differential analysis between wt and *rrp6*Δ strain was conducted under the R environment using the DESeq2 package. Resulting log_2FC were used to construct heatmaps using the ggplot2 and complexHeatmap packages.

Analysis of the degree of glycosylation of periplasmic invertase

The secretory invertase was analyzed as described in Hashimoto *et al*. (1997). Briefly, invertase expression was induced by incubating midlogarithmic phase cells in medium that contains sucrose instead of glucose for 2 h at 30°C or 37°C. Cells were treated with zymolyase and the periplasmic fraction containing invertase was separated from spheroplasts by centrifugation. The periplasmic fraction was subjected to 7.5% SDS–PAGE, gel was bathed in 0.1 M sodium acetate, pH 5.1, containing 0.1 M sucrose at 37°C for 1 h to carry out the enzymatic reaction of invertase, and then washed with water, placed in 0.1% 2,3,5- triphenyltetrazolium chloride, 0.5 M NaOH, and boiled to detect red bands. After staining, gel was washed with 7.5% acetic acid.

RNA isolation and RT-qPCR analysis

Total RNA was extracted by the hot phenol method (Schmitt *et al.*, 1990) and column purified with DNase treatment using a NucleoSpin RNA kit (Macherey Nagel) according to manufacturer instructions. RNA was quantified with a Nanodrop spectrophotometer and 1 µg was used in a strand-specific reverse-transcription reaction with a ProtoScript First Strand cDNA Synthesis Kit (New England Biolabs) with 0.1 µM gene-specific oligonucleotides and supplemented with actinomycin D (Sigma) to final concentration 5 µg/ml to ensure strand specificity. Twofold diluted cDNA (1 µl) was then amplified in Roche LightCycler 480 with the Maxima SYBR Green qPCR Master Mix detection kit from Thermo Scientific as recommended by the supplier. The qPCR datasets were analyzed using the ΔΔCt method, and the results were normalized to *PMA1* mRNA RT-qPCR amplification, which was used as internal control. The level of a certain transcript for each sample was expressed relative to its abundance in wild-type cells at 30°C, which was set as 1. Amplifications were done in duplicate for each sample, and three independent RNA extractions were analyzed.

Western blot analysis

Total proteins were obtained as described in Kushnirov (2000), resolved on SDS 10% polyacrylamide electrophoresis gels, and analyzed by Western blotting according to standard procedures. Myc-tagged Psa1 was probed with anti-c-Myc (9E10; Santa Cruz Biotechnology) at 1:1000 dilution and Pgk1 with anti-PGK1 (22C5D8; Abcam) at 1:5000 dilution. In both cases, mouse IgG kappa-binding protein HRP (Santa Cruz Biotechnology) at 1:50,000 dilution was used to detect the primary antibody. Blots were developed using Biorad Clarity Western ECL substrates and visualized with a C-DiGit Blot scanner (LI-COR Biosciences). Band intensity was quantified with GelAnalyzer 19.1 software and the results were normalized to Pgk1. The level of protein was expressed relative to its abundance in wild-type cells at 30°C, which was set as 1. Three independent protein extractions were analyzed for each sample.

Chromatin immunoprecipitation

Chromatin immunoprecipitation (ChIP) was performed similarly as described in Stuparevic *et al.* (2013). Forty milliliters of cells were fixed with 1% formaldehyde for 20 min. After glycine addition to stop the reaction, the cells were washed and lysed with glass beads to isolate chromatin. The cross-linked chromatin was sheared by sonication with a Vibra-Cell sonicator to reduce average fragment size to approximately 500 base pairs. Chromatin fractions of 400 µl were taken for each immunoprecipitation reaction and incubated with 4 µl of anti-RNA polymerase II antibodies (8WG16, sc-56767; Santa Cruz Biotechnology) at 4°C overnight. After incubation, 40 µl of protein G PLUS-agarose beads (sc-2002; Santa Cruz Biotechnology) were added and incubated for 2 h at 4°C. The beads were then washed extensively, and the chromatin was eluted. Eluted supernatants (output) and the input controls were hydrolyzed with Pronase (0.8 mg/ml final concentration; Sigma) for 2 h at 42°C, followed by 7 h incubation at 65°C to reverse cross-linked DNA complexes. DNA was extracted using the Macherey Nagel Nucleospin Gel & PCR Cleanup Kit. The immunoprecipitated DNAs (output) were quantified by qPCR in Roche LightCycler 480 with the Maxima SYBR Green qPCR Master Mix detection kit from Thermo Scientific as recommended by the supplier. Amplifications were done in triplicate for each sample. Immunoprecipitated samples (output) were normalized to input.

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Paper 2

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RESEARCH ARTICLE

Interplay of the RNA Exosome Complex and RNA-Binding Protein Ssd1 in Maintaining Cell Wall Stability in Yeast

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ABSTRACT Yeast cell wall stability is important for cell division and survival under stress conditions. The expression of cell-wall-related proteins is regulated by several pathways involving RNA-binding proteins and RNases. The multiprotein RNA exosome complex provides the 3′→5′ exoribonuclease activity that is critical for maintaining the stability and integrity of the yeast cell wall under stress conditions such as high temperatures. In this work, we show that the temperature sensitivity of RNA exosome mutants is most pronounced in the W303 genetic background due to the nonfunctional *ssd1-d* allele. This gene encodes the RNA-binding protein Ssd1, which is involved in the posttranscriptional regulation of cell-wall-related genes. Expression of the functional *SSD1-V* allele from its native genomic locus or from a centromeric plasmid suppresses the growth defects and aberrant morphology of RNA exosome mutant cells at high temperatures or upon treatment with cell wall stressors. Moreover, combined inactivation of the RNA exosome catalytic subunit Rrp6 and Ssd1 results in a synthetically sick phenotype of cell wall instability, as these proteins may function in parallel pathways (i.e., via different mRNA targets) to maintain cell wall stability.

IMPORTANCE Stressful conditions such as high temperatures can compromise cellular integrity and cause bursting. In microorganisms surrounded by a cell wall, such as yeast, the cell wall is the primary shield that protects cells from environmental stress. Therefore, remodeling its structure requires inputs from multiple signaling pathways and regulators. In this work, we identify the interplay of the RNA exosome complex and the RNA-binding protein Ssd1 as an important factor in the yeast cell wall stress response. These proteins operate in independent pathways to support yeast cell wall stability. This work highlights the contribution of RNA-binding proteins in the regulation of yeast cell wall structure, providing new insights into yeast physiology.

KEYWORDS RNA metabolism, yeast cell wall, RNA exosome, Rrp6, Dis3, Ssd1, RNA metabolism

The yeast cell wall is an essential structure that determines the shape of the cell and shields it from environmental stress (1). It is a cross-linked network composed of β-1,3-glucan, β-1,6-glucan, chitin and mannoproteins. Cell wall stability is particularly important for cell division and survival under stress conditions, so cell wall remodeling needs to be tightly regulated by regulating the expression of cell-wall-related proteins. Expression of cell wall proteins is a complex process because these proteins must be transported to the cell periphery via the secretory pathway and simultaneously with their transport undergo various posttranslational modifications. Accordingly, their expression is often regulated at the transcriptional and posttranscriptional levels by multiple signaling pathways and RNA-binding proteins (1–5).

The RNA-binding protein Ssd1 regulates the localization and suppresses translation of mRNAs encoding cell morphogenesis proteins by directing their incorporation into

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This new paper demonstrates the interplay between RNA exosome and Ssd1 which work via different mRNA targets to maintain cell wall stability in yeast.

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P-bodies and stress granules (6, 7). Ssd1 exerts its regulatory effects primarily by binding to the 5′ and 3′ untranslated regions (UTRs) of its mRNA targets, which primarily encode hydrolytic enzymes localized in the yeast cell wall (5, 6, 8). Ssd1 itself is negatively regulated by phosphorylation through the LATS/NDR protein kinase Cbk1, which is localized to regions of cell growth and cytokinesis (i.e., the bud and bud neck), where expression of cell wall hydrolases is necessary to enable cell wall remodeling and expansion (6). The *SSD1* gene is hypomorphic in a commonly used laboratory strain, W303, which accounts for a number of phenotypic differences between W303 and other strain backgrounds (9–13).

A key regulator of RNA metabolism in eukaryotic cells is the RNA exosome, an essential multiprotein complex involved in 3′→5′ RNA degradation and processing (14). Targets of RNA exosome processing include rRNAs, snoRNAs, and snRNAs, whereas the exosome degrades aberrant tRNAs, mRNAs and noncoding RNAs termed cryptic unstable transcripts (CUTs) (14, 15). Nine RNA exosome subunits form a catalytically inactive core through whose channel single-stranded RNA substrates are threaded to the processive exoribonuclease subunit Dis3 (16, 17). The only nonessential subunit of the RNA exosome, Rrp6, binds to the top of the core and provides distributive exoribonucleolytic activity (16, 17). The roles of the core subunits and Rrp6 also include stimulation of the exoribonuclease activity of the Dis3 subunit (18). We have recently shown that the exoribonuclease activity of Dis3 and a noncatalytic function of Rrp6 are involved in maintaining cell wall stability in yeast, which is why RNA exosome mutant cells display temperature-sensitive phenotypes (19). We now show that the reason that the temperature sensitivity of RNA exosome mutant cells is most pronounced in the W303 genetic background is the presence of the nonfunctional *ssd1-d* allele. A negative genetic interaction, previously identified for *RRP6* and *SSD1* genes in a large genetic screen (20), was demonstrated to stem from a synthetic phenotype of cell wall instability, since these proteins may function in parallel pathways (i.e., via different mRNA targets) to maintain cell wall stability.

RESULTS

We recently identified the nuclear RNA exosome complex as an important yeast cell wall regulator (19). The absence of the RNA exosome catalytic subunit Rrp6 leads to cell wall instability, which manifests as cell lysis and inviability under conditions of high temperature or other forms of cell wall stress (19). The temperature sensitivity of *rrp6*Δ mutant cells can be completely suppressed at high temperatures by providing the cells with osmotic support (e.g., by adding 1 M sorbitol to the growth medium), and this suppression was found to be consistent for *rrp6*Δ mutants from different genetic backgrounds (19). However, it was striking that the temperature-sensitive phenotype of *rrp6*Δ mutant cells was most pronounced in the W303 genetic background, making it the primary choice for studying Rrp6-related phenotypes (18, 21). As exemplified in Fig. 1A, the *rrp6*Δ mutant of the BY4741 genetic background grows better at 37°C, compared with its wild-type counterpart, than is the case for the equivalent mutant of the W303-derived BMA41 genetic background. The association between the temperature-sensitive phenotype of this mutant and cell wall instability prompted us to investigate which W303-specific alleles might be enhancing this phenotype. One possible candidate was the polymorphic *SSD1* gene, which encodes the RNA-binding protein Ssd1, which binds 5′ and 3′ UTRs of mRNAs encoding cell wall morphogenesis proteins and represses their translation (5, 6). The Ssd1 family is closely related to the Dis3L2 3′→5′ exonucleases, which belong to the same RNase II/RNB family as the catalytic subunit of the RNA exosome Dis3 (22). In the genetic background BY4741, the active *SSD1-V* allele encodes the full-length protein, whereas strains of genetic background W303 carry the *ssd1-d* allele, which contains a premature stop codon due to a C-to-G transversion at nucleotide 2094, resulting in termination of the Ssd1 protein at the beginning of its RNB domain (Fig. 1B) (12). This truncation renders Ssd1 inactive: i.e., W303-derived strains behave as deficient for Ssd1 function.

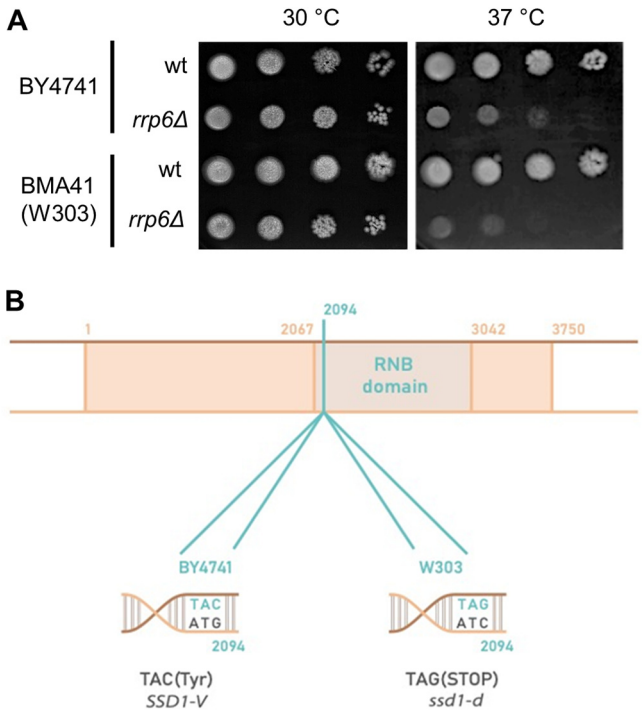

FIG 1 (A) The *rrp6*Δ mutation results in a stronger temperature-sensitive phenotype in BMA41 (W303) than in the BY4741 genetic background. (B) The *SSD1-V* allele of the BY4741 strain encodes a functional Ssd1 protein, whereas the *ssd1-d* allele of the W303 strain encodes a truncated nonfunctional Ssd1 protein. wt, wild type.

To test whether the nonfunctional *ssd1-d* allele causes the more severe temperature-sensitive phenotype of W303 *rrp6*Δ mutant cells, we deleted the *RRP6* gene in the W303 *SSD1-V* strain and its isogenic *ssd1-d* strain and tested cell viability at 37°C (Fig. 2A). As expected, the *SSD1-V* and *ssd1-d* strains displayed normal growth at the physiological and high temperatures, whereas the *rrp6*Δ mutation impaired growth of both strains at high temperature. Crucially, the growth of the *ssd1-d rrp6*Δ mutant was significantly more impaired than that of the *SSD1-V rrp6*Δ mutant at 37°C, clearly showing that the *ssd1-d* mutation enhances its temperature sensitivity (Fig. 2B). The cell morphology of these mutants was visualized by fluorescence microscopy after calcofluor white (CFW) staining of cell wall chitin (Fig. 2C). At high temperatures, *ssd1-d rrp6*Δ mutant cells are enlarged, aberrantly shaped, grow in clumps, and stain very brightly, which indicates increased chitin deposition, whereas *SSD1-V rrp6*Δ mutant cells have a more typical shape and size under the same conditions, while still showing some defects in cell separation, as evident by the prominent staining of the cell septa. In summary, the severity of the *rrp6*Δ temperature-sensitive phenotype depends on the functionality of the accompanying *SSD1* allele.

To test whether the functional *SSD1-V* allele is also able to partially suppress the temperature-sensitive phenotypes of RNA exosome mutants when expressed from a plasmid, we introduced a centromeric plasmid carrying the *SSD1-V* allele into these cells and tested their viability at high temperatures. We found that *rrp6*Δ cells carrying a centromeric plasmid with the *SSD1-V* allele grew significantly better at 36°C than the corresponding cells carrying the empty plasmid (Fig. 3A). Importantly, expression of the *SSD1-V* allele partially suppressed the temperature-sensitive phenotype of *dis3 exo*$^{-}$ cells, which are defective for the exoribonuclease activity of the essential RNA exosome catalytic subunit Dis3 (Fig. 3A).

We next tested the viability of these strains at 30°C upon exposure to different concentrations of the cell wall stressors calcofluor white (CFW) and caffeine (Fig. 3B). Exposure to CFW or caffeine severely impaired growth of the *ssd1-d rrp6*Δ mutant,

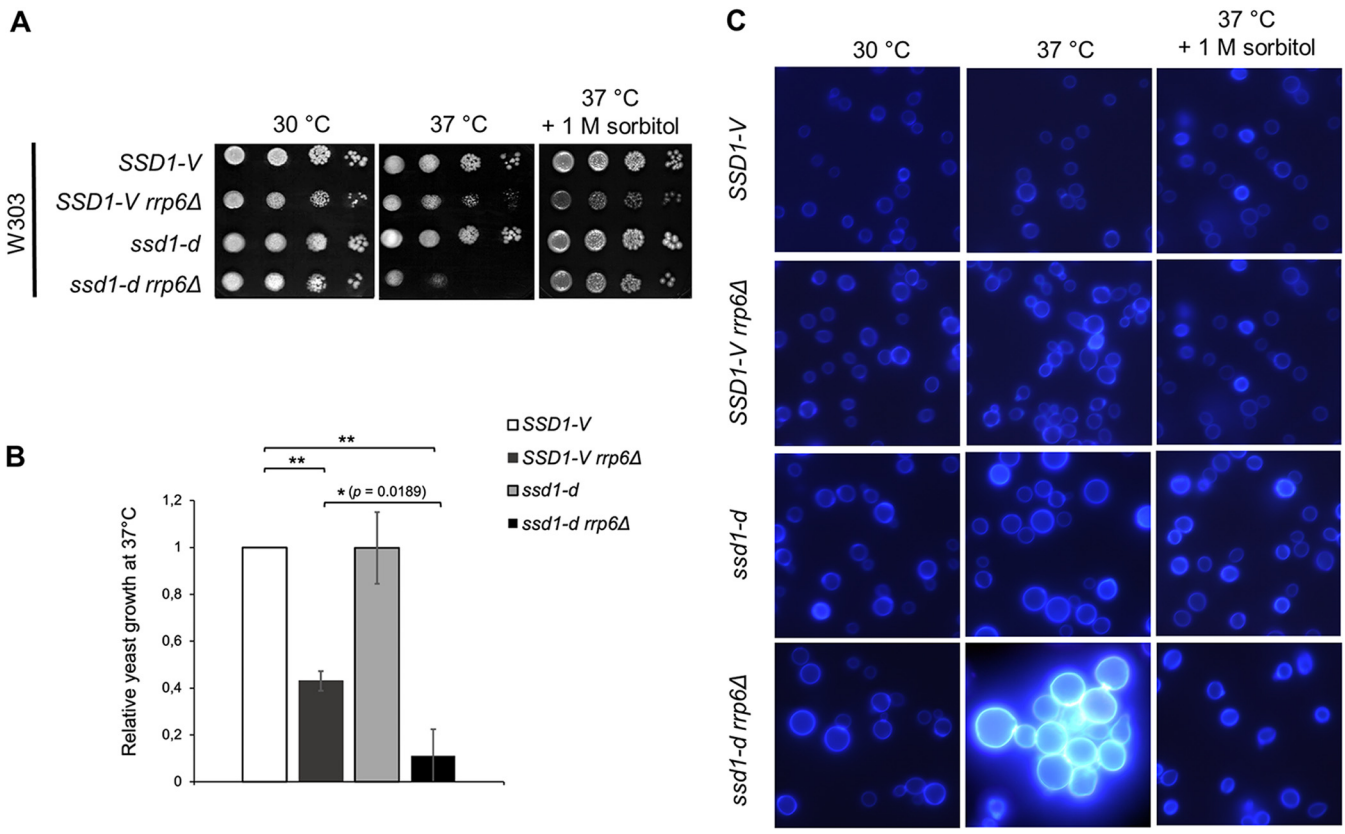

FIG 2 (A) The severity of the temperature-sensitive phenotype of the W303 *rrp6*Δ mutant depends on the functionality of the *SSD1* allele. (B) Strains were grown at 37°C as in panel A, and relative yeast growth on agar plates was quantified using the first dilution. The values shown represent the means and standard deviations from three independent experiments ($n = 3$). Indicated differences show the significant differences using a paired Student's *t* test. Asterisks indicate *P* values of ≤0.05 (*) and ≤0.01 (**). (C) Strains were grown as in panel A and visualized by fluorescence microscopy after calcofluor white staining of cell wall chitin.

while growth of the *SSD1-V rrp6*Δ mutant was much less affected. Interestingly, *ssd1-d* and *SSD1-V rrp6*Δ mutants, in which either Ssd1 or Rrp6, respectively, is nonfunctional, were less sensitive to CFW than the *ssd1-d rrp6*Δ double mutant, in which both proteins are nonfunctional. The synthetically sick phenotype of the double mutant cells upon cell wall stress suggests that Ssd1 and Rrp6 proteins function synergistically to promote cell wall integrity.

Double mutant cells in which Rrp6 and Ssd1 are inactivated show a more severe cell wall instability phenotype than corresponding single mutant cells. The most straightforward interpretation of this phenomenon is that the Rrp6-containing RNA exosome and Ssd1 operate through parallel pathways—that is, by regulating different cell-wall-related transcripts. However, since Rrp6 and Ssd1 are involved in RNA decay and translational repression of mRNAs, respectively, another possibility is that they regulate a common set of cell-wall-related transcripts. Inactivation of both proteins would then have a sequential, rather than a parallel, effect on cell wall instability at high temperatures. To this end, we examined relevant transcript levels in the RNA sequencing data set of BY4741 *rrp6*Δ mutant cells (10 min at 42°C) (23) and the enrichment of Ssd1 binding to mRNAs from the CRAC (cross-linking and analysis of cDNAs) data set (16 min at 42°C) (24). The first question we asked was how many cell-wall-related transcripts are significantly enriched for Ssd1 binding at high temperature? The second was how many are significantly dysregulated in *rrp6*Δ mutant cells compared with wild-type cells under similar conditions? The pool of mRNAs we focused on was divided into four main categories (see the supplemental material): those encoding cell-wall-anchored proteins, those directly or indirectly involved in cell wall assembly, and those related to osmoregulation, as Ssd1 has recently been shown to bind mRNAs

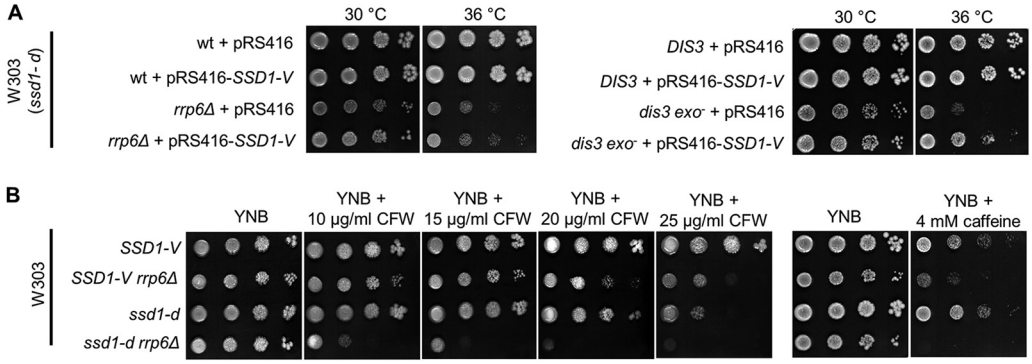

FIG 3 (A) Plasmid-borne expression of the *SSD1-V* allele partially suppresses the temperature-sensitive phenotype of the *rrp6*Δ mutant and the mutant for the exoribonuclease activity of the other RNA exosome catalytic subunit, Dis3. The spotting assay was performed on plates without uracil or leucine to select for the plasmid-bearing strains. (B) W303 *rrp6*Δ mutant cells are more sensitive to the cell wall stressors calcofluor white and caffeine when carrying the *ssd1-d* allele. The indicated amount of each cell wall stressor was added to plates, which were incubated at 30°C. wt, wild type.

encoding key osmotic response regulators (9). A large number of mRNAs significantly enriched for binding to Ssd1 represented transcripts related to cell wall construction, of which transcripts encoding cell-wall-anchored proteins and proteins indirectly involved in cell wall assembly were the most abundant (Fig. 4A). This is consistent with previous studies showing that, although Ssd1 binds various mRNAs, its major targets encode proteins involved in cell wall morphogenesis (5, 6, 24), making it a more cell-wall-specific regulator. Conversely, of the significantly dysregulated mRNAs in *rrp6*Δ mutant cells in comparison to wild-type cells at high temperature, only a small fraction represented transcripts related to cell wall construction (Fig. 4B). This is consistent with the direct or indirect involvement of the nuclear RNA exosome participating in the regulation of a variety of mRNA transcripts, as well as other RNA biotypes (25–27).

Second, we correlated the differentially expressed genes between *rrp6*Δ mutant and wild-type cells at 25°C (*x* axis) and at 42°C (*y* axis) and projected the enrichment of Ssd1 binding at 42°C as a color scheme on top of it (Fig. 4C). This enabled us to determine whether Ssd1 and Rrp6 share any common cell-wall-related targets. It is evident that the majority of transcripts bound by Ssd1 are not dysregulated in *rrp6*Δ mutant cells at 25 or 42°C, as they are localized in the middle quadrant of the correlation diagram. Furthermore, of the Ssd1 targets that are dysregulated in *rrp6*Δ mutant cells at both temperatures, only *TOS1*, *TIR1*, and *FIT3* encode cell wall proteins. Levels of Ssd1 targets that are also direct Rrp6 targets (i.e., are degraded by Rrp6) are expected to increase upon depletion of Rrp6; however, none of the transcripts whose levels follow this trend are related to cell wall assembly. Instead, all cell-wall-related targets shared by Ssd1 and Rrp6 are downregulated in *rrp6*Δ mutant cells compared with wild-type cells and therefore represent indirect targets of Rrp6. For example, *FIT3* encodes a glycosylphosphatidylinositol (GPI)-anchored cell wall mannoprotein involved in iron metabolism, a process regulated by the nuclear RNA exosome (28), and the *TIR1* gene, which encodes a serine-rich cell wall mannoprotein, is known to be negatively regulated by noncoding antisense transcription (29), which may explain its low mRNA levels in *rrp6*Δ mutant cells compared with wild-type cells. Thus, the levels of these mRNA targets are positively regulated by Rrp6 in wild-type cells and therefore do not explain the reason for the cell wall instability phenotype of *ssd1-d rrp6*Δ mutant cells. Taken together, this analysis shows that Rrp6 and Ssd1 do not have significantly overlapping cell-wall-related targets, strengthening the overall conclusion that these proteins act in independent, parallel pathways to maintain cell wall stability in yeast.

DISCUSSION

In this work, we investigated the interplay between the RNA-binding protein Ssd1 and the RNase subunits of the RNA exosome complex, Rrp6 and Dis3, in the

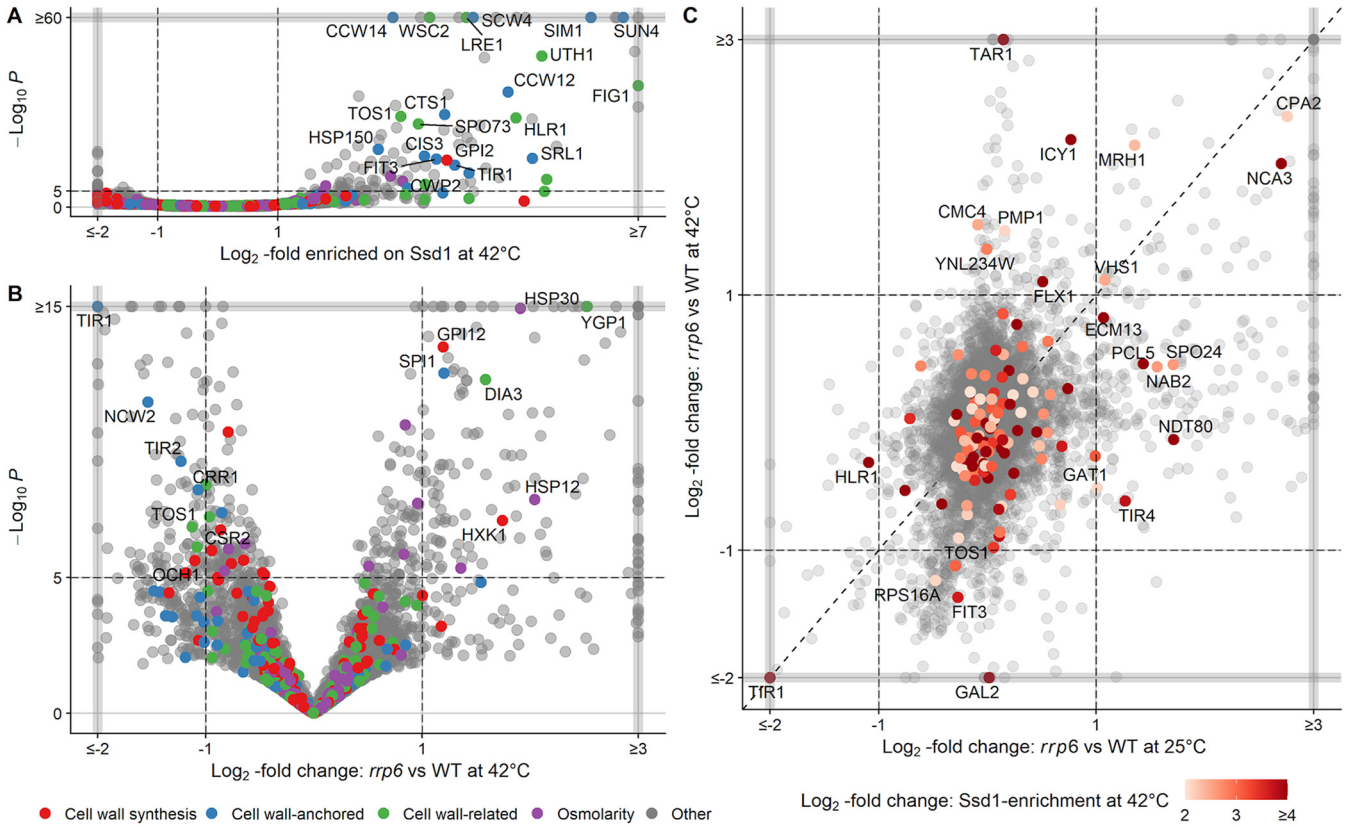

FIG 4 Volcano plots (A) showing which mRNAs are enriched for Ssd1 binding after heat stress (42°C for 16 min) (24) and (B) comparing transcriptomes of *rrp6*Δ versus wild-type (WT) yeast after heat stress (42°C for 10 min) (23). Different colors highlight four groups of cell-wall-associated proteins (see supplemental material). Only mRNAs belonging to these four groups of genes and more than 2-fold enriched/upregulated or depleted/downregulated ($|\log_2|$ fold change of ≥ 1, $-\log_{10} P \geq 5$) are highlighted. Genes within gray lines are more up- or downregulated than shown but were placed within the lines to save space. (C) Comparison of *rrp6*Δ versus wild-type yeast transcriptomes at 25°C (*x* axis) (23) and 42°C (*y* axis, 10 min) (23), with mRNAs highlighted in the shades of red if they are enriched for Ssd1 binding more than 4-fold ($\log_2$ fold change of ≥ 2) during heat shock (42°C for 16 min) (24) and labeled only if they are up- or downregulated by more than 2-fold ($|\log_2|$ fold change of ≥ 1).

maintenance of yeast cell wall stability. Ssd1 is closely related to the Dis3L2 RNase family, but its RNB domain is inactive, so it does not degrade mRNAs, but suppresses their translation by sequestering them into P-bodies (6, 7). Since proteins of the Dis3L2 family lack the PIN domain that anchors Dis3 and Dis3L to the exosome core, they do not physically interact with the RNA exosome and have been shown to act in exosome-independent pathways (30, 31). In line with this, Ssd1 and the RNA exosome appear to act in independent pathways to promote cell wall stability in yeast, as simultaneous inactivation of both factors results in a synthetically sick phenotype of cell wall instability. This finding is further supported by our analysis of the mRNA targets of Rrp6 and Ssd1, which show very little overlap for mRNAs encoding cell-wall-related proteins and none that could explain the severe cell wall instability phenotype of the *ssd1-d rrp6*Δ double mutant. The absence of Ssd1 activates the cell wall integrity (CWI) pathway, a signaling cascade that is a major regulator of the cell wall stress response in yeast (32). Because the absence of Rrp6 and the CWI kinase Mpk1 results in an additive phenotype of cell wall instability (23), Rrp6 may act in a signaling pathway that parallels Ssd1 and CWI signaling. When a cell is confronted with environmental stress, a rapid response leading to cell wall remodeling is critical for cell survival. Accordingly, cell wall damage triggers signal transduction through multiple pathways (33, 34). Because of their roles in all types of RNA-based gene regulatory mechanisms, RNA-binding proteins are emerging as major players in the cell wall stress response in yeast (2, 3, 35).

In line with this, we demonstrate that the severity of the temperature-sensitive phenotype of RNA exosome mutants is modified according to the functionality of the accompanying *SSD1* allele: i.e., it is enhanced in the W303 genetic background, where it is nonfunctional. Due to the basal level of cell wall destabilization caused by the

TABLE 1 *S. cerevisiae* strains used in this study

Strain	Genotype	Source
BY4741		
Wild type	*MAT***a** *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*	43
rrp6Δ	BY4741 with *rrp6*::*KanMX4*	19
BMA41		
Wild type	*MAT***a** *ade2-1 ura3-1 leu2-3,112 his3-11,15 trp1Δ can1-100*	44
rrp6Δ	BMA41 with *rrp6*::*KanMX4*	38
DIS3	*MAT***a** *ade2-1 ura3-1 leu2-3,112 his3-11,15 trp1-1 can1-100 dis3*::*KanMX4* [pBS3269-*DIS3*, *LEU2*]	45
dis3 exo^{-}	*MAT***a** *ade2-1 ura3-1 leu2-3,112 his3-11,15 trp1-1 can1-100 dis3*::*KanMX4* [pBS3270-*dis3D551N*, *LEU2*]	45
W303		
SSD1-V	*MAT***a** *ade2-1 trp1-1 leu2-3,112 his3-11,15 ura3-1 SSD1-V*	13
SSD1-V rrp6Δ	W303 *SSD1-V* with *rrp6*::*KanMX4*	This work
ssd1-d	*MAT***a** *ade2-1 trp1-1 leu2-3,112 his3-11,15 ura3-1 ssd1-d*	13
ssd1-d rrp6Δ	W303 *ssd1-d* with *rrp6*::*KanMX4*	This work

ssd1-d allele, RNA exosome mutations leading to temperature sensitivity have a more pronounced phenotype in this commonly used laboratory strain. Hypomorphism of Ssd1 in the W303 background has been shown to affect numerous yeast phenotypes in addition to cell wall stability, including aneuploidy tolerance, chronological life span, and transcription by all three RNA polymerases (9, 13, 36). Besides basic research on the RNA exosome complex, yeast cells are also used when modeling clinically relevant pathogenic mutations in genes encoding subunits of the RNA exosome complex to understand how these mutations alter its function (37). This study therefore highlights the need for careful interpretation of biological effects, especially when comparing data obtained with yeast strains of different origins.

MATERIALS AND METHODS

Strains, media, strain construction, and plasmids. The yeast strains used in this study are listed in Table 1. Yeast strains were grown in yeast nitrogen base (YNB) medium (described in reference 19) supplemented with the required amino acids and uracil (80 mg/liter each), with addition of 16 g/liter agar for plates. The *RRP6* gene was deleted in W303 *ssd1-d* and *SSD1-V* strains using a disruption cassette generated by PCR with primers RRP6-Kan1 and RRP6-Kan2 (38). Transformants were selected on G418 plates (0.2 mg/ml; Sigma), and gene deletion was confirmed by PCR. pRS416-*SSD1-V* is a centromeric vector that carries the *SSD1-V* allele under regulation of its native promoter, and pRS416 is its corresponding empty vector (39).

Spotting assays. Exponential-phase cultures were adjusted to an optical density at 600 nm (OD_{600}) of 1, and four 10-fold serial dilutions of that sample were spotted onto YNB plates. Plates were incubated at indicated temperatures for 3 days and photographed using a UVIDOC HD6 camera (Uvitec, Cambridge, United Kingdom). Relative yeast growth on agar plates was quantified by densitometry as described in reference 40.

Fluorescence microscopy. Cells were stained with calcofluor white (CFW) stain (Sigma) and visualized using the Olympus BX51 fluorescence microscope under identical conditions and settings for all samples. The fluorescence from CFW was filtered with a DAPI (4′,6-diamidino-2-phenylindole) filter.

Bioinformatic analysis and data availability. The bioinformatic analysis was conducted in the R computing environment (R Core Team, 2020). Comparison of the *rrp6Δ* mutant with the wild type at 25 and 42°C for 10 min was based on data from reference 23 obtained from the Gene Expression Omnibus (GEO accession no. GSE140504), while the data set quantifying Ssd1-enriched transcripts (24) was obtained from Github (https://github.com/ewallace/Ssd1_CRACanalysis_2020). Both sets of data were processed as in the original publications, using DESeq2 (41), followed by shrinking $\log_2$ fold change with the adaptive Student's *t* prior shrinkage estimator from the "apeglm" package (42). Detailed results of the DESeq and lfcShrink functions, used to plot Fig. 4 and exported from the R programming environment as .xlsx files, are given in the supplemental material, together with lists of genes that comprise four color-coded groups in panels A and B.

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

SUPPLEMENTAL FILE 1, XLSX file, 1.4 MB.

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We declare no conflicts of interest.

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Paper 3

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REVIEW ARTICLE

Non-coding RNAs as cell wall regulators in *Saccharomyces cerevisiae*

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ABSTRACT

The cell wall of *Saccharomyces cerevisiae* is an extracellular organelle crucial for preserving its cellular integrity and detecting environmental cues. The cell wall is composed of mannoproteins attached to a polysaccharide network and is continuously remodelled as cells undergo cell division, mating, gametogenesis or adapt to stressors. This makes yeast an excellent model to study the regulation of genes important for cell wall formation and maintenance. Given that certain yeast strains are pathogenic, a better understanding of their life cycle is of clinical relevance. This is why transcriptional regulatory mechanisms governing genes involved in cell wall biogenesis or maintenance have been the focus of numerous studies. However, little is known about the roles of long non-coding RNAs (lncRNAs), a class of transcripts that are thought to possess little or no protein coding potential, in controlling the expression of cell wall-related genes. This review outlines currently known mechanisms of lncRNA-mediated regulation of gene expression in *S. cerevisiae* and describes examples of lncRNA-regulated genes encoding cell wall proteins. We suggest that the association of currently annotated lncRNAs with the coding sequences and/or promoters of cell wall-related genes highlights a potential role for lncRNAs as important regulators of the yeast cell wall structure.

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Yeast; cell wall; long non-coding RNAs (lncRNAs); gene regulation

Introduction

The budding yeast *Saccharomyces cerevisiae* is a unicellular eukaryote encapsulated by a multi-layered cell wall that acts as an extracellular organelle important for the cell's protection and the detection of environmental cues. Given that the cell wall represents a cell's interface with the environment, a more detailed understanding of its dynamics enables engineering of the yeast cell surface to make this organism a better tool for biotechnology and synthetic biology (Tanaka and Kondo 2015; Lozancic et al. 2019). Moreover, the cell wall is a critical target for antifungal therapies since it is essential for the yeast cell and an equivalent structure is absent from mammalian host cells (Cortés et al. 2019). The cell wall of vegetative yeast cells is a polysaccharide network built out of β-1,3-glucan, β-1,6-glucan and chitin, to which mannoproteins are bound (Nguyen et al. 1998). As it is crucial for maintaining optimal integrity of the yeast cell, the cell wall has to be continuously remodelled as cells progress through their life cycle and mitotic cell cycle phases and while they are confronted with various environmental stressors. This is accomplished by modifying the polysaccharide network through coordinated action of glycoside hydrolases, glycosyltransferases, and transglycosylases, as well as incorporation or shedding of cell wall mannoproteins (Klis et al. 2002; Hurtado-Guerrero et al. 2009; Teparić and Mrša 2013). The expression of genes encoding cell wall-related proteins is therefore tightly regulated. This often occurs *via* various mechanisms to achieve fine-tuning of regulation, depending on the type and strength of environmental stimuli that the cells must respond to. Indeed, several regulatory strategies have been implicated in cell wall gene expression, e.g. transcriptional control imposed by the cell wall integrity (CWI) pathway and other major signalling pathways (Klis et al. 2002; Sanz et al. 2017), chromatin-based regulation of promoter structure (Barrales et al. 2012; Sanz et al. 2018), regulation of mRNA stability and localization (Catala et al. 2012; Cohen-Zontag et al. 2019) and proteolytic processing (Gagnon-Arsenault et al. 2008; Grbavac et al. 2017). In this review, we summarize the current knowledge of how the transcription of long

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Supplemental data for this article can be accessed here.

non-coding RNAs regulates yeast gene expression and provide arguments in favour of ncRNAs as important new regulators of genes involved in establishing the yeast cell wall structure. In particular, we discuss the important question if the ncRNA's synthesis or the RNA molecule itself is critical for its regulatory role.

Transcriptional regulatory roles of non-coding RNAs in yeast

Recent studies using DNA strand-specific tiling microarrays and RNA-sequencing discovered pervasive transcription across eukaryotic genomes, that results in transcription of numerous non-coding RNAs (ncRNAs) (Shoemaker et al. 2001; Wheelan et al. 2008; Granovskaia et al. 2010; Lardenois et al. 2011). These transcripts have little or no protein-coding potential and, based on their length, are defined as either small (<200 nt) or long (≥200 nt). The budding yeast *Saccharomyces cerevisiae* is a unicellular eukaryote that lost the RNA interference (RNAi) pathway during evolution and therefore completely lacks small ncRNAs (Fink et al. 2014). Loss of RNAi permitted an expansion of its long non-coding RNA (lncRNA) transcriptome, which shows unusually high expression levels, extensive transcript lengths and high degrees of overlap with protein-coding genes in the case of sense/antisense pairs (Alcid and Tsukiyama 2016). Yeast lncRNAs are further classified on the basis of their sensitivity to RNA-degradation pathways, or conditions in which they are transcribed. In this regard, stable unannotated transcripts (SUTs) can be detected in wild type cells but cryptic unstable transcripts (CUTs) and Xrn1-sensitive unstable transcripts (XUTs) can only be detected upon inactivation of Rrp6 or Xrn1 exoribonucleases, respectively (Wyers et al. 2005; Davis and Ares 2006; Xu et al. 2009; Van Dijk et al. 2011). Furthermore, meiotic unannotated transcripts (MUTs) show peak expression during early, middle or late meiosis (Lardenois et al. 2011).

The functions of most ncRNAs are currently not known; however, some examples in yeast are well studied and exemplify mechanisms of transcriptional regulation *via* non-coding transcription. ncRNAs are transcribed on the same (sense ncRNAs) or opposite strand (antisense ncRNAs) of a protein-coding gene. Some ncRNAs overlap gene open reading frames (ORFs) and others are intergenic, e.g. their transcription occurs at 5′ (promoter) or 3′ regions of a gene. As a general rule, regulatory ncRNAs are more or less unstable and some are known to control the expression of their target genes at transcriptional or post-transcriptional levels. These transcripts most often act *in cis* at their genomic loci, to either positively or negatively influence protein-coding gene expression. Known mechanisms for gene regulation through ncRNAs include (1) *sense/antisense transcriptional interference*, that is to say, their transcription interferes with the synthesis of the sense transcript at the level of transcriptional initiation or elongation (Figure 1(A)) (Bumgarner et al. 2009; Donaldson and Saville 2012; Till et al. 2018), and (2) *promoter interference*, whereby their transcription across a target gene's promoter influences binding of transcriptional (co)factors and/or assembly of the preinitiation complex (Figure 1(B)) (Bumgarner et al. 2009; Donaldson and Saville 2012; Niederer et al. 2017; Till et al. 2018). In such cases it is the transcriptional synthesis of ncRNAs alone that elicits a regulatory effect. This is consistent with the fact that these RNAs are typically unstable because they are targeted by the nuclear RNA exosome for rapid degradation. Well studied examples include ncRNA-mediated transcription interference *in cis* at *SER3* and *IME1* loci and *trans*-acting ncRNAs at Ty1 and *PHO84* loci, reviewed in Niederer et al. (2017) and Till et al. (2018).

However, another interesting class of ncRNAs can also influence chromatin structure by recruiting chromatin-modifying or -remodelling complexes. This indicates a regulatory effect beyond RNA transcription that involves the ncRNA itself, by indirectly stabilizing a given chromatin conformation at their respective target loci (Donaldson and Saville 2012; Till et al. 2018). In other recent work, it was proposed that sense/antisense overlapping pairs of mRNA/lncRNA transcripts could

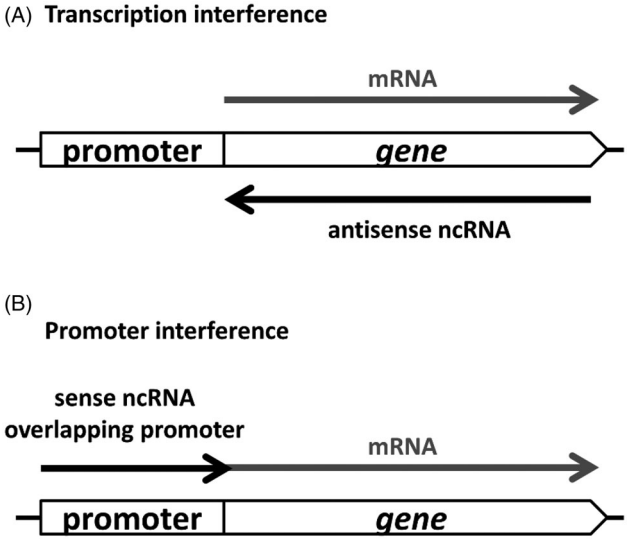

Figure 1. Synthesis of non-coding transcripts. Two schematics show (A) antisense and (B) sense lncRNA synthesis that could influence gene expression. Arrows represent mRNA or lncRNA transcripts. Promoters and genes are indicated. A black line represents DNA.

form double-stranded RNAs (dsRNAs) that may negatively regulate the mRNA-encoded protein levels (Becker et al., 2017). This points to an interesting novel regulatory role for antisense lncRNAs in controlling mRNA localization and/or translation. Again, in such cases it is not just the synthesis of antisense RNA but its ability to form dsRNAs that mediates its effect. We propose that interacting (that is, protein/RNA-binding) regulatory ncRNAs are an emerging critical class of regulatory transcripts in yeast and likely also in multicellular eukaryotes.

lncRNA-associated genes encoding cell wall-related proteins

The PIR gene family encodes the so-called Proteins with Internal Repeats, which are bound to the cell wall covalently through an alkali-labile linkage, presumably formed between their internal repeat unit and β-1,3-glucan (Ecker et al. 2006). *Saccharomyces cerevisiae*'s genome encodes five Pir proteins (Pir1–5), which differ in the number of internal repeats (1–10) (Table 1). These proteins are non-essential for growth in rich medium and their physiological roles are still poorly defined. They seem to have largely redundant functions, since the quadruple disruption of *PIR1-4* additively leads to a fragile cell wall phenotype that causes slow growth, osmotic instability and sensitivity to cell wall-disturbing agents (Mrša and Tanner 1999). These four genes are induced by the cell wall integrity pathway (CWI) through Mpk1 (Jung and Levin 1999). Furthermore, *PIR1-3* were also shown to be among the most highly regulated genes in the cell cycle (Spellman et al. 1998), while *PIR5* seems to be required only for sporulation (Enyenihi and Saunders 2003). Importantly, all *PIR* loci except *PIR4/CIS3* are associated with non-coding transcription (Xu et al. 2009; Lardenois et al. 2011). *PIR1* entirely overlaps with antisense transcript SUT227 and this transcript pair shows antagonistic expression during meiosis and sporulation; however, this does not appear to affect Pir1 protein levels (Becker et al. 2017). At the same time, antisense transcription *PIR2/HSP150* decreases when cells switch from respiration to sporulation, while Pir2 protein levels increase (Becker et al. 2017). Conversely, the non-coding intergenic transcript SUT228 is transcribed upstream of *PIR3*'s ORF in the sense direction, through its promoter region (Xu et al. 2009). Similarly to *SER3* regulation by SRG1 (Winston et al. 2005), *PIR3* ncRNA acts *in cis* and has a negative effect on respective coding transcription: its abrogation by insertion of a transcription termination site leads to a 2-fold increase in *PIR3* expression (Ceschin 2012). Curiously, both *PIR3* ncRNA and *PIR3* mRNA show increased levels under cell wall stress conditions, such as elevated temperature or treatment with caffeine, which argues against an antagonistic role of non-coding transcription in these conditions (Ceschin 2012). Nevers et al. studied quiescence-specific gene expression and found that a significant proportion was silenced during exponential growth by non-coding transcription. Since that study also found strong induction of *PIR3* upon quiescence (Nevers et al. 2018) and we observed that HA tagged Pir3 is readily detectable by Western blotting in stationary but not exponential cultures (unpublished results from the I. Stuparević laboratory) it would be interesting to test the impact of its non-coding transcription in these conditions.

TIR1, previously identified as *SRP1* (Serine-rich protein 1), is a non-essential gene induced by glucose, low temperature, anaerobiosis and static culture conditions (Marguet and Lauquin 1986; Donzeau et al. 1996; Kitagaki et al. 1997) (Table 1). It encodes a GPI-anchored cell wall mannoprotein rich in clustered serine and alanine residues. Tir1 probably participates in sustaining anaerobic β-1,3-glucan assembly (Bourdineaud et al. 1998). Importantly, *TIR1* is strongly silenced upon inactivation of the 5′–3′ cytoplasmic exonuclease Xrn1, presumably due to stabilization of its non-coding antisense transcript TIR1axut (van Dijk et al. 2011). Indeed, abrogation of TIR1axut transcription by insertion of a KANMX cassette re-establishes 70% of the *TIR1* mRNA level in the *xrn1* mutant (van Dijk et al. 2011). *TIR1* silencing was also shown to be mediated by methylation of histone H3 lysine 4 by Set1, as disrupting *SET1* results in high levels of *TIR1* mRNA in the *xrn1* mutant (van Dijk et al. 2011). Additionally, two other genes from the same family, *TIR2* and *TIR3*, are significantly downregulated in *xrn1* cells (van Dijk et al. 2011).

Table 1. Examples of well-studied cell wall-related genes regulated by lncRNA transcription.

Gene	ncRNA	cis/trans	sense/antisense	Effect on transcription	Mechanism of regulation
FLO11	*ICR1*	*cis*	Sense	Negative	Promoter interference
	PWR1	*cis*	Antisense	Positive	Transcription interference
ECM3	*EUC1*	*cis*	Sense	Positive	N.D.
SPS100	*SUT169*	*cis*	Antisense	Positive	mRNA isoform regulation
PIR3	*SUT228*	*cis*	Sense	Negative	N.D.
TIR1	*TIR1axut*	*cis*	Antisense	Negative	N.D.

A major cell wall mannoprotein required for cell-cell and cell-surface adhesion is encoded by *FLO11/MUC1* (Flocculation 11), which is regulated by the largest known promoter in the *S. cerevisiae* genome (Lo and Dranginis 1998; Barrales et al. 2012) (Table 1). *FLO11* 5′ regulatory region comprises about 3.4 kb, integrates regulatory signals from at least three signalling pathways (MAPK and cAMP/PKA pathways and Gcn4-controlled signalling) and is bound by numerous chromatin factors (Rupp et al. 1999; Halme et al. 2004; Barrales et al. 2008, 2012; Wang et al. 2015). Another aspect of its regulatory complexity came to light when a *cis*-acting two-component ncRNA "toggle switch" was discovered to be of central importance for its regulation (Bumgarner et al. 2009). Two ncRNAs are antagonistically transcribed in the *FLO11* 5′ regulatory region: a 3,2 kb sense transcript called ICR1 (interfering Crick RNA) and a 1,2 kb antisense transcript called PWR1 (promoting Watson RNA) (Bumgarner et al. 2009) (Figure 2(A)). Transcription of ICR1 inhibits *FLO11* transcription through a promoter interference mechanism, while transcription of PWR1 inhibits transcription of ICR1 and consequently has a net positive effect on *FLO11* transcription (Bumgarner et al. 2009). Single-cell analysis supports this model and expands on it to explain how this toggle switch contributes to clonal heterogeneity of *FLO11* expression, i.e. why some cells in a population strongly induce *FLO11*, while in others it is fully repressed (Grisafi et al. 2013). The current model presents a role for transcription factors Flo8 and Sfl1 in promoting transcription of PWR1 or ICR1, respectively. Depending on their competitive binding either one or the other ncRNA is transcribed and activating or silencing factors are subsequently recruited to the *FLO11* promoter (Bumgarner et al. 2009; Grisafi et al. 2013). Transcription of these ncRNAs is also influenced by local chromatin structure. Curiously, the histone-deacetylase Rpd3L and the histone-acetylase Gcn5 are both implicated in repressing ICR1 transcription and thereby promote *FLO11* expression under certain conditions (Bumgarner et al. 2009; Wang et al. 2015). Recent genome-wide RNA profiling studies based on RNA-sequencing clearly identified both *FLO11* mRNA and upstream lncRNAs and also revealed partially overlapping antisense transcripts (SUT194 and NUT0373; Figure 2(A)) (Van Dijk et al. 2011). Critically, a study using engineered yeast cells that express Dicer and Argonaut (required for RNAi) detected the formation of double-stranded RNAs that likely involve *FLO11* at the 5′ and 3′ regions (Wery et al. 2016) (Figure 2(B)). We propose that such structures might influence mRNA stability/localization and they could interfere with ribosome binding and/or elongation.

A comparable configuration resembling a two-component ncRNA toggle switch was reported for the promoter of *FLO10*, which belongs to the same gene family as *FLO11* and also shows heterogeneous expression within populations. However, *FLO10*'s regulation was not studied in detail (Bumgarner et al. 2009). Intriguingly, an antisense upstream lncRNA (XUT1464) covers almost the entire large 5′-UTR of *FLO10* and forms a dsRNA with it (Figure 3(A,B)). It is conceivable that such a structure has an effect on the translation of *FLO10* by interfering with ribosome binding. We note that repression of *FLO1*, *FLO5*, *FLO9* and *FLO10* was shown to require the NNS (Nrd1-Nab3-Sen1) complex and the endoribonuclease Rnt1 for transcriptional termination and mRNA degradation, respectively (Singh et al. 2015). These genes were also found to be significantly upregulated upon inactivation of the exoribonuclease Rrp6, however, this upregulation did not cause a flocculation phenotype (Singh et al. 2015).

The non-essential *ECM3* (Extra cellular mutant 3) gene was first identified in a large-scale screen for yeast genes involved in cell surface biosynthesis and architecture, based on the sensitivity of the corresponding mutant to the cell wall stressor Calcofluor White (Lussier et al. 1997) (Table 1). The non-coding intergenic transcript EUC1 (*ECM3* upstream CUT) is transcribed across the *ECM3* promoter in the sense direction (Raupach et al. 2016), resembling the well-studied mechanism of *SER3* regulation via the *cis*-acting intergenic transcript *SRG1* (Winston et al. 2005; Hainer et al. 2011). Stabilization of EUC1 upon inactivation of the exosome complex does not affect the level of *ECM3* mRNA; however, reducing EUC1 transcription by deletions in its promoter region decreases the level of *ECM3* mRNA, arguing for a positive role of intergenic transcription in controlling the expression of *ECM3* (Raupach et al. 2016). Expression of *ECM3* is also positively regulated by the Paf1 complex, which associates with RNA Polymerase II during transcriptional elongation and plays a crucial role in the co-transcriptional establishment of histone modifications, of which ubiquitination of histone H2B lysine 123 and methylation of histone H3 lysine 4 are required for *ECM3* expression (Raupach et al. 2016). The Paf1 complex has recently been implicated in broadly affecting transcription of non-coding RNAs (Ellison et al. 2019). Interestingly, both inactivation of the Paf1 complex and abrogation of EUC1 transcription reduce methylation of histone H3 lysine 4 in the 5′ region of *ECM3*'s ORF. However, their combined inactivation causes a greater defect in *ECM3*

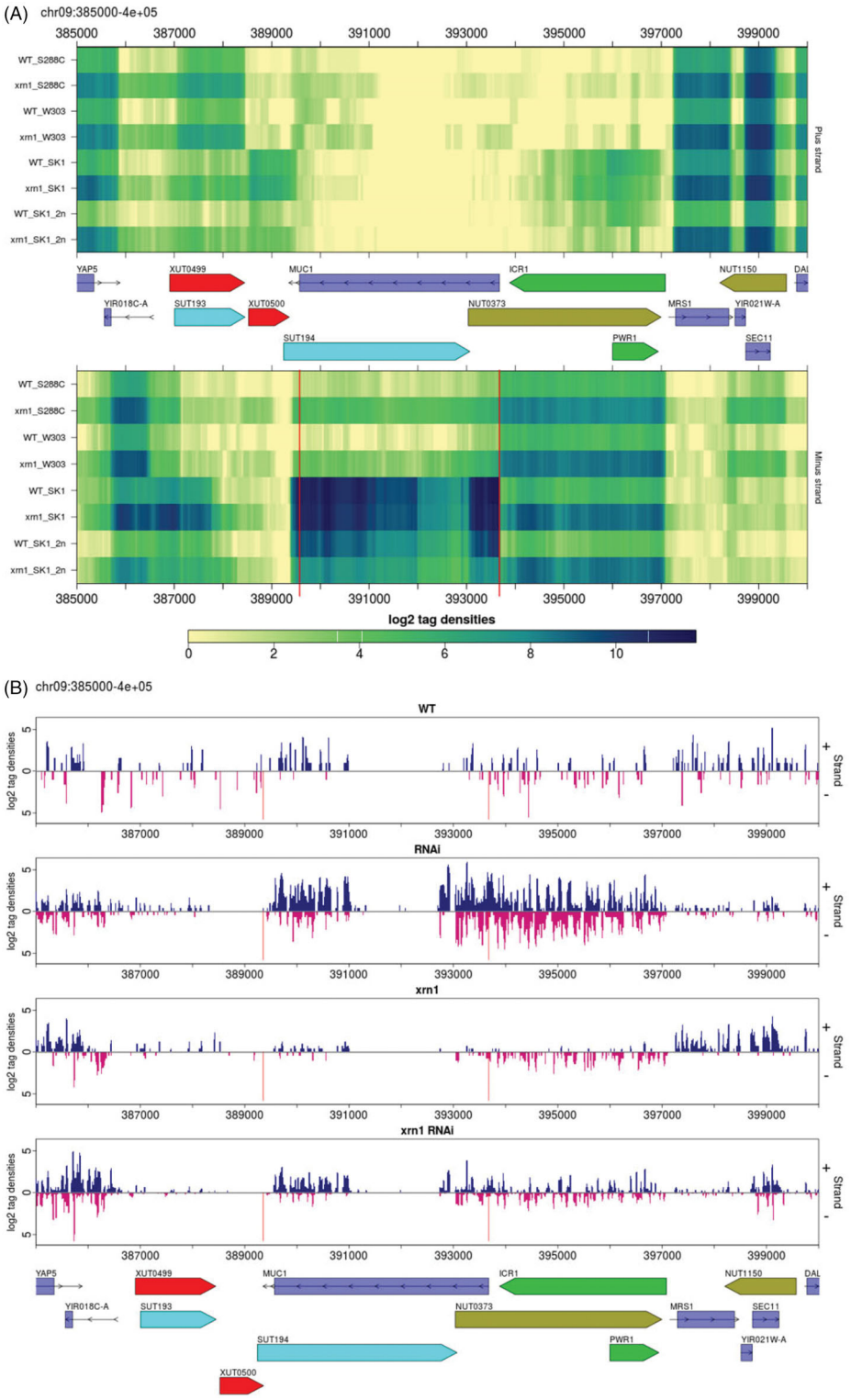

Figure 2. RNA and dsRNA profiling data for *FLO11* (*MUC1*). (A) The online version of the paper shows a color coded heatmap for the genomic region including *FLO11* (*MUC1*). RNA-Sequencing data are shown for ORFs (violet), XUTs (red), SUTs (light blue), NUTs (olive green), and annotated lncRNAs (green) as rectangles. For ORF, an arrow indicates the direction of transcription. Wild type (WT) and mutant strains lacking XRN1 (*xrn1*) strains in different genetic backgrounds (haploid strains S288C, W303, SK1 and diploid strain SK1 2n) are shown to the left and top (plus) and bottom (minus) strands are given to the right. Genome coordinates and chromosome numbers are shown. A scale for log 2 transformed expression data is shown at the bottom. Vertical lines delineate the target gene. The RNA profiling data were published by van Dijk et al. (2011). (B) A bar diagram summarizes dsRNA data for the *FLO11* (*MUC1*) locus. Log-transformed signals and DNA strands are indicated to the left and right, respectively. Genome annotation is like in panel A. dsRNA signals for the top strand (+) are in blue and for the bottom (−) strand are in purple. The data were published by Wery et al. (2016). A genomics viewer is available at http://vm-gb.curie.fr/mw2/ (follow *XUT lncRNAs landscape* for RNA data and *genome wide mapping of double stranded RNA* for dsRNA data).

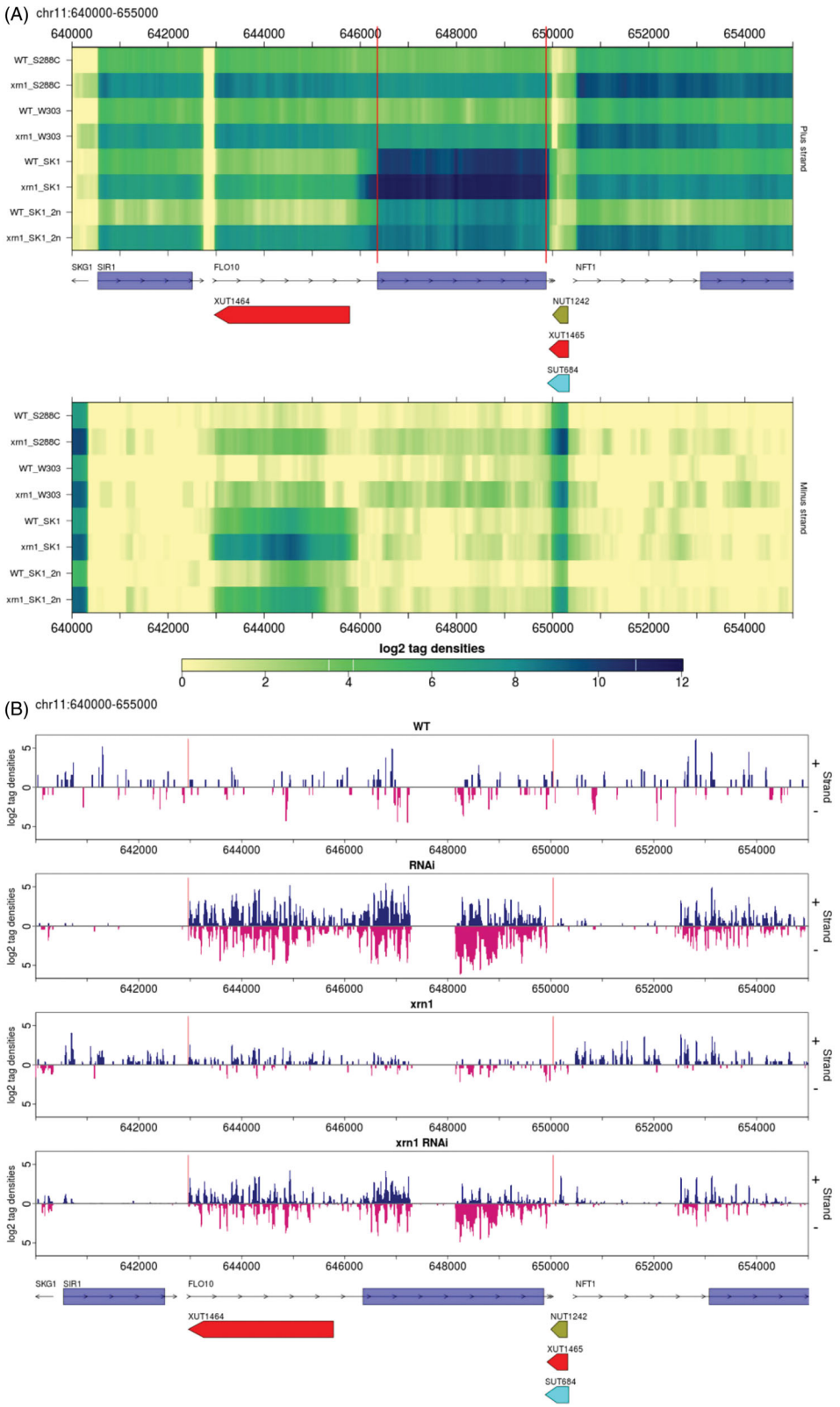

Figure 3. RNA and dsRNA profiling data for *FLO10*. (A, B) Data for RNA and dsRNA signals are shown as in Figure 2.

expression than either mutation alone. This indicates that they have non-overlapping synergistic roles in this process (Raupach et al. 2016).

Like in the cases of *FLO10* and *FLO11*, profiling data show that *ECM3* overlaps an antisense lncRNA (NUT1420) and that haploid cells undergoing rapid

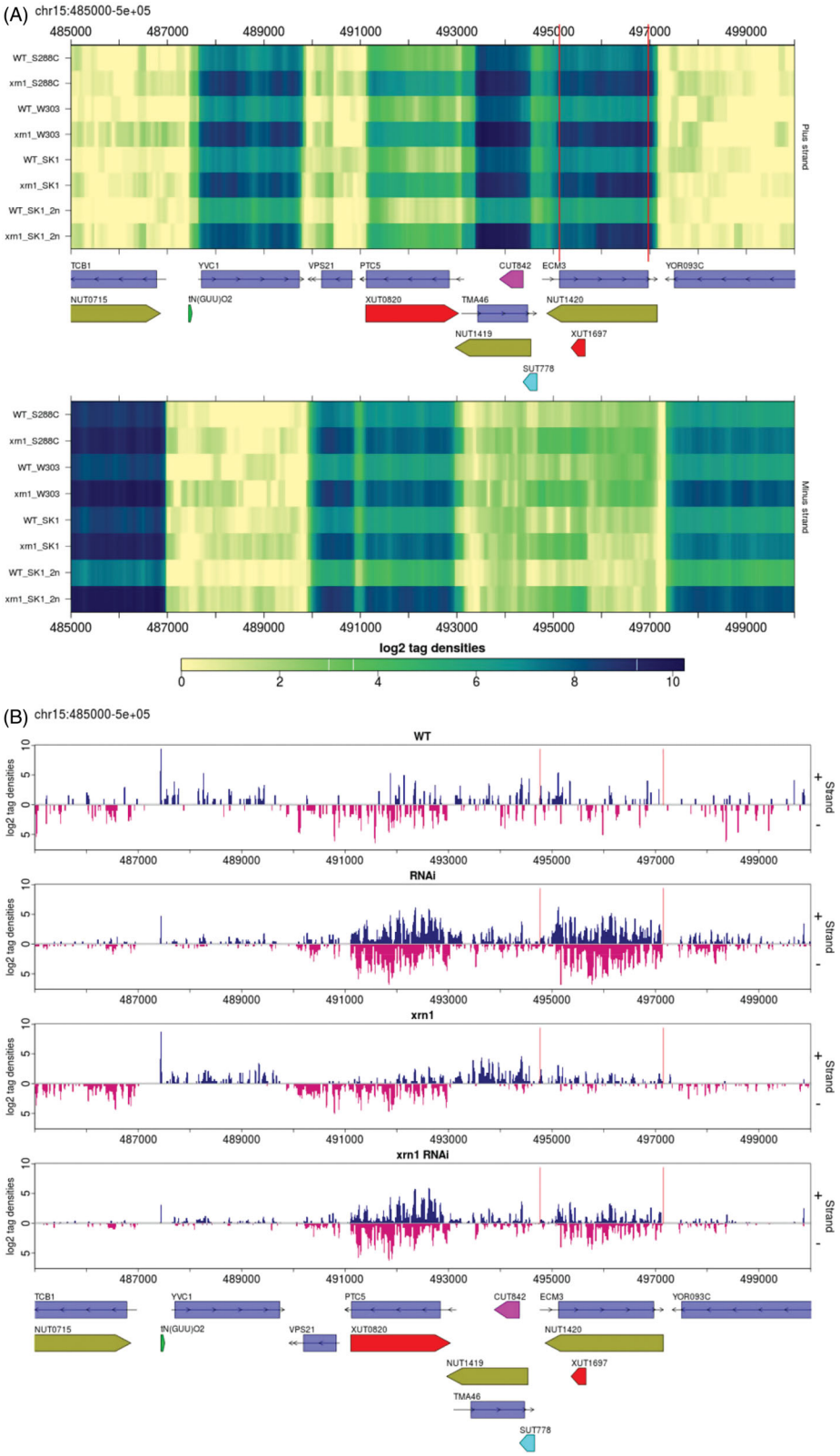

Figure 4. RNA and dsRNA profiling data for *ECM3*. (A, B) Data for RNA and dsRNA signals are shown as in Figure 2.

growth in rich medium (YPD) form a stable dsRNA at the *ECM3*/NUT1420 locus (Figure 4(A,B)). Such a configuration is consistent with rapid growth under optimal conditions where no stress signal requires large quantities of Ecm3 protein. Consistently, S288C yeast cells growing in rich medium (YPD) contain 55 molecules per cell, while cells cultured in synthetic complete medium (SC) contain 1785 molecules per cell (Ho et al.

2018). It is tempting to speculate that NUT1420 and, as a consequence, dsRNA formation are down-regulated in minimal media, enabling more efficient *ECM3* mRNA translation.

SPS100 (Sporulation specific 100) gene is induced late in sporulation and encodes a spore wall protein required for timely spore wall maturation (Law and Segall 1988) (Table 1). Normal expression of *SPS100* during sporulation requires the putative Ser/Thr protein kinase Sps1 (Friesen et al. 1994). During nutrient starvation (3 days in liquid SC medium with 0,1% glucose), expression of *SPS100* is positively regulated *in cis* through transcription of the non-coding antisense RNA SUT169 (Bunina et al. 2017). Surprisingly, SUT169 does not influence the activation of *SPS100*'s promoter, but instead regulates the ratio of *SPS100* 3′ mRNA isoforms that show different half-lives (Bunina et al. 2017). Transcription of SUT169 promotes expression of the long *SPS100* mRNA isoform, which is more stable than the short isoform. This effect requires the $(AAAAAC)_8$ tandem repeat in SUT169 to promote its stability and/or regulate the mRNA isoform switch (Bunina et al. 2017). Remarkably, the 3′-intergenic region (IGR) of *SPS100*, from which SUT169 transcription is initiated, is a context-independent regulatory element, as replacing the 3′-IGR of a gene of interest by *SPS100*'s 3′-IGR leads to an antisense-dependent upregulation of the corresponding gene (Bunina et al. 2017).

Cell wall-related loci are associated with antisense lncRNAs

At least 201 genes encode proteins connected to the cell wall structure, its biosynthesis or remodelling in vegetative yeast cells (Orlean 2012). Manual inspection of these genes using data provided by online viewers of genome-wide ncRNA expression levels (http://sgv.genouest.org/; Xu et al. 2009; Granovskaia et al. 2010; Lardenois et al. 2011; http://vm-gb.curie.fr/mprimig/5FU/; Xie et al. 2019; and http://vm-gb.curie.fr/mw2 Wery et al. 2016) shows that many cell wall related loci are associated with ncRNAs. We first focussed on antisense transcripts, which overlap sense ORFs and found that 88 of 201 loci (44%) exhibit antisense non-coding transcription (Figure 5(A); Supplemental File 1). A detailed classification reveals that the group contains 57 SUTs, 12 CUTs, 48 XUTs and 11 MUTs (the sum of which exceeds 88 because many RNAs bear multiple annotations). Interestingly, a genome wide analysis shows that some differentially expressed mRNAs of protein-coding genes related to the cell wall show opposed expression profiles when compared to their antisense ncRNAs (Lardenois et al. 2011). For example, the transcriptional level of *SCW11*, encoding a cell wall protein similar to glucanases, decreases during meiosis, while the level of its antisense transcript SUT1580 increases. Similar expression profiles are observed for *SSG1*/SUT785, *PIR1*/SUT227, *and SPS22*/SUT1024 loci. On the other hand, expression of *KNH1* and its antisense SUT1240 increases simultaneously during meiosis (see http://sgv.genouest.org).

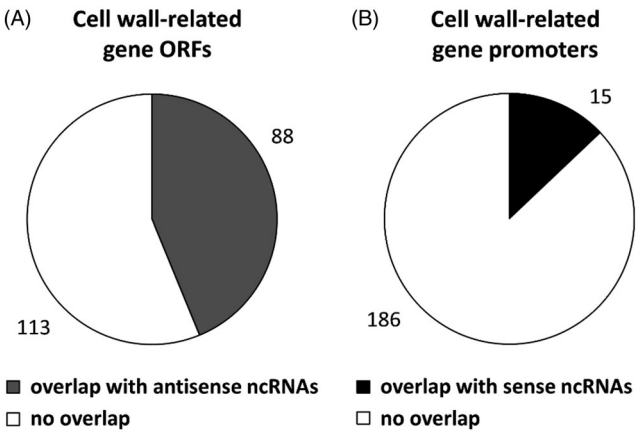

Figure 5. Non-coding transcripts related to cell wall-related loci. (A) Number of antisense ncRNAs overlapping cell wall related ORFs; (B) number of sense ncRNAs transcribed over promoter regions of cell wall related genes.

A survey of annotated sense non-coding transcripts which overlap putative promoter regions (500 bp upstream of ORFs; Lubliner et al. 2015) and do not overlap another gene's ORF, showed that 15 of the 201 cell wall-related gene loci exhibit sense non-coding transcription over promoter regions (e.g. *PIR2*/MUT847, *PIR3*/SUT228, *VRG4*/SUT111) (Figure 5(B)). Detailed classification resulted in 5 SUTs, 7 CUTs, 3 XUTs and 3 MUTs. Of note, the numbers of genes exhibiting non-coding transcription does not correspond to the sum of transcripts found in the detailed classification, because some loci express two different transcripts in the same region, e.g. *SPS2*/SUT081, CUT100, XUT0249; *CHS7*/SUT588, XUT1308 and *ENG1*/XUT0781, XUT0782 (Xie et al. 2019). We also found that most cell wall related non-coding antisense RNAs (e.g. *SED1*/SUT1135, *YPS2*/XUT0191, *FKS3*/CUT792) form double-stranded RNAs with coding transcripts upon reconstitution of RNAi pathway in *S. cerevisiae* (Wery et al. 2016) (and unpublished data from M. Primig's laboratory), which also argues in favour of the idea that they have regulatory roles.

In addition, Wilkinson et al. used RNA sequencing to study the differential expression of lncRNAs within differentiated cell subpopulations of colonies and biofilms and found significant differences between cells located

at upper (U) and lower (L) parts of a 15-day-old colony (Wilkinson et al. 2018). A large number of gene/lncRNA pairs were either co- or anti-regulated in U as opposed to L cells, which included genes with roles in cell wall organization (Wilkinson et al. 2018). This supports a role for non-coding transcripts in cell wall remodelling, as U cells are known to resemble starved and quiescent cells which have thickened cell walls, which is in contrast to L cells that mobilize carbohydrates stored in the cell wall by activating cell wall-degrading enzymes (Traven et al. 2012).

Perspectives

It is estimated that approximately 20% of all currently annotated yeast genes are broadly involved in cell wall formation and maintenance (De Groot et al. 2001). This reflects how important it is for yeast cells to be able to quickly and thoroughly adapt their cell wall structure in response to environmental cues that stimulate cell division, mating, gametogenesis, stress response or quiescence. Among 201 genes directly involved in cell wall formation, maintenance or remodelling, we selected 88 loci that exhibit transcription of antisense ncRNAs overlapping ORFs and 14 loci that display promoter-associated sense ncRNAs. We propose that the former group typically contains antisense lncRNAs that bind sense mRNAs and thereby exert a biological function themselves, while the latter tend to influence promoter activity *via* their transcription alone. However, it remains to be determined how many of these protein-coding loci indeed are associated with non-coding transcripts that have physiologically relevant roles in regulating genes critical for cell wall formation, remodelling and maintenance. The potential importance of antisense lncRNA transcription was highlighted by Huber et al. who measured protein levels in strains in which transcription of 162 antisense SUTs was prematurely terminated. The authors found that around 25% of these genes are regulated by antisense lncRNAs transcription under exponential growth conditions, whereby the effects of these lncRNAs are typically to reduce the expression level of weakly expressed genes (Huber et al. 2016). Moreover, Nevers et al. showed that up to 30% of quiescence-specific genes are repressed during exponential growth, *via* transcriptional interference by antisense ncRNAs which are normally targeted by the nonsense-mediated decay (NMD) pathway (Nevers et al. 2018). The fact that only few of the target genes were identified in both studies (Nevers et al. 2018) demonstrates the importance of growth conditions and the genetic background when studying the molecular consequences of ncRNA transcription. We note that this is especially important since cell wall-related transcriptome dynamics are not characterized comprehensively as yet.

Taken together, available evidence presented in this review is consistent with the idea that a sub-class of cell wall-related genes may at least in part be controlled by overlapping antisense lncRNAs transcription and sense lncRNAs transcription that overlap 5′ regulatory regions. We therefore propose that the question merits further experimental analyses both at the genome-wide level and at specific loci, to obtain a more complete picture of the interplay between cell wall-related genes and their associated lncRNAs. These questions are pertinent for the development of future antifungal therapies that target the cell wall and for approaches in the fields of biotechnology and synthetic biology that aim at engineering yeast cells with specific growth properties.

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Paper 4

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RESEARCH ARTICLE

Antisense non-coding transcription represses the *PHO5* model gene at the level of promoter chromatin structure

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Abstract

Pervasive transcription of eukaryotic genomes generates non-coding transcripts with regulatory potential. We examined the effects of non-coding antisense transcription on the regulation of expression of the yeast *PHO5* gene, a paradigmatic case for gene regulation through promoter chromatin remodeling. A negative role for antisense transcription at the *PHO5* gene locus was demonstrated by leveraging the level of overlapping antisense transcription through specific mutant backgrounds, expression from a strong promoter *in cis*, and use of the CRISPRi system. Furthermore, we showed that enhanced elongation of *PHO5* antisense leads to a more repressive chromatin conformation at the *PHO5* gene promoter, which is more slowly remodeled upon gene induction. The negative effect of antisense transcription on *PHO5* gene transcription is mitigated upon inactivation of the histone deacetylase Rpd3, showing that *PHO5* antisense RNA acts *via* histone deacetylation. This regulatory pathway leads to Rpd3-dependent decreased recruitment of the RSC chromatin remodeling complex to the *PHO5* gene promoter upon induction of antisense transcription. Overall, the data in this work reveal an additional level in the complex regulatory mechanism of *PHO5* gene expression by showing antisense transcription-mediated repression at the level of promoter chromatin structure remodeling.

Author summary

Non-coding transcripts synthesized by RNA Poll are short-lived because they are rapidly terminated by the NNS complex and degraded by the RNA exosome. However, some non-coding transcripts can regulate the expression of coding genes at whose loci they are initiated. In this work, we show that a non-coding antisense transcript regulates the expression of the yeast *S. cerevisiae PHO5* gene that encodes a periplasmic acid phosphatase. Furthermore, we show that the repressive effect of antisense transcription at the *PHO5* gene locus is mediated by chromatin-modifying complexes, such as the histone deacetylase Rrp3 and the chromatin-remodeling complex RSC. Consequently, elongation

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of the antisense transcript through the *PHO5* promoter region results in a chromatin conformation that is more difficult to remodel upon gene induction and therefore in slower kinetics of gene expression. Overall, this work paints a more complete picture of the regulatory process involved in repression of the yeast *PHO5* promoter, a well-studied model for gene regulation through chromatin structure remodeling.

Introduction

The canonical view of eukaryotic transcription has evolved from being considered a highly regulated process initiated from specialized genomic regions, such as gene promoters, to a process that permeates the entire genome [1]. In addition to gene promoters, transcription often initiates from intergenic and intragenic regions, as well as regulatory regions such as gene enhancers. Most of the transcripts originating from these regions are non-coding RNAs that are usually rapidly degraded after synthesis, suggesting that the act of transcription has more potential to exert important biological functions compared to the transcripts themselves [2].

In eukaryotic cells, promoter activation occurs in the context of a repressive chromatin structure, *i.e*. the packing of DNA with histone proteins into nucleosomal arrays [3]. Since chromatinized DNA is not accessible for interaction with the transcriptional machinery, activators work in concert with chromatin-modifying and -remodelling factors to expose regulatory sites and allow promoter activation. Chromatin modifiers catalyze covalent modifications of histones, such as acetylation, methylation, and phosphorylation, whereas chromatin remodelers use the energy of ATP hydrolysis to slide histones along the DNA or evict them from the DNA [4,5]. Dynamic changes in chromatin conformation also require histone chaperones, which act as histone acceptors and bind them when they are not incorporated into nucleosomes [6]. In gene-dense genomes such as that of yeast, transcription often initiates at the 3' end of genes, leading to the production of antisense (AS) non-coding transcripts [2]. AS read-through transcription invades the promoter region of the corresponding gene, where it can exert regulatory effects that are usually repressive to transcription of the coding gene [7–10]. Genome-wide and single gene studies have shown that promoters invaded by AS transcription read-through have high nucleosome occupancy and narrow nucleosome-depleted regions (NDRs) [11,12]. Our recent genome-wide study showed that induced elongation of non-coding antisense transcription into coding gene promoters results in increased deacetylation of promoter nucleosomes by Rpd3. Histone deacetylation leads to decreased recruitment of the major chromatin remodeler RSC and consequently to NDR closure, which represses transcription [13]. However, there are still few examples of *bona fide* effects of specific AS RNAs on transcriptional regulation of their respective genes, such as the yeast *PHO84* gene.

Studies with the *PHO84* gene have been highly instructive in elucidating the mechanisms of transcriptional regulation through AS non-coding RNAs [14–16]. These studies converged on a model in which *PHO84* AS transcription is rapidly terminated in wild-type cells by the NNS (Nrd1-Nab3-Sen1) complex and degraded by the activity of the Rrp6-containing nuclear RNA exosome. Inactivation of any of these crucial factors, such as in *rrp6Δ* mutant cells, leads to transcriptional read-through of *PHO84* AS transcripts, allowing recruitment of histone deacetylases (HDACs) Hda1 or Rpd3 to the *PHO84* promoter. Histone deacetylation is thought to lock the chromatin structure of the promoter in a repressed conformation, thereby negatively regulating transcription of the sense transcript, *i.e. PHO84* mRNA. This mechanism was subsequently explored genome-wide in yeast, which revealed a group of genes that accumulate AS RNAs in the absence of Rrp6 and are silenced in an HDAC-dependent manner [15]. Genes of

this class are characterized by AS transcripts that span the entire gene length, extend beyond the TSS and are enriched for so-called 'closed' promoters. These promoters are typical of inducible or stress-activated genes, and are characterized by precisely positioned nucleosomes whose remodeling is a prerequisite for transcriptional activation [17,18]. A paradigmatic closed promoter that also belongs to this gene class is that of the *PHO5* gene, which is a member of the same (PHO) regulon as *PHO84* [19].

The *PHO5* gene encodes the secreted non-specific acid phosphatase which is located in the periplasmic space and has a role in phosphate metabolism. Accordingly, expression of the *PHO5* gene is regulated in response to intracellular phosphate concentration through the PHO signalling pathway, so that it is repressed when the intracellular concentration is abundant and induced under phosphate starvation conditions [19]. This regulation is primarily achieved through phosphorylation of the specific activator Pho4. Under a high phosphate concentration Pho4 undergoes phosphorylation by the cyclin-dependent-kinase Pho80-Pho85, preventing its accumulation in the nucleus and transcriptional activation of the *PHO5* gene. In low phosphate, Pho4 is imported into the nucleus and activates transcription. From the early days of chromatin research in the 1980s until now, the *PHO5* gene promoter has been and continues to be instrumental in the discovery of numerous fundamental principles and mechanisms of chromatin structure remodeling (reviewed in [19]). In the repressed state, the *PHO5* promoter features five precisely positioned nucleosomes, which upon induction are remodelled into a broad nucleosome-depleted region of ∼600 bp [20]. This massive chromatin transition requires the concerted action of a large network of chromatin-modifying and -remodeling complexes as well as histone chaperones. The repressive chromatin conformation is maintained by H3K4 methylation catalyzed by Set1, a mark that recruits the histone deacetylase Rpd3 to the *PHO5* promoter [21,22]. Another histone deacetylase, Hda1, plays a minor role in this process [23]. When the intracellular phosphate concentration is limited, signal transduction *via* the PHO signaling pathway leads to the accumulation of the unphosphorylated transcriptional activator Pho4 in the nucleus [19,24]. The first step in transcriptional activation of the *PHO5* gene is the binding of Pho4 to the UASp1 (Upstream activating sequence phosphate 1) site, which is located in the short nucleosome-depleted region between nucleosomes -2 and -3 of the *PHO5* gene promoter. Pho4 recruits histone acetyltransferases, such as the Gcn5-containing SAGA complex, which establish a hyperacetylated promoter configuration [25,26]. Acetylated histones are read by chromatin-remodeling complexes containing bromodomains [27,28]. Alternatively, these remodelers can be recruited to the *PHO5* promoter by direct interaction with Pho4 [29]. Five remodelers (SWI/SNF, RSC, INO80, Isw1, Chd1) from all four yeast remodeler families cooperate to catalyze the chromatin opening at the *PHO5* promoter [30,31], with the most abundant remodeler, RSC, providing the crucial share of the remodeling activity required for this transition [31]. Histone eviction allows Pho4 to bind to the UASp2 site otherwise covered by nucleosome -2, which is ultimatively required for full transcriptional activation [32–34].

Another level of *PHO5* promoter regulatory complexity was revealed upon mapping of the *PHO5* AS transcript, CUT025 [35,36]. This transcript initiates from the 3' region of the *PHO5* ORF and extends through its promoter region, spanning ∼2.4 kb in size. It is produced only in cells growing under repressive (phosphate-rich) conditions and is more abundant in *rrp6Δ* mutants compared to wild-type cells, indicating its degradation by the nuclear RNA exosome [35]. AS transcription is generally associated with a repressive effect on transcription of the corresponding genes, and the *PHO5* gene is among the rare examples for which AS transcription is proposed to have a positive effect [35]. In this work, we examined the effect of non-coding AS transcription on *PHO5* gene expression by enhancing or impairing elongation of the *PHO5* AS transcript. In both cases, our results argue in favour of antisense transcription

having a negative effect on *PHO5* gene expression. Moreover, we provide evidence that this negative effect occurs through a chromatin-remodeling based mechanism mediated by AS transcription which decreases the accessibility of the chromatin structure at the *PHO5* gene promoter.

Materials and methods

Yeast strains and primer sequences used in this study are listed in S1 and S2 Tables, respectively.

Strains, media, plasmids and strain construction

Yeast *Saccharomyces cerevisiae* strains used in this study are listed in S1 Table. All strains were grown at 30˚C. For repressive conditions (high phosphate, $+P_i$), yeast strains were grown in YNB medium supplemented with 1 g/l KH_2PO_4 (YNBP) with or without lack of amino acids for plasmid selection. For gene induction by phosphate starvation ($-P_i$), cells were washed in water and resuspended in the phosphate-free synthetic medium with or without lack of amino acids for plasmid selection [30,31,37]. Anchor-away of Nrd1-AA and Sth1-AA was induced by adding 1 μg/ml of rapamycin (Sigma) to the medium. The *RRP6* gene was deleted using a disruption cassette generated by PCR with the primer pairs RRP6-Kan1 and RRP6-Kan2 or RRP6hph_fwd and RRP6hph_rev and the BMA41 *rrp6*::*KanMX4* genomic DNA or the *hph*-carrying pYM16 plasmid from [38] as template, respectively. The *GCN5* gene was deleted using a disruption cassette generated by PCR with the primer pair gcn5HIS_fwd and gcn5HIS_rev and the *SpHIS5*-carrying pKT101 plasmid from [39] as template. Transformants were selected on G-418 (0.2 mg/ml, Sigma), Hygromycin B (0.3 mg/ml, Sigma) or -His plates, depending on the marker, and gene deletion was confirmed by PCR. The BMA41 *TEF1-PHO5 AS* strain was constructed by transformation with a cassette generated by PCR with primers TEF1PHO5AS_fwd and TEF1PHO5AS_rev and the pYM-N18 plasmid from [38] as template. Transformants were selected on G-418 plates, and correct insertion of the cassette was confirmed by PCR. The pP5Z reporter plasmid is centromeric vector that carries a *PHO5* promoter-*lacZ* gene fusion and is described in [40]. The pCEN-RRP6 plasmid was previously constructed by Gateway cloning from the pAG416GPD backbone [41]. Plasmid pTDH3-dCas9 (pFS3891) [42] was obtained from Addgene (Plasmid #46920). Plasmid pFS3892, which contains the guide RNA scaffold, was generated by one-step isothermal Gibson assembly reaction (New England BioLabs) using two fragments, one obtained by PCR on pRPR1_gRNA_handle_RPR1t (Addgene Plasmid #49014) using OFS_2869 and OFS_2870 oligonucleotides, the other by PCR on YCpLac33 using OFS_2871 and OFS_2872 oligonucleotides. Plasmid *PHO5 AS gDNA-URA3* was then obtained by Gibson assembly reaction (NEB) using OFS_2886 and OFS_2887 to amplify pFS3892 backbone and OFS_2888 and OFS_3095 for gDNA cloning. To test the putative *in trans* activity of the *PHO5* AS RNA, strains were designed as following. First, the *PHO5* ORF was replaced by the *URA3* marker in either a *MATA* FSY6857 or a *MATα* FSY5439 strain (see S1 Table). This was performed by amplification of the *URA3* marker from the pUG72 plasmid with OFS5084 and OFS5085 primers and the resulting amplicon was transformed in FSY6857 and FSY5439 strains following selection in a -Ura medium. The *MATA* and *MATα* strains deleted for *PHO5* were named FSY9286 and FSY9287. We then amplified the wild-type *PHO5* gene with either the OFS5086 and OFS5087 primer pair or OFS5088 and OFS5089 primer pair in order to insert a terminator for the *PHO5* mRNA (sense) or the AS transcript, respectively. The PCR products targeting either the sense or the antisense transcription were transformed in the FSY9286 and FSY9287 and counter-selected on a 5-FOA medium. The strains targeting the *PHO5* mRNA or AS RNA

were named FSY9288 and FSY9291. Finally, the 3 different diploids (AS in *cis*, AS blocked and AS in *trans*) were generated after crossing FSY6857 with FSY9287, FSY9286 with FSY9291 and FSY9288 with FSY9291, respectively, and selection on -His-Trp medium.

Enzyme activity assays, RNA isolation, Nothern blot and RT-qPCR

Acid phosphatase and beta-galactosidase activity assays were done with intact yeast cells, exactly as described in [31]. Total RNA was extracted by the hot phenol method [43], treated with RNAse-free DNAse I (New England Biolabs) and purified by phenol/chloroform extraction. Strand-specific reverse transcription was performed using 1 μg of RNA and strand-specific oligonucleotides (0.1 μM each) with the ProtoScript First Strand cDNA Synthesis Kit (New England Biolabs) supplemented with actinomycin D (Sigma) to final concentration 5 μg/ml to ensure strand specificity. cDNAs were amplified in Roche LightCycler 480 with the Maxima SYBR Green qPCR Master Mix detection kit (Thermo Scientific). The qPCR datasets were analysed using the ΔΔCt method, and the results were normalized to either *PMA1*, *ACT1* or *SCR1* RNAs amplification, which were used as internal controls. To test the putative *in trans* activity of the *PHO5* AS RNA, OFS2522 and OFS2523 were used to measure *PHO5* mRNA and AS RNA levels. Amplifications were done in duplicate for each sample, and three independent RNA extractions were analysed. For the Northern blot, total RNA (10 μg for each sample) was run on a 1% denaturing formaldehyde agarose gel and transferred to nylon membranes (Amersham Hybondtm-N+). Membranes were crosslinked and incubated overnight at 60˚C with 100μg/ml boiled salmon sperm DNA in 50% formamide, 5x standard saline citrate (SSC), 20% dextran sulfate sodium, 1% sodium dodecyl sulfate (SDS). Subsequently, membrane wered hybridized with 32P-labeled SP6/T7 riboprobes in 50% formamide, 7% SDS, 0.2 M NaCl, 80 mM sodium phosphate (pH 7.4), and 100 μg/ml boiled salmon sperm DNA for 6h. All blots were washed with 2X SSC and 0.1% SDS for 5 minutes at60˚C and then with 0.5X SSC and 0.1% SDS for 45 minutes at 60˚C. Riboprobes were obtained by SP6/T7 in vitro transcription of gene-specific PCR fragments containing an SP6/T7 promoter. Quantifications were performed with a Phosphor Imager machine.

Chromatin analysis

For anti-histone H3 chromatin immunoprecipitation (ChIP), forty millilitres of cells were fixed with 1% formaldehyde for 20 min. After quenching with 400 mM glycine to stop the reaction, the cells were washed and lysed with glass beads to isolate chromatin. Sonication of cell lysates was performed with a Vibra-Cell sonicator in 1.2 mL of FA150 buffer (50 mM Hepes pH 7.5, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.1% Sodium deoxycholate and 0.1% SDS) to reduce average fragment size to approximately 500 base pairs. The samples were centrifuged at 2500 g and the supernatant recovered. Chromatin fractions of 400 μl were taken for each immunoprecipitation reaction and incubated with 4 μl of anti-histone H3 antibodies (ab1791, Abcam) at 4˚C overnight. After incubation, 40 μl of protein G PLUS-agarose beads (sc-2002, Santa Cruz Biotechnology) were added and incubated at 4˚C for 2 h. The beads were washed extensively by successive washing steps: 3 times with FA150 lysis buffer, 3 times with FA500 lysis buffer (similar to FA150 but with 500 mM NaCl), 1 time with washing buffer 1 (10 mM Tris-HCl pH 8, 250 mM LiCl, 1 mM EDTA, 0.5% NP-40, 0.5% Sodium deoxycholate) and 1 time with washing buffer 2 (10 mM Tris-HCl pH 8, 1 mM EDTA, 1% SDS). Chromatin was eluted at 80˚C in elution buffer (50 mM Tris-HCl pH 8, 10 mM EDTA, 1% SDS) during 20 minutes. Samples (regardless of Input or IP) were reverse cross-linked at 65˚C overnight. Eluted supernatants (output) and the input controls were hydrolysed with Pronase (0.8 mg/ml final concentration, Sigma) at 42˚C for 2 h, followed by incubation at 65˚C for 7 h to reverse

cross-linked DNA complexes. DNA was extracted using the Macherey Nagel Nucleospin Gel & PCR Cleanup Kit. The immunoprecipitated DNAs (output) were quantified by qPCR in Roche LightCycler 480 with the Maxima SYBR Green qPCR Master Mix detection kit (Thermo Scientific). Amplifications were done in triplicate for each sample. Immunoprecipitated samples (output) were normalized to input and to a *PHO5*-adjacent control region which does not show chromatin signatures similar to the *PHO5* gene promoter, as described in [33]. Chromatin analysis of yeast nuclei by restriction nuclease accessibility assay was done as described previously [31,37,44]. 120 U of the ClaI restriction enzyme (New England Biolabs) was used for chromatin analysis of nuclei and 40 U of HaeIII (New England Biolabs) was used for secondary cleavage. Probe for hybridization was as described previously [31,37,45]. Quantification of the percentage of cleaved DNA was done by PhosphorImager analysis (Fuji FLA3000). ChIP of dCas9 was essentially perfomed as in [13] without addition a *S. pombe* spike-in. An anti-Cas9 antibody (Diagenode #C15310258) was used for the immunoprecipitation step.

Downloaded data sets

For RNA-seq and RNAPII PAR-CLIP, data were retrieved from [46] (GEO: GSE175991) and from [47] (GEO: GSE56435). Data of MNase-seq, ATAC-seq and Sth1 ChEC-seq were reanalyzed from [13] (GEO: GSE130946).

Results

AS transcription is involved in regulation of *PHO5* gene expression

The product of antisense transcription at the *PHO5* model gene locus, CUT025 (hereafter referred to as *PHO5* AS), is initiated at the 3' end of the gene ORF in the antisense direction and extends through the *PHO5* promoter region (Fig 1A). The 3'-5' exoribonuclease Rrp6, which is the catalytic subunit of the nuclear RNA exosome complex, degrades this transcript in wild-type (wt) cells, consistent with the increased level of this transcript in *rrp6Δ* mutant cells (S1A Fig). We confirmed the increased level of the *PHO5 AS* transcript at the *PHO5* promoter region in *rrp6Δ* compared to wt cells by strand-specific reverse-transcription quantitative PCR (RT-qPCR) upon shifting the cells from repressive (phosphate-rich, $+P_i$; YNB with additional 1 g/l KH_2PO_4) to inducing (no phosphate, $-P_i$) conditions. *PHO5* AS accumulation in *rrp6Δ* was most pronounced under repressive conditions (Fig 1A, 0 h of induction), consistent with [35]. After shifting to inducing conditions, the level of *PHO5* AS gradually decreased in both wild-type and *rrp6Δ* cells, however the increased level in *rrp6Δ* cells was still present at an early time point of gene induction (Fig 1A). The *PHO5* AS transcript has a much lower steady-state level than the corresponding *PHO5* mRNA transcript, as observed by RNA-seq, which measures steady-state RNA levels, *i.e.*, takes into account both the level of nascent transcription and RNA degradation. However, the RNAPII photoactivatable ribonucleoside-enhanced crosslinking and immunoprecipitation (PAR-CLIP) signal, which measures only nascent transcription, is comparable or even higher for the AS transcript than for the mRNA transcript under same growth conditions (4 mM P_i), showing that the AS transcript is being produced to a potentially significant level (Fig 1B).

Whole-genome tiling array datasets revealed production of another non-coding transcript at the *PHO5* gene locus, SUT446, transcribed in the sense direction through the *PHO5* promoter region, which appears not to be accumulated in *rrp6Δ* mutant cells and is weakly expressed ([15,36]; S1A Fig). It was determined by RT-qPCR that the level of SUT446 was not significantly increased in *rrp6Δ* compared to wild-type cells neither in repressive nor inducing conditions (S1B Fig), arguing against its gene-regulatory function. Overall, these data support

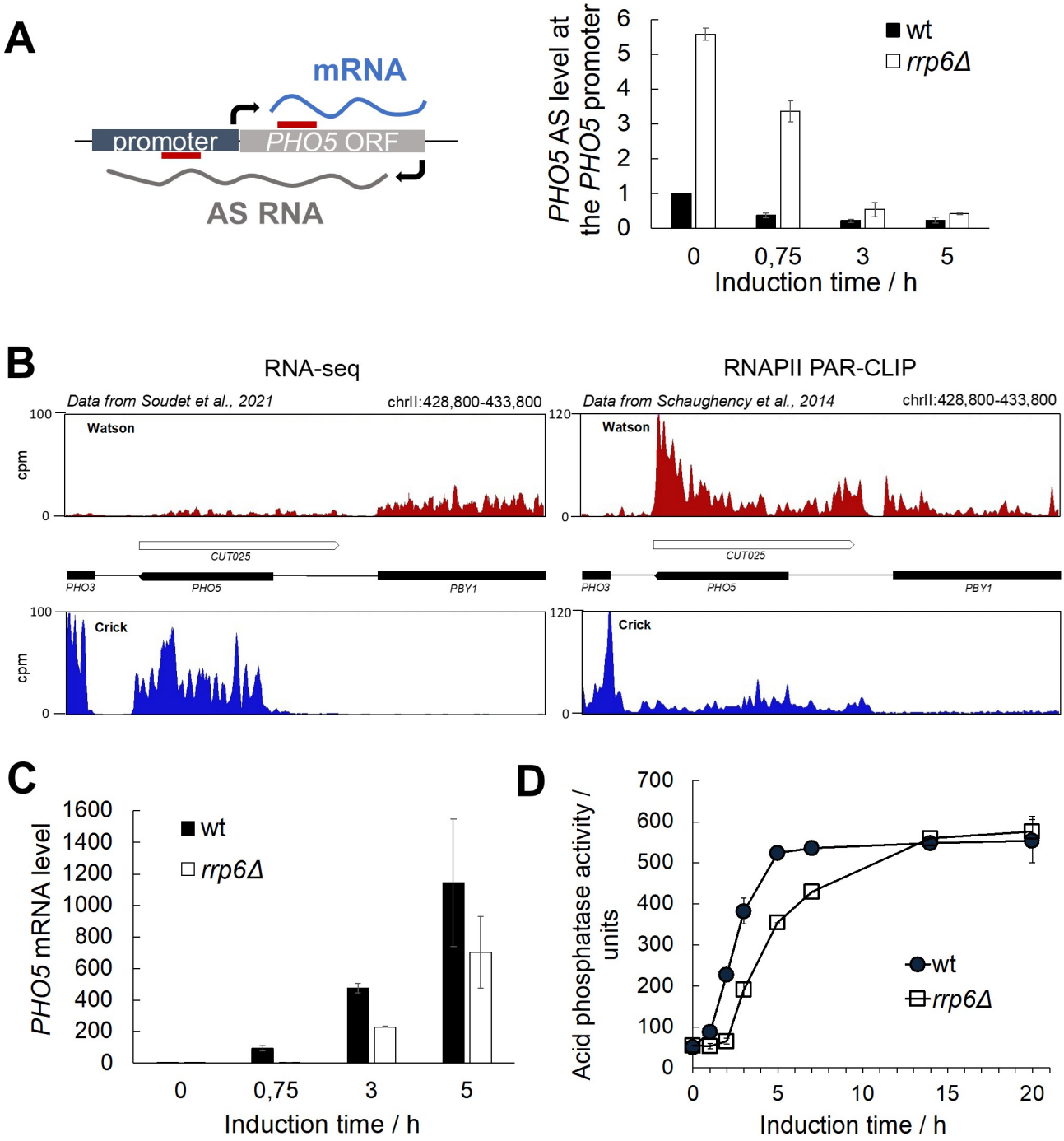

Fig 1. Kinetics of *PHO5* gene expression are inversely correlated with level of the corresponding antisense transcript. **(A)** Scheme showing transcription of an antisense (AS) RNA at the *PHO5* gene locus with thick straight red lines denoting the the regions used for qPCR (left) and AS RNA levels at the *PHO5* promoter region in wild-type BMA41 (wt) and corresponding *rrp6Δ* mutant cells upon induction through phosphate starvation, monitored by strand-specific reverse transcription quantitative PCR (RT-qPCR) (right). RT-qPCR values were normalized to *PMA1* RNA and expressed relative to transcript abundance in wild-type cells under repressive conditions (0 h of induction), which was set to 1. **(B)** The left panel shows RNA-seq signal from an Nrd1-AA strain in the absence of rapamycin (wild-type equivalent) at the *PHO5* locus. The right panel represents RNAPII PAR-CLIP signal or nascent transcription signal in the same conditions. Data were retrieved from [46] and [47], respectively. **(C)** Levels of *PHO5* mRNA in wild-type BMA41 (wt) and corresponding *rrp6Δ* mutant cells upon induction through phosphate starvation. RT-qPCR values were normalized to *PMA1* RNA and expressed relative to transcript abundance in wild-type cells at repressive conditions (0 h of induction), which was set to 1. **(D)** Same as (C), but acid phosphatase induction kinetics were monitored by measuring acid phosphatase activity with whole cells. Reported values represent the means and standard deviations of three independent experiments (n = 3).

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a possible regulatory role of the CUT025 AS non-coding transcript, but not the SUT446 promoter non-coding transcript, in regulation of *PHO5* gene transcription.

We further investigated whether the increased level of the *PHO5* AS transcript under repressive conditions and during early gene induction in *rrp6Δ* cells correlates with a change in *PHO5* mRNA level. *PHO5* mRNA was quantified by RT-qPCR upon gene induction and a strong delay in its expression was observed in *rrp6Δ* cells compared to wild-type cells (Fig 1C). This delay persisted during the first hours of gene induction and corresponded to a delay in expression of the Pho5 acid phosphatase, as determined by measuring its enzymatic activity (Fig 1D). However, after prolonged induction, the level of acid phosphatase in *rrp6Δ* cells reached that of wild-type cells (Fig 1D). The observed delay in gene expression was dependent on the catalytic activity of Rrp6, because the catalytically dead *rrp6Y361A* mutant cells also exhibited delayed *PHO5* gene expression, and acid phosphatase activity was brought to wild-type levels when a functional *RRP6* gene was expressed from a centromeric plasmid in *rrp6Δ* cells (S2A Fig). A similar delay was also measured with *rrp6Δ* cells of two other genetic backgrounds (S2B and S2C Fig), showing that it is not specific to the W303-derived strain used in these experiments.

We also performed a control experiment to test whether the observed kinetic delay in *PHO5* expression in *rrp6Δ* cells is caused by an indirect effect due to compromised signal transduction through the PHO signaling pathway. We made use of a construct in which expression of the *lacZ* reporter gene was driven by the *PHO5* promoter and monitored its expression by measuring beta-galactosidase activity upon induction (no phosphate, $-P_i$) in wild-type and *rrp6Δ* cells (S2D Fig). Expression kinetics of the *PHO5* promoter-*lacZ* construct were similar in wild-type and *rrp6Δ* cells, arguing that PHO signaling is not compromised in *rrp6Δ* cells. This result demonstrates that the kinetic delay in *PHO5* expression observed with the *rrp6Δ* strain (Fig 1C and 1D) was not an indirect effect caused by compromised induction strength and consequently impaired *PHO5* transcriptional activation. Additionally, this result speaks in favour of a possible regulatory role of the AS transcript originating from the *PHO5* ORF.

PHO5 gene expression kinetics are delayed upon induction in mutants related to RNA exosome function

Rrp6 is the nuclear-specific catalytic subunit of the RNA exosome complex. To determine the involvement of other RNA exosome subunits and cofactors in the regulation of *PHO5* gene expression, we examined the kinetics of *PHO5* gene expression using appropriate mutant cells. Deletion mutants for the monomeric cofactors of the nuclear exosome, Rrp47 and Mpp6, also showed delayed acid phosphatase expression kinetics (Fig 2A). The TRAMP complex is another cofactor of the nuclear RNA exosome and consists of a non-canonical poly(A) polymerase (Trf4 or Trf5), an RNA-binding subunit (Air1 or Air2), and the essential helicase Mtr4 [48,49]. Interestingly, single *air1Δ* and *air2Δ* mutant cells showed no delay, whereas the *air1Δ-air2Δ* double mutant showed an even greater delay than the *rrp6Δ* mutant (Fig 2A), consistent with a high degree of redundancy between homologous TRAMP subunits [50]. Somewhat surprisingly, acid phosphatase activity measured after overnight induction was increased in some mutants compared with wild-type cells. It is possible that this may reflect specialized cofactor requirements that support the specific conditions of prolonged gene induction and was not pursued further. The mutant for the exonuclease activity of the essential RNA exosome catalytic subunit Dis3 (*dis3Δ* + *pDis3-exo*$^-$) also showed delayed kinetics compared with the corresponding wild-type cells (*dis3Δ* + *pDis3*) and with the mutant for its endoribonuclease activity (*dis3Δ* + *pDis3-endo*$^-$) (Fig 2B). These results demonstrate the involvement of the second

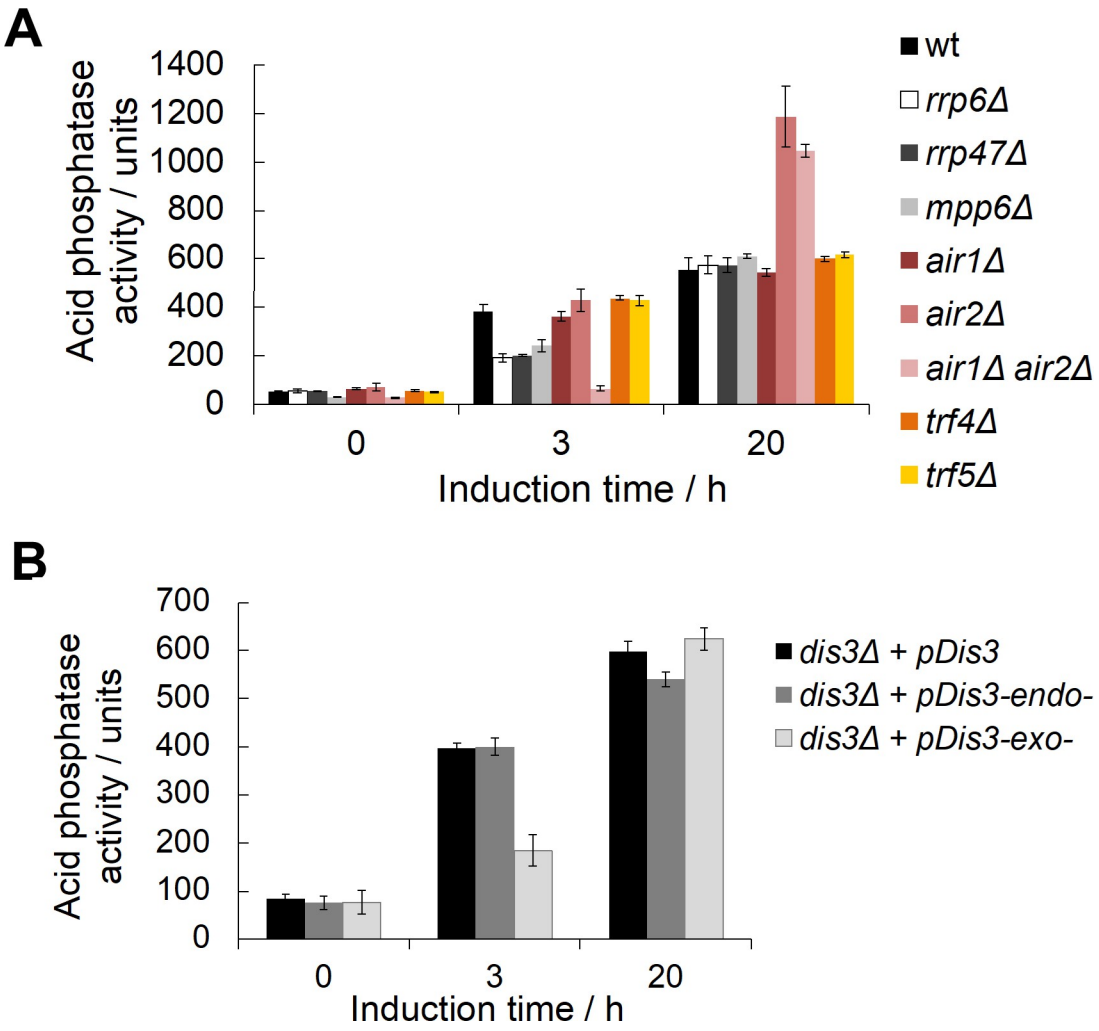

Fig 2. Expression of the *PHO5* gene is negatively affected in RNA exosome mutant cells. (A) Acid phosphatase induction kinetics in wild-type BMA41 (wt) and corresponding deletion mutant cells for Rrp6 and RNA exosome cofactors upon induction through phosphate starvation. Reported values represent the means and standard deviations of three independent experiments (n = 3). **(B)** Same as (A), but for W303-derived strains with genomic copy of *DIS3* gene deleted but bearing a centromeric plasmid that carries the wild-type copy of *DIS3* gene (*dis3Δ* + *pDis3*) or its alleles with abolished endonuclease (*dis3Δ* + *pDis3-endo*$^-$, D171N) or exonuclease (*dis3Δ* + *pDis3-exo*$^-$, D551N) activity.

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catalytic subunit of the RNA exosome, Dis3, as well as the nuclear RNA exosome cofactors Rrp47, Mpp6 and the TRAMP complex in the regulation of *PHO5* gene expression.

In *rrp6Δ* and other RNA exosome deletion mutant backgrounds, AS transcription is constitutively induced due to sequestration of the NNS (Nrd1-Nab3-Sen1) termination complex by stabilised non-coding RNAs. The NNS complex cannot be efficiently recycled to sites of transcription, inducing termination defects at non-coding RNA loci and resulting in their increased elongation frequency [51]. To rule out possible indirect effects on transcription of the *PHO5* gene due to gene deletion mutant backgrounds in which AS transcription is constitutively elongated, we turned to a system in which AS elongation is inducible. To this end, we used the Anchor Away (AA) system to rapidly deplete Nrd1 protein from the nucleus by rapamycin treatment [52]. Since Nrd1 belongs to the NNS surveillance system, its removal is expected to trigger transcriptional read-through of non-coding RNAs [51]. Indeed, treatment

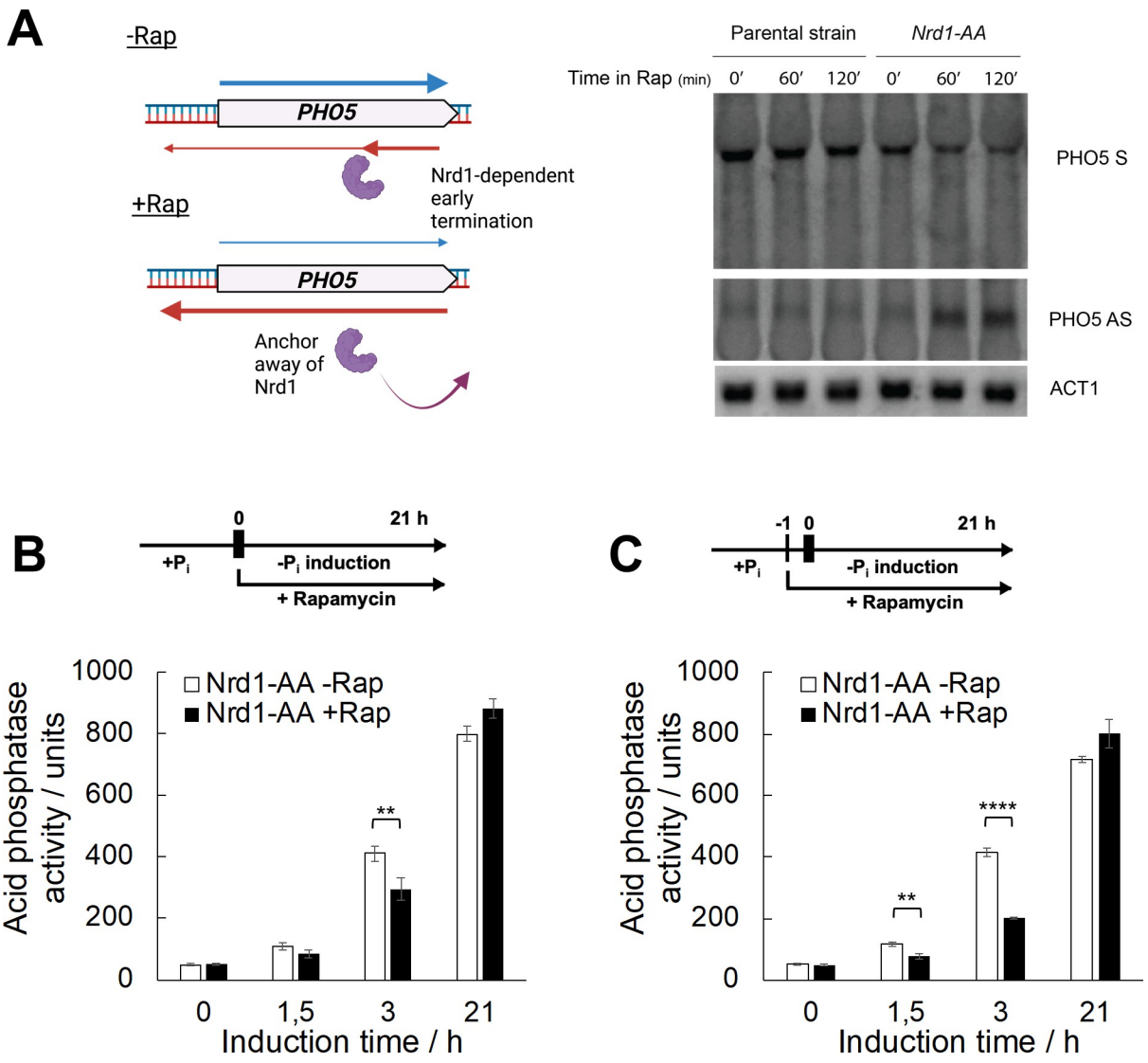

Fig 3. Induction of *PHO5* AS elongation by depletion of Nrd1 from the nucleus delays expression of the *PHO5* gene. (A) Nothern blot analysis of total RNA from the parental Anchor Away (AA) and the corresponding Nrd1-AA strains upon addition of rapamycin to the growth medium. Nothern blots were probed specifically for sense and antisense *PHO5* transcripts, while *ACT1* RNA was used as a loading control. **(B)** Acid phosphatase induction kinetics in Nrd1-AA strain upon induction through phosphate starvation with (+Rap) or without addition of rapamycin (-Rap). Reported values represent the means and standard deviations of three independent experiments (n = 3). Indicated differences show the significant differences using an unpaired Student's t test. Two (**) and four (****) asterisks denote a p-value lower than or equal to 0.01 and 0.0001, respectively. **(C)** Same as (B), but rapamycin was added one hour before induction.

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of Nrd1-AA cells with rapamycin resulted in rapid induction of the *PHO5* AS transcript production, clearly demonstrating that the NNS complex is important for its early termination in wild-type cells. Importantly, even under *PHO5* repressive conditions, induction of *PHO5* AS transcript production through the Nrd1-AA system was accompanied by downregulation of *PHO5* mRNA levels, as shown by Northern blot (Fig 3A). Furthermore, with the Nrd1-AA system, it was possible to induce elongation of AS transcription by adding rapamycin simultaneously when shifting the cells to *PHO5* inducing conditions (*i.e.* phosphate free medium) (Fig 3B) or an hour before the shift (Fig 3C). Consistently, the kinetics of Pho5 expression monitored by measuring acid phosphatase activity showed a kinetic delay which was dependent on

the timing of rapamycin addition during cultivation (Fig 3B and 3C). The results of this experiment demonstrated that the negative correlation between *PHO5* AS and mRNA transcript levels is not an indirect consequence of gene deletion mutant backgrounds, since it is also seen upon induced Nrd1 depletion.

Transcription of *PHO5* AS RNA regulates *PHO5* gene expression *in cis*

To increase the transcription level of the *PHO5* AS transcript without using RNA degradation/termination mutant backgrounds, we inserted the strong constitutive *TEF1* promoter in the antisense configuration downstream of the *PHO5* gene ORF (Fig 4A). We confirmed that this resulted in the *TEF1* promoter driving AS transcription at the *PHO5* gene locus by RT-qPCR, as the level of *PHO5* AS transcript in these mutant cells was ≈20 times higher than in the corresponding wild-type cells. Impressively, even under $+P_i$ conditions in which the *PHO5* mRNA is only basaly expressed, *TEF1*-induced overexpression of the AS transcript caused a severalfold decrease in *PHO5* mRNA level, confirming the negative correlation between *PHO5* AS and mRNA transcript levels. What is more, Pho5 expression in these cells was delayed compared with wild-type cells and did not reach full expression level even after overnight induction (Fig 4B). This result indicates that an artificially induced high constitutive level of AS transcription at the *PHO5* locus drives repression of the *PHO5* gene even after prolonged induction.

Furthermore, we tested whether the *PHO5* AS transcript can regulate *PHO5* gene expression when expressed *in trans*, *i.e*. whether the AS transcript itself has a regulatory function. We constructed diploid strains (as in [10]) in which only one copy of the *PHO5* AS transcript was expressed either *in cis* (from the same chromosome as *PHO5* mRNA), *in trans* (from the opposite chromosome) and another one in which AS transcription in *cis* was blocked (Fig 4C). Insertion of a terminator sequence to block AS transcription *in cis* resulted in only partial downregulation of *PHO5* AS level as shown by RT-qPCR. However, there was a marked increase in *PHO5* mRNA level in this diploid strain compared with the strain with native *PHO5* AS levels expressed *in cis* (Fig 4C). Crucially, when *PHO5* AS was expressed *in trans* in addition to downregulation of its level *in cis*, *PHO5* S expression was higher than for the native locus indicating no repressive effect of the AS. These results argue that the act of AS transcription, rather than the AS RNA transcript itself, represses transcription of the *PHO5* gene.

Block of AS transcription through dCas9 enhances the kinetics of *PHO5* gene expression

Given that accumulation of the *PHO5* AS transcript negatively affects *PHO5* gene transcription kinetics, blocking AS transcript production should enhance it. To specifically target *PHO5* AS transcription, we undertook a CRISPRi approach in which a catalytically dead Cas9 protein (dCas9) is directed by a guide RNA (gRNA) to interfere with AS transcription at the *PHO5* gene locus. The CRISPRi system blocks transcription due to physical collision between the elongating RNA Polymerase and the dCas9:gRNA complex [53]. Furthermore, this system was shown to function in a strand-specific manner, by blocking transcription only when the nontemplate DNA strand of a transcription unit is targeted [53,54]. Therefore, we targeted dCas9 to the nontemplate strand of the AS transcription unit at the *PHO5* gene locus to block only AS transcription. First, we confirmed the presence of the dCas9 protein at the *PHO5* ORF by anti-Cas9 chromatin immunoprecipitation (ChIP). Notably, a strong peak of dCas9 binding at the *PHO5* gene ORF compared to a control strain not expressing the gRNA was observed (Fig 5A), while no dCas9 binding could be detected at the *PHO5* promoter region covered by nucleosomes -4 and -1 (Fig 5A). RNA levels in the Nrd1-AA strain with the active CRISPRi

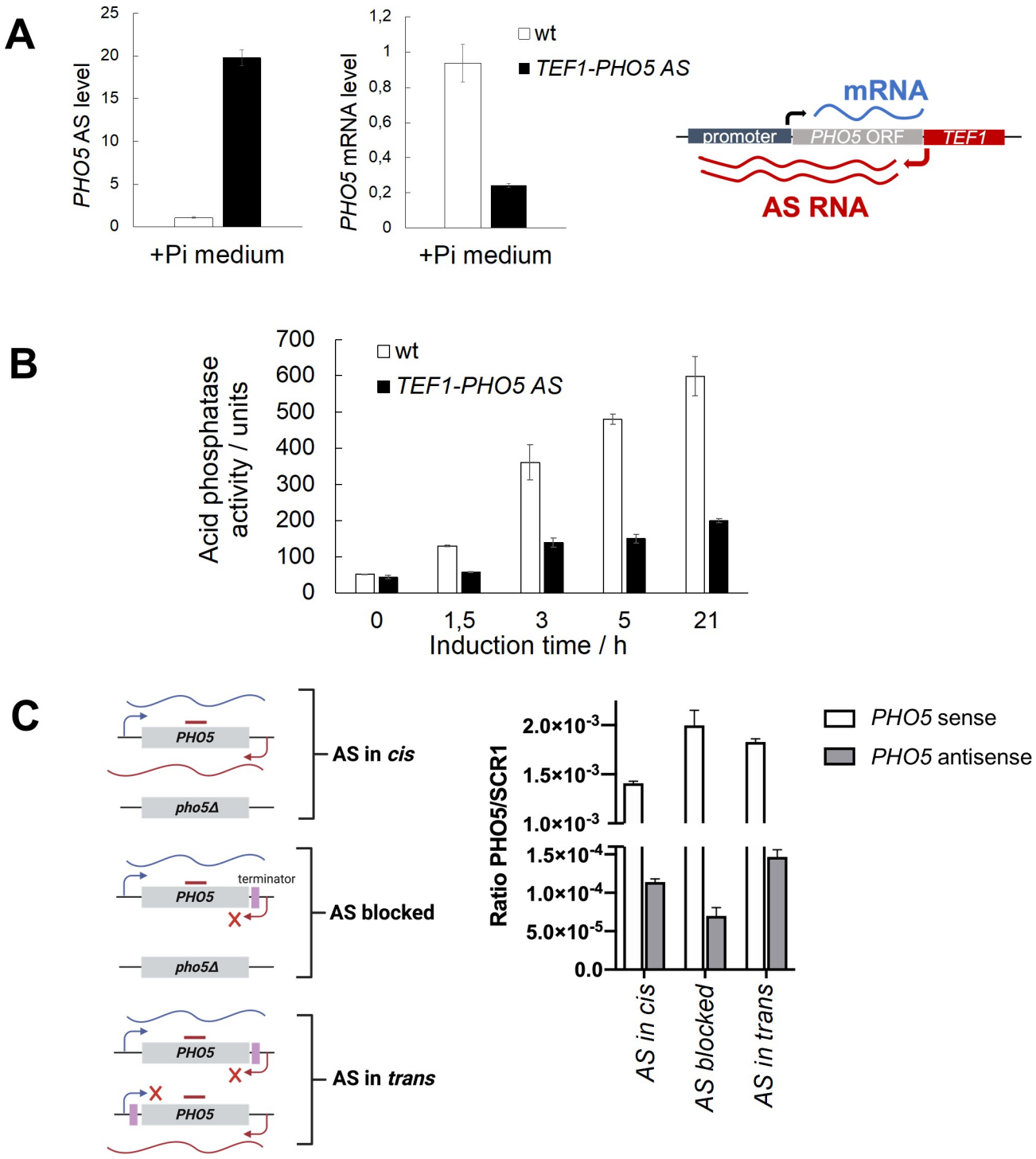

Fig 4. AS transcription represses the *PHO5* gene *in cis*. **(A)** Levels of *PHO5* AS and mRNA transcripts in the BMA41 wild-type and the corresponding *TEF1-PHO5 AS* strain at +P_i conditions, monitored by RT-qPCR. Values were normalized to *ACT1* RNA. Right: Scheme of the *PHO5* gene locus in the *TEF1-PHO5 AS* strain. **(B)** Acid phosphatase induction kinetics upon induction through phosphate starvation in wild-type BMA41 (wt) and corresponding *TEF1-PHO5 AS* mutant cells. Reported values represent the means and standard deviations of three independent experiments (n = 3). **(C)** Left: Scheme showing the *PHO5* gene locus in diploid strains in which *PHO5* AS is transcribed *in cis*, *in trans* or its transcription is blocked. The position of terminator sequences is denoted by purple boxes and a thick straight red line denotes the region used for qPCR. Right: Levels of *PHO5* AS and S transcripts monitored by RT-qPCR in these strains. Values were normalized to *SCR1* RNA. Reported values represent the means and standard deviations of two independent experiments (n = 2).

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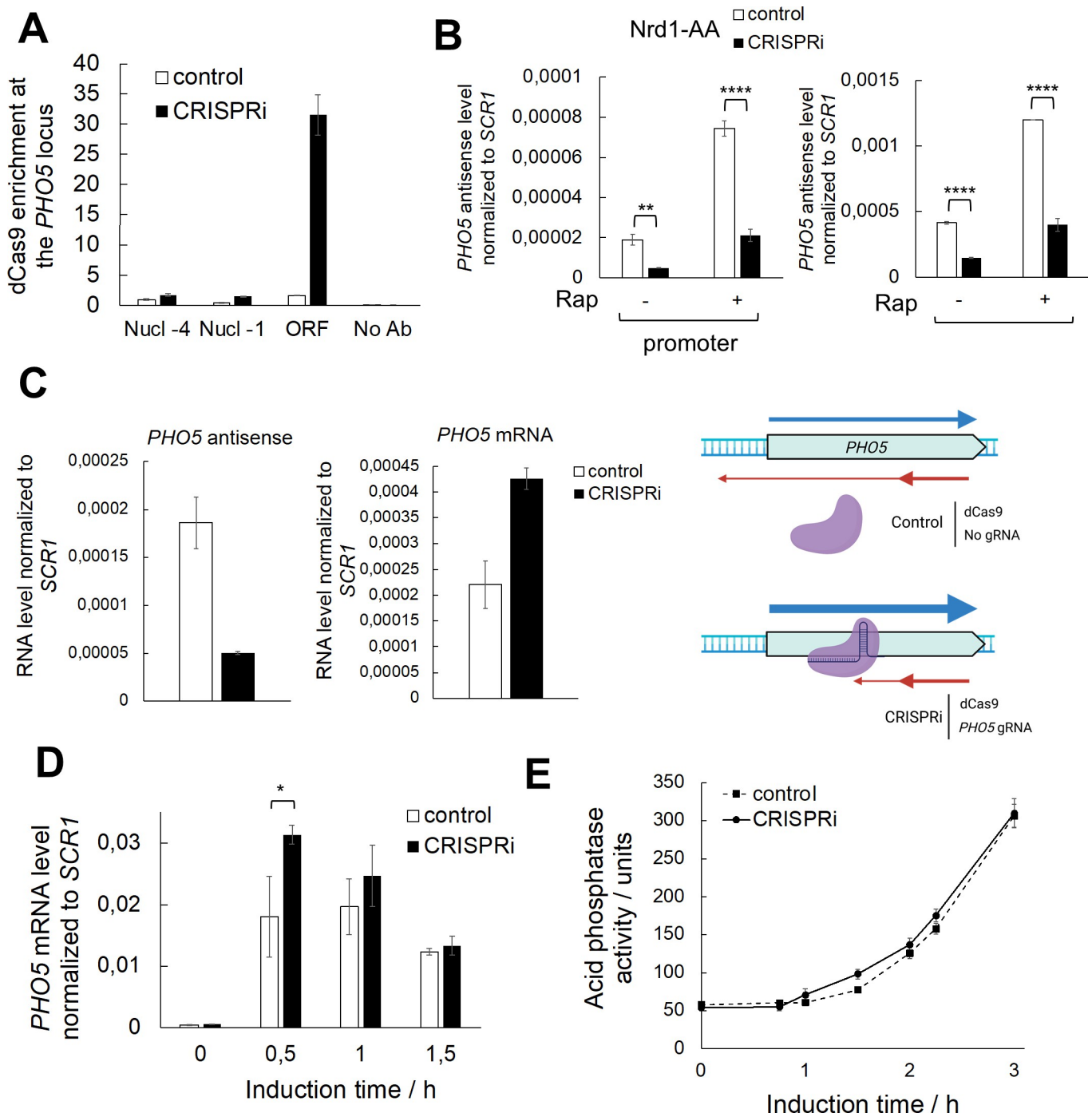

Fig 5. Targeting dCas9 to specifically block *PHO5* AS transcription enhances expression kinetics of the *PHO5* gene. (A) Chromatin immunoprecipitation (ChIP) analysis of dCas9 binding at the *PHO5* gene locus. Immunoprecipitated DNA was quantified by qPCR with primers specific for different regions of the *PHO5* promoter (Nucleosomes -4 and -1) and ORF regions. Both strains were transformed with a dCas9 expressing plasmid, while the CRISPRi strain was additionaly transformed with a plasmid expressing a gRNA targeted to strand-specifically block *PHO5* AS transcription and the control strain with the corresponding empty plasmid. Nucl—nucleosome, No Ab—no antibody ChIP control. **(B)** Levels of *PHO5* AS transcribed at the *PHO5* promoter and ORF regions at 0 h of induction in Nrd1-AA strain with or without addition of rapamycin (for 1 hour; to deplete Nrd1 and induce AS transcription) and an active CRISPRi system. RT-qPCR values were normalized to *SCR1* RNA. Reported values represent the means and standard deviations of three independent experiments (n = 3). Indicated differences show the significant differences using an unpaired Student's t test. Two (**) and four (****) asterisks denote a p-value lower than or equal to 0.01 and 0.0001, respectively. **(C)** Levels of *PHO5* AS and mRNA transcripts in the CRISPRi and the corresponding control strain, monitored by RT-qPCR at 0 h of induction as in (B). Strains are Nrd1-AA with the absence of rapamycin (wild-type equivalent). Right: Scheme of the CRISPRi strategy used to block *PHO5* AS transcription. Strains were transformed with two plasmids, one expressing dCas9, and the other expressing or not a gRNA

targeting the non-template strand of the *PHO5* AS transcription unit. **(D)** Levels of *PHO5* mRNA in the CRISPRi and the corresponding control strain upon induction through phosphate starvation monitored by RT-qPCR as in (B). Strains are same as in (C). Reported values represent the means and standard deviations of three independent experiments (n = 3). Indicated differences show the significant differences using an unpaired Student's t test. One (*) and two (**) asterisks denote a p-value lower than or equal to 0.05 and 0.01, respectively. **(E)** Same as (D), but acid phosphatase induction kinetics were monitored by measuring acid phosphatase activity with whole cells.

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system were monitored by RT-qPCR and showed a highly reproducible decrease in *PHO5* AS levels compared to the control strain (Fig 5B). This decrease was significant at the *PHO5* promoter and ORF regions without rapamycin addition or with rapamycin (*i.e.*, depletion of Nrd1 which induces AS transcription). These results are consistent with a dCas9-mediated transcriptional roadblock of AS transcription at the *PHO5* gene locus. After the addition of rapamycin, *PHO5* AS levels were increased in both the CRISPRi Nrd1-AA strain and the control Nrd1-AA strain. However, its levels in the CRISPRi strain remained significantly lower, maintaining the difference in levels already observed without the addition of rapamycin (Fig 5B). These results demonstrated that the dCas9-mediated roadblock of AS transcription at the *PHO5* gene locus is robust and maintained after global induction of AS transcription, although AS transcription was not completely abolished.

Importantly, impairment of *PHO5* AS RNA elongation led to an increase in *PHO5* mRNA levels (Fig 5C), clearly demonstrating the direct role of AS transcription in *PHO5* gene repression. Also, it argues in favour that the CRISPRi system strand-specifically blocked only AS transcription without significantly impacting mRNA transcription. We further tested if impairment of AS transcription with use of the CRISPRi system would result in enhanced kinetics of *PHO5* gene expression. As expected, the kinetics of *PHO5* gene expression upon gene induction were slightly faster when AS transcript production was impaired by dCas9 than in the control strain (Fig 5D and 5E). This effect was noticed only at very early timepoints of gene induction (30 min for mRNA levels and 1,5 h for acid phosphatase levels), possibly due to the dCas9 protein losing its roadblock function past a certain level of ongoing transcription.

AS RNA elongation affects *PHO5* promoter chromatin structure

Since transcriptional activation of the *PHO5* promoter requires a large transition of its chromatin structure, we investigated whether the kinetics of *PHO5* promoter chromatin opening upon gene induction also inversely correlate with *PHO5* AS transcription. To this end, we examined the chromatin structure at the *PHO5* promoter with anti-histone H3 ChIP at nucleosome -2, which covers the high-affinity Pho4 binding site and is considered the critical nucleosome for *PHO5* chromatin remodeling [19]. A higher histone occupancy was observed in *rrp6Δ* compared to wild-type cells already under repressive conditions (Fig 6A). Accordingly, histone removal from the *PHO5* promoter was slower in *rrp6Δ* than in wild-type cells during the first hours of gene induction and reached a similar final level after 5 hours (Fig 6A). To confirm the delayed kinetics of chromatin opening in *rrp6Δ* cells, we took advantage of the ClaI restriction enzyme accessibility assay, which quantifies the efficiency of cleavage by ClaI enzyme at nucleosome -2 of the *PHO5* promoter (Fig 6B). Consistent with the anti-histone H3 ChIP, the accessibility of the ClaI site at the *PHO5* promoter was lower in *rrp6Δ* and *air1Δ-air2Δ* than in wild-type cells during the first hours of gene induction (Fig 6C). These results show that AS transcription mediates a negative effect on *PHO5* transcriptional activation by influencing the chromatin structure at its promoter region.

Our results suggest that AS transcription at the *PHO5* gene locus locks the chromatin structure of the *PHO5* promoter in a more repressive configuration that is harder to remodel (Fig 6). This could be due to the activity of HDACs, which have been shown to negatively affect

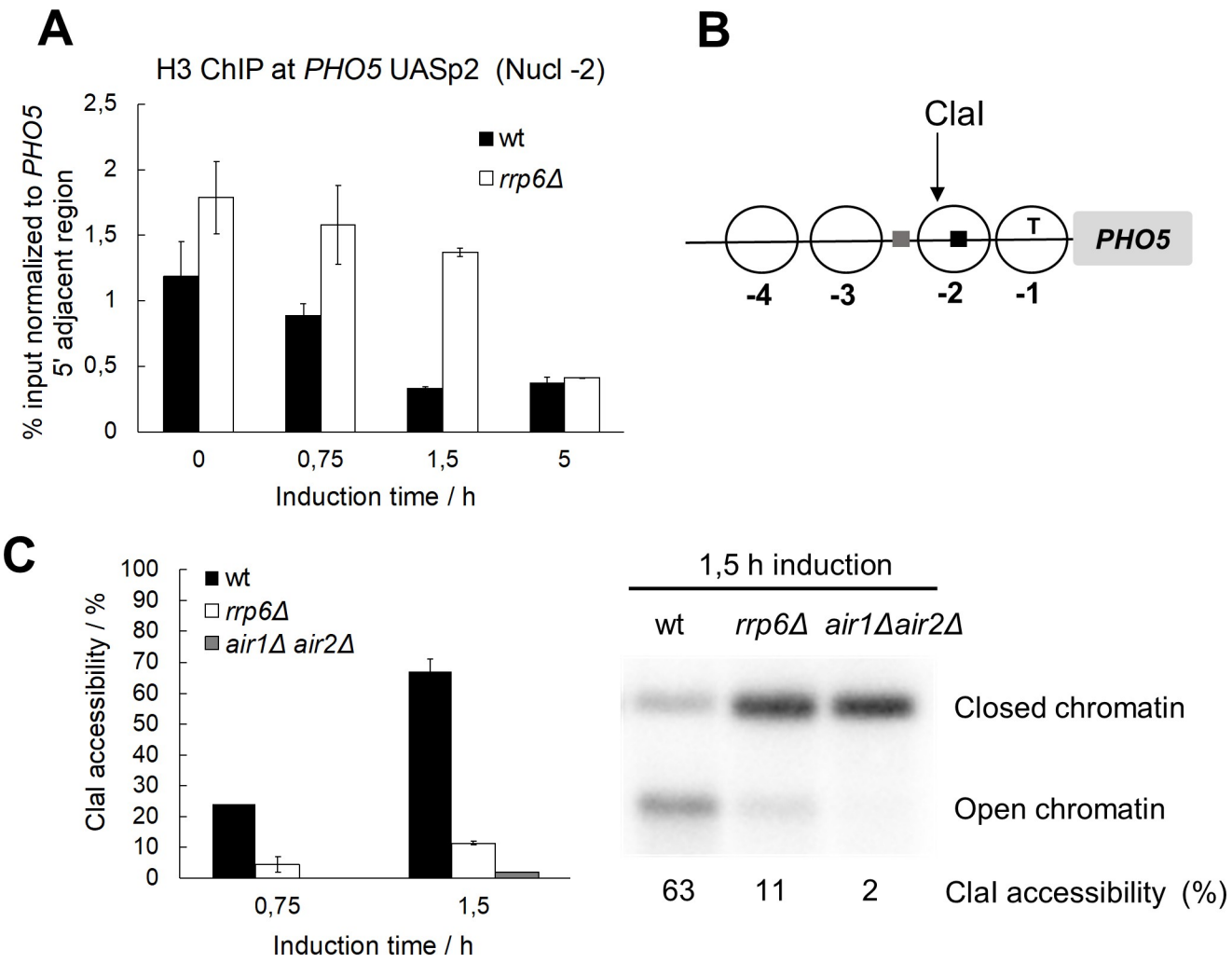

Fig 6. *PHO5* AS elongation negatively affects kinetics of histone removal at the *PHO5* gene promoter upon induction. **(A)** ChIP analysis of histone H3 binding at nucleosome -2 of the *PHO5* gene promoter in wild-type BMA41 (wt) and corresponding *rrp6Δ* cells upon induction through phosphate starvation. Immunoprecipitated DNA was quantified by qPCR and normalized to a control genomic region adjacent to the *PHO5* gene locus. **(B)** Scheme of the *PHO5* gene promoter region. Nucleosomes are denoted by circles, Pho4 binding sites by squares (gray—low affinity, black—high affinity) and the TATA box by the letter T. Site of cleavage with the ClaI restriction enzyme is denoted by a black arrow. **(C)** Kinetics of *PHO5* promoter opening monitored by ClaI accessibility at nucleosome -2 after induction as in (A).

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chromatin structure at the *PHO5* promoter [22,23]. Remarkably, inactivation of the HDAC Rpd3 in the *rrp6Δ* mutant background does not affect the level of the *PHO5* AS RNA, but it restores transcription activation of the *PHO5* gene to the level or even higher than in wild-type cells, as shown by tiling arrays and RT-qPCR with single and double deletion mutant cells ([15]; Fig 7A). Accordingly, the expression kinetics of acid phosphatase measured with the *rpd3Δ rrp6Δ* double mutant cells are not delayed compared to wild-type cells, in contrast to the corresponding *rrp6Δ* single mutant cells (Fig 7B). Consistent with this, expressing the *PHO5* AS-blocking CRISPRi system leads to faster gene expression kinetics in wild-type and *rrp6Δ*, but not in *rpd3Δ* and *rpd3Δ rrp6Δ* double mutant cells (S3 Fig). These results demonstrate that the *PHO5* AS transcript acts *via* a pathway that involves histone deacetylation. Gcn5, the catalytic subunit of the SAGA and ADA complexes, is known to be the major histone acetyltransferase that enables physiological gene induction kinetics at the *PHO5* promoter

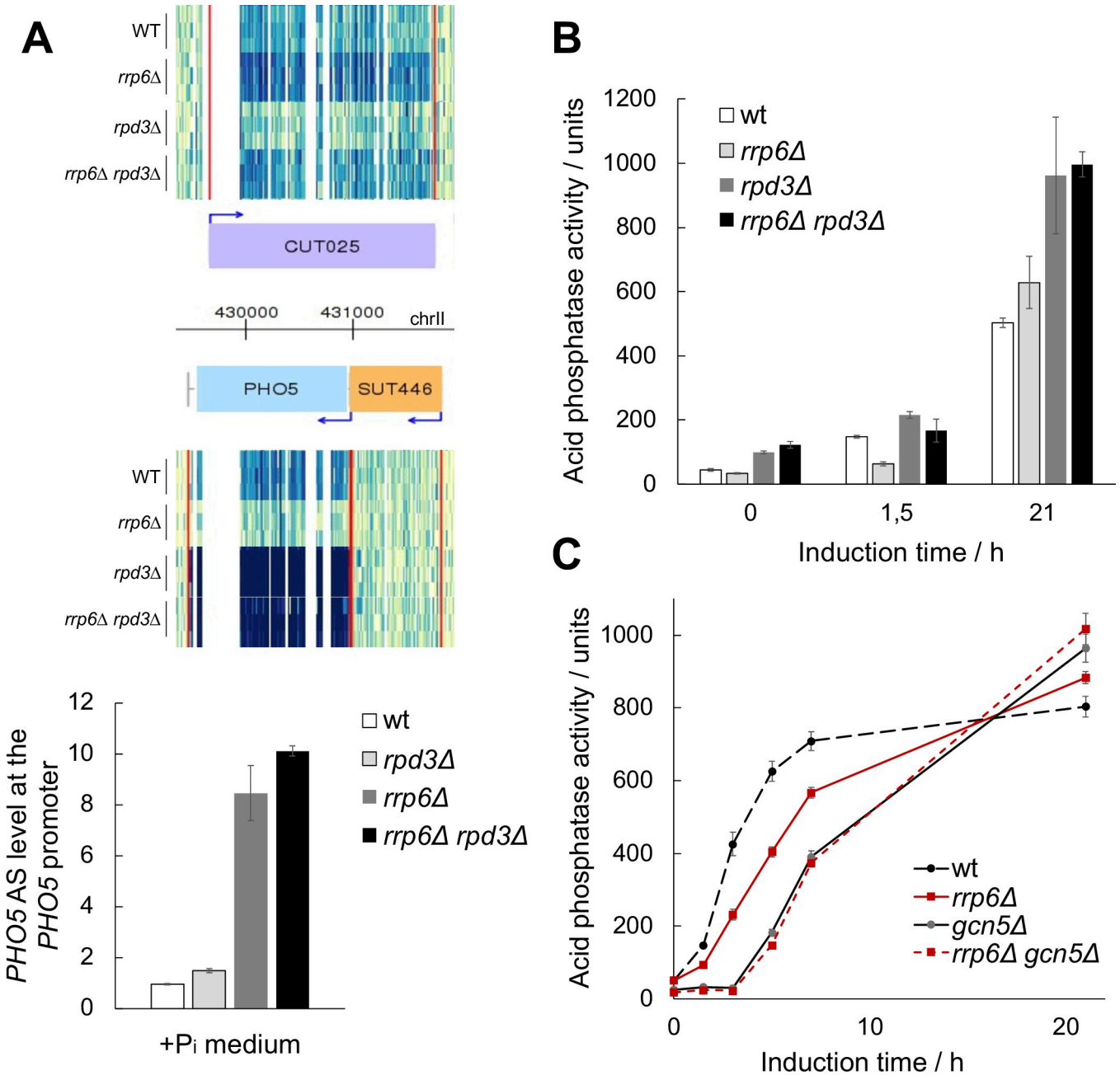

Fig 7. *PHO5* AS elongation affects *PHO5* gene expression *via* histone acetylation. (A) Heatmap of the *PHO5* gene locus in wild-type W303 (wt), *rpd3Δ*, *rrp6Δ*, and *rrp6Δrpd3Δ* mutant cells. Snapshot of tilling arrays intensities from [15] at the *PHO5* locus for the Watson (W, upper half) and the Crick (C, lower half) strands. Three replicates of each strain are represented. A darker signal depicts a higher score of RNA expression. The red vertical lines represent the inferred coding and non-coding genes boundaries. Below: Levels of *PHO5* AS transcript measured by RT-qPCR with the same strains at +Pi conditions. Values were normalized to *ACT1* RNA. **(B)** Acid phosphatase induction kinetics in wild-type W303 (wt) and the corresponding *rrp6Δ*, *rpd3Δ* and *rrp6Δrpd3Δ* cells upon induction through phosphate starvation. Reported values represent the means and standard deviations of three independent experiments (n = 3). **(C)** As in (B), but for wild-type BY4741 (wt) and the corresponding *rrp6Δ*, *gcn5Δ* and *rrp6Δgcn5Δ* mutant cells.

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[25,26]. We reasoned that in the absence of Gcn5, *i.e.* when the majority of histone acetylation normally present at the *PHO5* gene promoter is reduced, an *rrp6Δ* strain should have no additional effect on the kinetics of *PHO5* gene expression. Indeed, the kinetics of acid phosphatase expression in *gcn5Δrrp6Δ* double mutant strain are the same as in the *gcn5Δ* single mutant

throughout the induction period (Fig 7C). Taken together, these results support that AS transcription-mediated repression of *PHO5* gene expression occurs *via* histone deacetylation.

AS transcription negatively affects recruitment of RSC to the *PHO5* promoter

Histone acetylation plays two important roles in transcriptional activation. It neutralizes the positive charge of lysine groups, thereby weakening histone-DNA interactions, and it also provides docking sites for the bromodomains of proteins involved in transcriptional regulation. RSC (Remodels Structure of Chromatin) complex is the most abundant and the only essential remodeler in yeast and contains seven of the fourteen bromodomains identified in *S*. *cerevisiae* [28,55]. RSC was found to be the major remodeler among the five chromatin remodelers involved in the chromatin remodeling process at the *PHO5* promoter [31]. Its partial depletion, achieved by a temperature-sensitive mutant of its catalytic subunit $sth1^{td}$, resulted in a strong delay in promoter chromatin structure opening and, consequently, delayed kinetics of acid phosphatase expression upon *PHO5* gene induction.

To first test the hypothesis that histone acetylation recruits RSC to the *PHO5* gene promoter upon induction, we used the anchor away system to deplete its catalytic subunit Sth1 from the nucleus in Sth1-AA and corresponding *gcn5Δ* mutant cells. Because Sth1 is essential for cell viability, we first attempted to induce its depletion in parallel with the induction of the *PHO5* gene. Addition of rapamycin upon shifting the cells to phosphate-free medium caused a delay in acid phosphatase expression kinetics similar to the partial depletion through $sth1^{td}$ (Fig 8A). In *gcn5Δ* mutant cells, this partial depletion leads to an additive effect on acid phosphatase expression kinetics. However, since RSC is very abundant, it is possible that the partial depletion of Sth1 still leaves a lot of active RSC complex in the nucleus in the first hours of gene induction. We therefore added rapamycin two hours before *PHO5* gene induction to achieve more extensive RSC depletion before shifting the cells to phosphate-free medium. Addition of rapamycin two hours before gene induction resulted in an epistatic effect of the Sth1 depletion. Upon simultaneous inactivaton of Gcn5 and RSC, acid phosphatase expression kinetics were severely delayed, but reached overnight levels comparable to wild-type (Sth1-AA -Rap) cells (Fig 8A). This result positions Gcn5 and RSC in the same pathway of *PHO5* gene transcriptional activation and speaks in favour of a link between RSC recruitment and Gcn5-mediated acetylation upon induction.

To test the effect of Sth1-AA depletion in *rrp6Δ* mutant cells, we monitored acid phosphatase expression kinetics upon addition of rapamycin (S4 Fig). Even when rapamycin was added two hours before induction to achieve more complete inactivation of RSC, it resulted in an additive effect on acid phosphatase expression kinetics with the *rrp6Δ* mutation. It is possible that Rrp6 and RSC regulate *PHO5* gene expression through at least partially independent pathways. However, because these cells barely induced the *PHO5* gene, as indicated by the levels of acid phosphatase activity measured after overnight induction, we cannot rule out the possibility that this additive effect is due to the severely impaired cell viability because of the Sth1-AA depletion in the slow-growing *rrp6Δ* background.

To directly answer the question of whether AS-induced deacetylation of the *PHO5* promoter may inhibit the recruitment of RSC, resulting in a more closed chromatin conformation, we took advantage of our genomic analyses recently performed with Nrd1-AA and Nrd1-AA *rpd3Δ* cells with and without the addition of rapamycin [13]. We examined the *PHO5* gene locus in the Micrococcal Nuclease sequencing (MNase-seq), Assay for Transposase-Accessible Chromatin using sequencing (ATAC-seq) and Sth1-Chromatin Endogenous Cleavage-sequencing (Sth1-ChEC-seq) datasets, which give us information about the

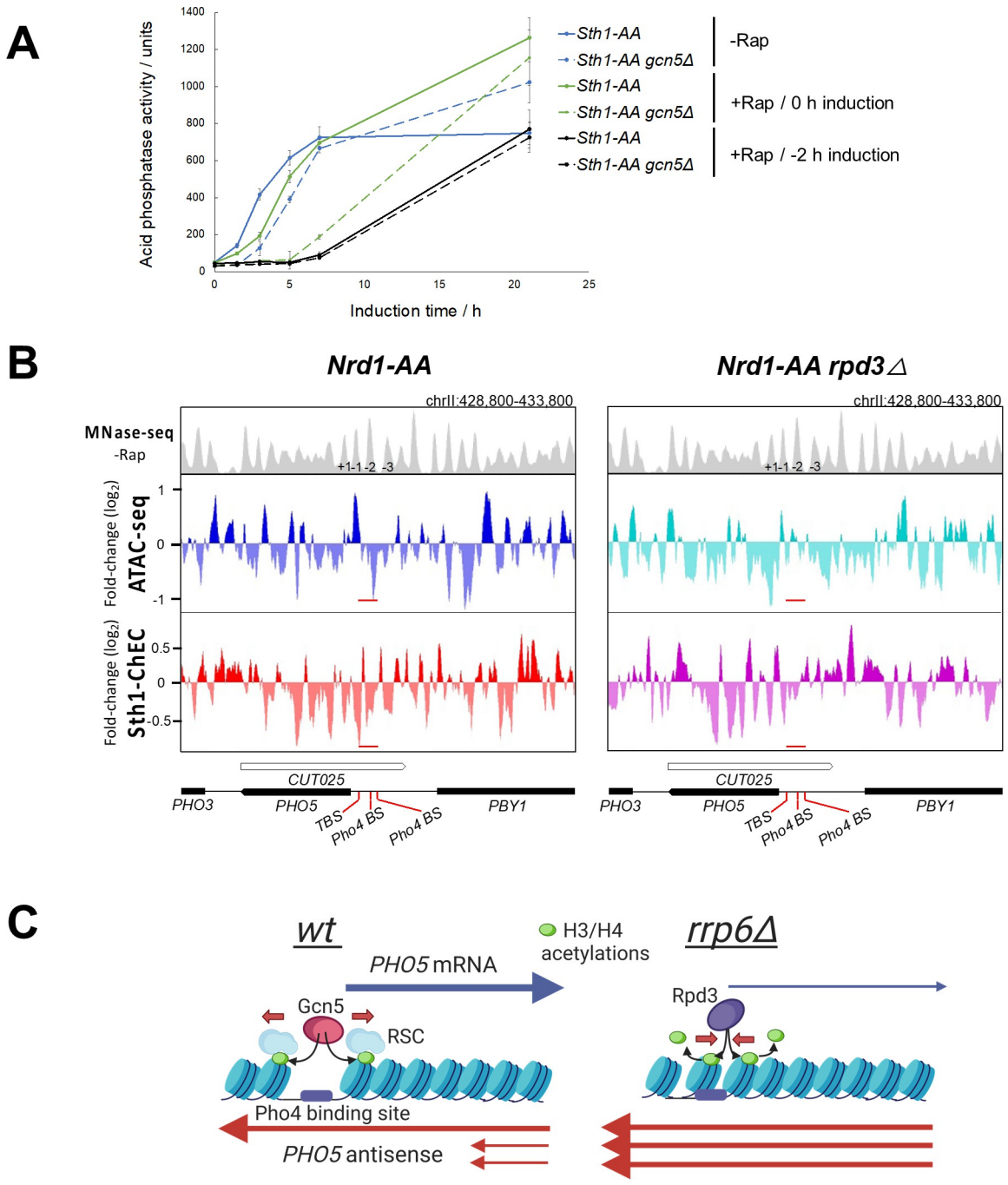

Fig 8. Chromatin remodeling at the *PHO5* gene promoter is negatively affected by *PHO5* AS elongation. (A) Acid phosphatase induction kinetics in Sth1-AA and the corresponding *gcn5Δ* cells upon induction through phosphate starvation without (-Rap) or with addition of rapamycin (+Rap) at indicated times. Reported values represent the means and standard deviations of three independent experiments (n = 3). **(B)** Snapshot of the *PHO5* gene locus in Nrd1-AA and the corresponding *rpd3Δ* strain from MNase-seq (-Rap), ATAC-seq (+Rap/-Rap) and Sth1-ChEC (+Rap/-Rap) experiments. Data is from [13]. **(C)** Proposed model for how AS RNA regulates transcription of the *PHO5* gene at the level of promoter chromatin structure. In wild-type cells, antisense RNA transcription is terminated by the NNS complex and degraded by the RNA exosome. Histones at the *PHO5* gene promoter are acetylated by Gcn5 and serve as docking sites for recruitment of the chromatin remodeling complex RSC, thus enabling physiological kinetics of promoter opening and gene induction. In *rrp6Δ* cells, read-through of the AS transcript into the *PHO5* promoter region results in increased recruitment of the histone deacetylase Rpd3 and subsequently in hypoacetylation and decreased recruitment of RSC. This results in delayed kinetics of promoter opening and induction of the *PHO5* gene.

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chromatin conformation and Sth1 binding at the *PHO5* promoter upon induction of AS transcription (+Rap/-Rap) and depending on the presence of Rpd3 (Fig 8B). The ChEC-seq data show the fold change in association of Sth1, the ATP-ase subunit of RSC, with chromatin upon induction of AS transcription (+Rap/-Rap). In addition, the ATAC-seq data provide us with information about chromatin accessibility under the same conditions. In Nrd1-AA cells there is a negative fold change, *i.e*. a decrease in Sth1 binding, associated with a decrease in chromatin accessibility upon addition of rapamycin, for the region encompassing nucleosome -2 of the *PHO5* promoter (the position of which was determined using MNase-seq data in -Rap) (Fig 8B). Conversely, in isogenic *rpd3Δ* cells, addition of rapamycin has a much smaller effect on Sth1 binding or chromatin accessibility in this region (Fig 8B). When comparing the two biological experimental replicates, the log2 values for the change Sth1 binding (+Rap/-Rap) were consistently lower in Nrd1-AA compared to isogenic *rpd3Δ* cells (-0,3947 and -0,566 *vs*. -0,2057 and -0,2841, respectively, calculated over the middle 40 bp region of nucleosome -2). These data argue in favour of a model in which read-through of AS transcription acts *via* recruitment of histone deacetylases to the *PHO5* gene promoter, the activity of which results in decreased recruitment of the RSC complex (Fig 8C).

Discussion

The role of non-coding RNAs in regulation of gene expression could not be appreciated until recent advances in high-throughput methods facilitated their detection and characterization. From a gene-centered view, non-coding RNAs can be transcribed in tandem with genes, *i.e*. from the same strand as the gene, or from the opposite strand, resulting in production of antisense (AS) non-coding RNAs. Apart from a few isolated examples, production of AS non-coding RNAs is generally thought to have a repressive *cis*-regulatory effect on the expression of associated mRNAs [7,9,56]. This seems to be particularly the case when transcription of AS non-coding RNAs invades promoters of coding genes [10,15,57]. In light of this current view, we felt compelled to reexamine the role of AS transcription at the model yeast *PHO5* gene locus, which was originally suggested to support gene activation [35]. In this work, we show a clear negative role for AS transcription in *PHO5* gene expression. By leveraging mutant backgrounds in which AS transcription is constitutively enhanced or inducible and artificially driving its expression from a strong promoter *in cis*, we show that increased *PHO5* AS elongation frequency correlates with decreased expression of the corresponding mRNA. Furthermore, we demonstrate that the use of a CRISPRi system that specifically blocks AS transcription at the *PHO5* gene locus increases the level of *PHO5* mRNA and enhances its induction kinetics upon phosphate depletion. Importantly, these observations show that AS RNA transcription has an impact on *PHO5* gene expression in wild-type cells, and not only upon enhanced AS RNA stabilisation in strains mutant for RNA degradation factors. We also show that AS RNA transcription regulates expression of the *PHO5* gene only when transcribed *in cis*, and not *in trans*. The role of *PHO5* AS transcription is therefore reminiscent of the role of AS transcription in maintaining the tight repression of quiescence-related transcripts during the exponential growth phase, recently demonstrated by Nevers et al. [10]. A previous study suggesting a positive regulatory role for *PHO5* AS transcription achieved AS inactivation by incorporating a full-length marker gene sequence with its promoter region in the middle of the *PHO5* gene ORF [35]. This major perturbation of the *PHO5* gene locus may have resulted in experimental artefacts, highlighting the need for precise interventions, such as those achieved by the CRISPRi system, to perform functional analyses of AS transcripts [58].

There are now several well-described examples of yeast gene loci at which either antisense or upstream non-coding transcription that extends through a coding sense promoter has an

inhibitory effect on its transcription initiation [14,59–62]. In most cases, it is likely that elongation of non-coding transcription leads directly to displacement of transcription factors (TFs) and/or the preinitiation complex (PIC) or that the recruitment of TFs or the PIC to these gene promoters is decreased as a consequence of a more repressive chromatin configuration established at the promoter region due to elongation of non-coding transcription (see [63] for a review). This model is supported by whole-genome analyses showing that invasion of gene promoters by AS transcription leads to increased histone occupancy and altered recruitment of chromatin-modifying and -remodeling complexes [11,13,64]. At the tandemly transcribed *SRG1* lncRNA/*SER3* protein-coding gene locus, non-coding transcription has been shown to cause nucleosome deposition at the gene promoter, thereby repressing *SER3* transcription [65]. As another example, we have shown that AS transcription at the *PHO84* gene locus silences the corresponding gene by recruiting HDACs to its promoter region [14]. The AS RNA does not recruit the HDACs directly, but the act of its transcription promotes a histone methylation-based mechanism to restore the repressive chromatin structure in the wake of the elongating RNA Pol II. The histone methyltransferase Set2 associates with the elongating RNA Pol II and catalyses H3K36 methylation, a mark read by the Eaf3 chromodomain of the HDAC Rpd3 [66]. Consistent with this, our recent genome-wide study in yeast has shown that AS transcription leads to deacetylation of a subpopulation of -1/+1 nucleosomes associated with increased H3K36 methylation, which in turn leads to decreased binding of the RSC chromatin-remodeling complex and sliding of nucleosomes into previously nucleosome-depleted regions [13]. We have now shown that elongation of *PHO5* AS under repressive conditions leads to increased histone occupancy at the *PHO5* gene promoter and slower histone removal upon gene induction. Moreover, the negative effect of AS RNA elongation on *PHO5* gene activation is mitigated by inactivation of Rpd3, suggesting a histone acetylation-based regulatory mechanism that may affect the recruitment of RSC, a chromatin remodeler that plays an important role in *PHO5* gene promoter opening [31]. This is supported by ChEC-seq of Sth1, the catalytic subunit of RSC, showing a decrease in its recruitment to the *PHO5* gene promoter upon induction of antisense transcription, that is suppressed by inactivation of Rpd3.

PHO5 belongs to a group of ~100 genes that are more transcribed in AS direction as a non-coding transcript than in the sense orientation as an mRNA in a standard medium (Fig 1B). In such culture conditions, the Pho4 transcriptional activator is rarely located in the nucleus [19]. Thus, as we proposed in [46] for the SAGA-dependent gene class to which *PHO5* belongs, the steady-state chromatin structure of the promoter NDR might be maintained tightly closed by ongoing AS transcription. What may also be relevant to this mechanism is the recently discovered autoregulatory mechanism of the SAGA complex, which is induced in response to environmental changes such as phosphate starvation conditions [67]. The SAGA catalytic subunit Gcn5 has been shown to acetylate the Ada3 subunit, which promotes dimerization of the SAGA complex and in turn leads to higher efficiency of SAGA-catalysed histone acetylation. *PHO5* expression was shown to correlate negatively with decreasing levels of Ada3 acetylation and consequently lower efficiency of histone acetylation by Gcn5. The same was also found for *SUC2* transcription, which is induced during growth in sucrose-containing media. Of importance to our work is the finding that of the 8 known histone deacetylases, the Ada3 subunit is deacetylated only by Rpd3, but the mechanism of its recruitment to SAGA remains to be elucidated. Therefore, the enhanced recruitment of Rpd3 mediated by AS transcription may play a dual role in regulating *PHO5* gene expression, considering that Rpd3 deacetylates promoter histones and Gcn5, both of which contribute to transcriptional repression. It remains to be investigated whether such a regulatory mechanism of AS transcription-mediated repression could be a common mechanism for AS transcription-induced repression of stress-inducible and SAGA-dependent genes regulated by promoter chromatin structure remodeling.

The regulatory roles of non-coding RNAs are intertwined with that of chromatin structure. Not only does non-coding transcription affect chromatin structure, but chromatin structure also determines where and how often non-coding RNAs are transcribed. This fact is increasingly appreciated with respect to the directionality of transcription at promoters of coding genes. Specifically, chromatin modifiers such as the HDAC Hda1, and chromatin remodelers such as RSC, have been shown to dictate promoter directionality by attenuating divergent non-coding transcription [68,69]. Furthermore, chromatinization of DNA limits aberrant transcription that would otherwise occur on naked DNA, as was recently demonstrated through *in vitro* experiments by the Kornberg group [70]. In this study, a chromatinized *PHO5* gene locus fragment was transcribed seven times more from the physiological transcription start site than the same naked DNA locus, and also resulted in transcription patterns more similar to those seen *in vivo*. Although only chromatin was considered in this study, it would be interesting to also investigate non-coding transcription using a similar *in vitro* transcription system.

Chromatin remodeling complexes and non-coding RNAs are important regulators of gene expression, and therefore dysregulation of either of these factors may affect the development and progression of various cancers. The SWI/SNF family of chromatin remodeling complexes includes the SWI/SNF complex with its catalytic subunits BRG1 or BRM in humans, and the SWI/SNF and RSC complex with their catalytic subunits Snf2 and Sth1, respectively, in yeast. Numerous associations between chromatin remodelers of this family and long non-coding RNAs have been detected in human cancers (reviewed in [71]). These complexes and the corresponding regulatory non-coding RNAs therefore represent promising diagnostic and therapeutic targets. Transcription of long non-coding RNAs is particularly important for the yeast genome, which has a very high gene density, such that many of them overlap coding gene ORFs or promoter regions. Another reason why budding yeast is a good model for studying the transcription of such long ($\geq$200 nt) non-coding RNAs is that it exclusively synthesizes this non-coding transcript class since its divergence from other yeasts and the loss of the RNAi system that produces small non-coding RNAs [72]. In addition, extensively studied gene loci, such as the yeast *PHO5* gene, are invaluable for mechanistic studies of gene regulation. Studies of the *PHO5* gene and its promoter region made an immense contribution to deciphering the mechanisms of gene regulation through chromatin remodeling [19] and our study now opens the possibility to focus on non-coding transcription in this system.

Supporting information

S1 Fig. Non-coding transcripts CUT025 and SUT446 are transcribed at the *PHO5* gene locus. (A) A heatmap summarising tiling array expression data at the *PHO5* gene locus in wild-type W101 (wt) and corresponding *rrp6Δ* cells. Data is from [36] and is visualized with the SGV Genomics Viewer [73]. **(B)** Levels of SUT446 in wild-type BMA41 (wt) and corresponding *rrp6Δ* mutant cells upon induction through phosphate starvation. RT-qPCR values were normalized to *PMA1* RNA and expressed relative to transcript abundance in wild-type cells at repressive conditions (0 h of induction), which was set to 1.
(TIFF)

S2 Fig. Delayed expression kinetics of the *PHO5*, but not the *lacZ* gene under regulation of the *PHO5* promoter in *rrp6Δ* mutant cells. (A) Acid phosphatase induction kinetics in wild-type BMA41 (wt) and corresponding mutant cells upon induction through phosphate starvation. The strain *rrp6Y361A* carries a point mutation at the *RRP6* genomic locus which abolishes exonuclease activity of Rrp6. Plasmid pCEN-RRP6 is a centromeric plasmid which carries the *RRP6* gene under regulation of its native promoter. Reported values represent the

means and standard deviations of three independent experiments (n = 3). **(B)** Same as (A), but for wild type and corresponding *rrp6Δ* mutant cells from the BY4741 genetic background. **(C)** Same as (A), but for wild type and corresponding *rrp6Δ* mutant cells from the LPY917 genetic background. **(D)** Beta-galactosidase induction kinetics in wild-type BMA41 (wt) and corresponding *rrp6Δ* cells transformed with a reporter plasmid pP5Z carrying the *lacZ* gene under the control of the *PHO5* promoter upon induction through phosphate starvation. Reported values represent the means and standard deviations of three independent experiments (n = 3).
(TIFF)

S3 Fig. Expression of the *PHO5* AS-blocking CRISPRi system leads to faster gene expression kinetics in wt and *rrp6Δ*, but not in *rpd3Δ* and *rpd3Δ rrp6Δ* double mutant cells. Acid phosphatase induction kinetics in wild-type BMA41 (wt) and corresponding deletion mutant cells for Rrp6 and Rpd3, with and without expression of the CRISPRi system which blocks *PHO5* AS transcription, upon induction through phosphate starvation. Reported values represent the means and standard deviations of three independent experiments (n = 3).
(TIFF)

S4 Fig. Effect of simultaneous inactivation of Sth1 and Rrp6 on *PHO5* gene expression. Acid phosphatase induction kinetics in Sth1-AA and the corresponding *rrp6Δ* cells upon induction through phosphate starvation without (-Rap) or with addition of rapamycin (+Rap) at indicated times. Reported values represent the means and standard deviations of three independent experiments (n = 3).
(TIFF)

S1 Table. *S. cerevisiae* strains. Table includes names of strains, their genotypes and sources.
(PDF)

S2 Table. Primers. Table includes names and sequences of oligonucleotides used for strain construction, plasmid construction and RT-qPCR.
(PDF)

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4. GENERAL DISCUSSION

The 3'-to-5' exoribonucleolytic RNA exosome complex is involved in processing and degradation of practically all classes of RNA substrates in eukaryotic cells (Zinder and Lima 2017). The recently characterized substrates of the RNA exosome are long non-coding RNAs, transcripts of ≥200 nt which do not code for proteins but may exert important gene regulatory functions. As these transcripts are highly unstable due to their degradation by the RNA exosome complex, inactivation of the RNA exosome catalytic subunit Rrp6 was instrumental for enabling their detection on a transcriptome-wide scale. In yeast, these transcripts have been termed CUTs - cryptic unstable transcripts (Xu *et al.* 2009). In general, lncRNAs resemble mRNAs as they are transcribed by Pol II and modified through addition of the 5' cap and the 3' poly-A tail. How are then lncRNAs produced by pervasive transcription differentiated from mRNAs and targeted for this degradation pathway? They are targeted for early termination by the NNS complex, which associates with the CTD of the transcribing Pol II at the 5' regions of transcriptional units and recognizes specific motifs enriched in CUTs (Villa and Porrua 2022). NNS-terminated transcripts are substrates for the TRAMP complex, which catalyzes poly-A polymerization and RNA unwinding to make them accessible for degradation by the RNA exosome (LaCava *et al.* 2005). Why are these lncRNAs transcribed if they are to be degraded shortly after their synthesis? Detection of extensive amounts of lncRNAs transcribed from almost all regions of eukaryotic genomes was initially puzzling, because spurious transcription was previously disregarded as transcriptional noise (Tudek *et al.* 2015). However, since then, many studies have investigated the role of specific lncRNAs and lncRNA classes through single-gene and transcriptome-wide studies and revealed their functions in gene regulation through various molecular mechanisms. At most studied gene loci, non-coding transcription has been shown to have a negative regulatory role by interfering with the binding of the transcriptional machinery to the promoter of the corresponding coding gene, either by affecting the local chromatin structure or by interference mechanisms in which non-coding transcription more directly prevents PIC assembly (Soudet and Stutz 2019, Li *et al.* 2021). The RNA exosome complex can therefore indirectly regulate expression of certain genes at the level of transcription by affecting the levels of its direct targets - the corresponding regulatory lncRNAs. This helps explain the seemingly paradoxical finding that the levels of many mRNAs are downregulated upon inactivation of this ribonuclease complex (Schmid and Jensen 2018). However, non-coding transcription at many loci could be spurious and non-functional or even

have positive gene regulatory functions. Furthermore, the molecular mechanisms by which non-coding RNA and the RNA exosome regulate gene expression remain poorly defined and the effects of non-coding transcription at most eukaryotic gene loci are still largely uncharacterized.

This work (**PAPER 1**) shows that non-coding transcription at the *PSA1* gene promoter regulates the expression of Psa1, an essential enzyme involved in synthesis of the protein mannosylation precursor GDP-mannose. The lncRNA CUT488 is transcribed in the sense direction through the *PSA1* promoter region, and its levels were found to be anti-correlated with *PSA1* mRNA and Psa1 protein levels. Specifically, the absence of Rrp6 protein or exoribonuclease activity of the second catalytic subunit of the RNA exosome, Dis3, lead to an increase in CUT488 levels and decrease in Psa1 expression, which was particularly pronounced at high temperature. This was accompanied by a decrease in binding of Pol II to the core promoter of *PSA1*, which overlaps the CUT488 transcription unit, suggesting a regulatory role of the lncRNA at the level of transcriptional initiation. The general model that CUT levels are higher in *rrp6* mutant cells simply due to increased stabilization of these transcripts because they are not degraded by the RNA exosome was recently challenged by work from the groups of Rahmouni and Libri. They have shown that an increase in the levels of non-coding transcripts, accomplished by perturbation of RNA biogenesis (Moreau *et al.* 2019) or inactivation of the RNA exosome (Villa *et al.* 2020), lead to their sequestration of the NNS complex, which is required for early termination of lncRNA transcription. Sequestration of the NNS complex by lncRNAs impairs its recruitment to target lncRNA loci, leading to termination defects and, consequently, enhanced elongation of lncRNA transcription. On this basis, we propose a model for *PSA1* gene regulation, in which RNA exosome inactivation leads to increased elongation of the lncRNA CUT488 through the *PSA1* core promoter/TSS region which negatively influences PIC assembly and *PSA1* transcription. Interestingly, *PSA1* is a constitutively expressed gene, as protein mannosylation is an essential cellular process, so its expression is not condition specific. However, the lncRNA CUT488 may serve to fine tune the regulation of its transcription according to external conditions, such as high temperature, which causes stress to the cell wall structure that relies on the layer of mannosylated proteins to maintain its stability (Lehle *et al.* 2006). Interestingly, the *S. cerevisiae PSA1* gene seems to be regulated in a cell cycle dependent manner, as its expression is highest during S phase (Benton *et al.* 1996). Recent research suggest a link between cell cycle regulation and signaling pathways regulating yeast cell wall stability (Sato *et al.* 2022), but their significance and involvement of lncRNAs in these processes remain to be elucidated.

Role of the RNA exosome complex in regulation of protein mannosylation-related genes has implications for yeast physiology. Since the identification of the RNA exosome catalytic subunit Rrp6, it was known that its inactivation in *rrp6* mutant cells impairs their viability at high temperature (Briggs *et al.* 1998), however the underlying cause of this phenotype has not been explained. This work (**PAPER 1**) shows that downregulation of mannosylation-related genes such as *PSA1*, *DPM1* and *ALG7* in *rrp6* cells causes cell wall instability and impairs cell viability at high temperature. Accordingly, the temperature sensitive phenotype of *rrp6* mutant cells is suppressed by overexpression of Psa1 or by osmotic stabilization of the growth medium with 1 M sorbitol. An interesting finding is that this function is carried by a non-catalytic function of Rrp6, as cells containing catalytically inactive Rrp6 display a much less pronounced temperature sensitive phenotype. Furthermore, cells carrying the catalytic *Rrp6-Y361A* mutant do not overexpress the lncRNA CUT488, which downregulates *PSA1* expression, to the same extent as *rrp6* cells. In addition to the presence of the Rrp6 protein, the catalytic exoribonuclease activity of the Dis3 subunit is also required to maintain yeast cell wall stability upon stress, arguing in favor of a non-catalytic regulatory role of Rrp6 in RNA exosome activation. A similar non-catalytic function of Rrp6 was previously proposed by Conti and Lima laboratories. Structural and biochemical *in vitro* studies of the nuclear RNA exosome complex showed a role for Rrp6 in enhancing the activity of Dis3 by enlarging the pore of the S1/KH exosome cap structure and by contributing directly to RNA binding (Makino *et al.* 2015). A subsequent study built upon that model to pinpoint the unstructured C-terminal region of Rrp6, referred to as the lasso, as the region that mediates RNA binding and exosome activation (Wasmuth and Lima 2017). Consistent with our model of the noncatalytic role of Rrp6 in exosome activation mediating cell wall stability, the same study has shown that deletion of the C-terminal EAR region of Rrp6, responsible for interaction with the exosome is sufficient to cause temperature sensitivity (Wasmuth and Lima 2017).

Discovering the role of the RNA exosome in maintenance of yeast cell wall stability enabled us to explain the underlying cause of another characteristic phenotype of *rrp6* mutant cells. Several laboratories studying the RNA exosome found that the temperature sensitive phenotype of *rrp6* mutant cells was most pronounced in the W303 yeast strain background, which is why strains of this genetic background were preferentially used to study RNA exosome-related processes (Klauer and Van Hoof 2013, Wasmuth and Lima 2017). The correlation between the temperature sensitive phenotype of *rrp6* cells and cell wall instability led us to hypothesize that

W303 and its derived strains carry another mutation related to cell wall integrity that exhibits an additive cell wall instability phenotype with the *rrp6* mutation. In this work (**PAPER 2**), we show that the temperature sensitive phenotype of the *rrp6* mutation is augmented by a loss of function mutation in the *ssd1-d* allele, encoding the RNA-binding protein Ssd1 which regulates translation of cell wall-related mRNAs (Kurischko *et al.* 2011). The W303 *ssd1-d* allele contains a premature stop codon due to a C-to-G transversion at nucleotide 2094, resulting in truncation of the Ssd1 protein (Jorgensen *et al.* 2002). Upon expression of the functional *SSD1* allele from its native genomic locus or from a plasmid, the cell wall instability phenotype of *rrp6* mutant cells becomes less pronounced, arguing in favor of the synergistic cell wall instability phenotype of *rrp6* and *ssd1-d* mutations. Interestingly, previous work has linked Ssd1 function to the CWI pathway (Arias *et al.* 2011), which acts in parallel to Rrp6-mediated regulation (Wang *et al.* 2020). It is interesting to note that yeast cell wall stability is controlled by at least two parallel pathways regulated by RNA binding proteins, Ssd1 and the RNA exosome. RNA metabolism is emerging as an important factor in cell wall stress response, as many RNA binding proteins has been shown to regulate cell wall related gene expression at transcriptional and post-transcriptional levels (Hall and Wallace 2022). Another understudied feature of cell wall-related gene regulation is that based on mechanisms involving non-coding transcription. Accordingly, this work includes a literature review article on the topic of lncRNA-mediated cell wall regulation (**PAPER 3**), which outlines known examples of cell wall-related gene loci regulated by lncRNAs and discusses the broad potential of lncRNAs as cell wall regulators. In this work, we demonstrate that, out of 201 genes involved in cell wall biogenesis, 88 of their loci (44%) exhibit antisense non-coding transcription, while 15 of them are associated with non-coding transcription across their promoter regions. It remains to be elucidated how many of them have physiologically relevant roles, especially under stress conditions which necessitate cell wall remodeling.

Another stressful condition for yeast cells is phosphate depletion, which leads to upregulation of the *PHO* regulon genes. These genes encode proteins such as periplasmic phosphatases and membrane importers which scavenge inorganic phosphate (P_i) from extracellular substrates or import it into the cells, respectively (Austin and Mayer 2020). The gene encoding the high affinity P_i importer *PHO84* has been shown to be regulated by non-coding antisense transcription through a mechanism involving increased deposition of repressive histone marks at its promoter region. This regulation has been extensively studied by the Stutz group, so *PHO84* can be considered a model gene for regulation through non-coding transcription in yeast

(Camblong *et al.* 2007, 2009, Castelnuovo *et al.* 2013, 2014). Another *PHO* regulon gene, *PHO5*, has been of interest in the gene regulation field for more than 30 years due to the extensive transition of chromatin conformation at its promoter region upon gene induction (Korber and Barbaric 2014). Many basic principles of promoter chromatin structure remodeling have been established using the *PHO5* promoter model system, such as independence of the chromatin remodeling process on replication (Schmid *et al.* 1992) or gene transcription (Fascher *et al.* 1993), *in trans* removal of histones (Boeger *et al.* 2004, Korber *et al.* 2004), and redundancy of remodeling pathways that cooperate to open the same promoter region (Barbaric *et al.* 2007, Musladin *et al.* 2014). Interestingly, *PHO5* is one of the very rare examples where non-coding antisense transcription has been reported to play a positive gene regulatory role (Uhler *et al.* 2007). The Svejstrup group proposed a model in which transcription of antisense RNA through the *PHO5* promoter regions renders the chromatin structure more permissive to remodeling, probably due to the passing of the transcribing Pol II through this region. However, this work (**PAPER 4**) argues against this model and clearly shows that antisense non-coding transcription has a negative role in regulation of *PHO5* gene transcription. Increasing the level of *PHO5* antisense transcription by using strains mutant for the RNA exosome or the NNS complex, or through overexpression of this transcript *in cis*, impairs *PHO5* transcriptional kinetics upon gene induction. Partially blocking elongation of the *PHO5* antisense transcript by using the CRISPR interference (CRISPRi) system, containing the catalytically inactive dCas9, enhances transcriptional kinetics of the *PHO5* gene. The repressive effect of antisense transcription is relieved upon inactivation of the histone deacetylase Rpd3, showing that it is exerted *via* histone deacetylation. A similar mechanism regulates *PHO84* gene transcription, but implicates the histone deacetylase Hda1, as well as Rpd3 (Camblong *et al.* 2007, Castelnuovo *et al.* 2014). The *PHO84* antisense transcript can also regulate expression of the corresponding gene *in trans* (Camblong *et al.* 2009), while this work shows that *PHO5* antisense transcription regulates the corresponding gene only *in cis*. Furthermore, we have shown that a direct consequence of antisense transcription-induced deacetylation at the *PHO5* promoter region is decreased recruitment of the chromatin remodeling RSC complex, which contains several bromodomains that bind acetylated histones (Zhang *et al.* 2010). Consequently, antisense transcription leads to a more repressive chromatin conformation in the *PHO5* promoter region, which is more difficult to remodel upon inducing conditions. Recent genome-wide analyses from the Stutz group argue for global antisense transcription-mediated effects on chromatin, which function through the HIR histone chaperone complex that deposits histones on promoter regions upon induction of antisense transcription (Soudet *et al.* 2022),

leading to a global decrease in RSC complex recruitment (Gill *et al.* 2020). This study also found that the level of steady-state antisense transcription is significantly higher at promoters of SAGA-regulated genes compared to TFIID-dominated genes (Soudet *et al.* 2022). Accordingly, the SAGA complex with its histone acetyltransferase Gcn5 subunit is important for physiological kinetics of *PHO5* promoter chromatin opening (Barbaric *et al.* 2001, 2003). The activity of Gcn5 at the *PHO5* promoter is opposed by that of the histone deacetylase Rpd3 (Vogelauer *et al.* 2000, Wang *et al.* 2011). Recent work demonstrated an autoregulatory mechanism for SAGA activation that results from acetylation of the Ada3 subunit, which facilitates dimerization of the SAGA complex (Huang *et al.* 2022). The authors found that this autoregulatory mechanism is enhanced upon stress conditions such as phosphate starvation and is otherwise opposed by Rpd3, which deacetylates Ada3. This suggests a possible dual repressive role of Rpd3 at the *PHO5* promoter, in deacetylating both promoter histones and SAGA subunits. Together with our finding that antisense transcription affects acetylation of *PHO5* promoter chromatin structure, these studies paint a more complete picture of *PHO5* model gene transcriptional regulation.

This work also addresses the role of specific RNA exosome catalytic subunits and cofactors in two processes: maintenance of cell wall stability (**PAPER 1**) and regulation of *PHO5* gene expression (**PAPER 4**). The absence of Rrp6 protein or its catalytic exoribonuclease activity delays expression kinetics of the *PHO5* gene, implying that its activity is directly involved in degradation of the corresponding non-coding transcript. On the other hand, the presence of Rrp6 protein, but not its catalytic activity, is required for the proper regulation of glycosylation-related genes and the maintenance of cell wall stability upon stress, pointing to its non-catalytic role in this process. In contrast, both processes are affected by inactivation of the exoribonuclease activity of the essential exosome catalytic subunit Dis3 and not its endoribonuclease activity. Since both processes require the presence of Rrp6 protein, it is not surprising that they also depend on its cofactor Rrp47. It has been shown that the nuclear cofactor Rrp47 is responsible for stabilizing the Rrp6 protein, such that Rrp6 levels are severely impaired in the *rrp47* mutant (Feigenbutz *et al.* 2013, Stuparevic *et al.* 2013). It has also been shown that another monomeric exosome cofactor Mpp6, plays a role in both processes. The TRAMP complex is a heterotrimeric cofactor of the nuclear exosome which contains the essential helicase Mtr4, a poly-A polymerase Trf4 or Trf5 and an RNA-binding protein Air1 or Air2. The poly-A polymerase Trf subunits do not appear to play significant non-overlapping roles in either process, but their redundant roles could not be studied because the double mutant

trf4 trf5 is not viable (Castaño *et al.* 1996). The Air subunits were found to play redundant roles in both processes, as cell wall stability and *PHO5* gene expression were not affected in any of the single mutants but were severely impaired in the *air1 air2* double mutant. An interesting Air2-specific effect was observed, as only the *air2* mutation resulted in higher *PHO5* gene expression upon overnight induction, independent of the *air1* mutation. This is consistent with important roles of the TRAMP complex in both processes and the fact that their paralogous subunits have both redundant and specific functions in various processes (Schmidt *et al.* 2012, Stuparevic *et al.* 2013, Delan-Forino *et al.* 2020).

The cell wall and periplasm compartments are essential for yeast cells because they represent their interface with the environment and are therefore particularly important under stress conditions. However, these compartments are not present in mammalian cells. Nevertheless, the RNA exosome is a highly conserved complex important for viability and cellular homeostasis in all eukaryotes (Zinder and Lima 2017). The finding that the RNA exosome indirectly regulates protein glycosylation, more specifically the highly conserved first steps of the mannosylation pathway (**PAPER 1**) may have implications for cells of higher eukaryotes. Defects in protein glycosylation have been linked to several multisystem human diseases, known as congenital disorders of glycosylation (Chang *et al.* 2018), and glycoproteins are emerging as potential biomarkers for cancer, diabetes, and other complex diseases (Kailemia and Lebrilla 2017). In humans, mutations in genes encoding exosome subunits cause exosomopathies, a recently discovered group of rare diseases which primarily cause neurological defects (Morton *et al.* 2020). Yeast cells are used as a model for exosomopathies because mutations present in patients generally cause growth defects in yeast (Amorim *et al.* 2020). In this context, the finding that the effects of exosome mutations are amplified in W303-derived genetic backgrounds due to the nonfunctional *ssd1-d* allele (**PAPER 2**) should be considered when interpreting results with these models. The RNA exosome is also an important regulator of gene expression in both yeast and human cells and its functions in degrading regulatory non-coding transcripts have been well described (Preker *et al.* 2008, Xu *et al.* 2009, Flynn *et al.* 2011). This work describes the molecular mechanisms through which lncRNAs regulate transcription at specific stress-related yeast gene loci, *PSA1* (**PAPER 1)** and *PHO5* (**PAPER 4**). At both gene loci, the lncRNA is transcribed through the coding gene's promoter region, either in the sense or antisense direction, and has been shown to play a negative *cis*-regulatory role. The *PHO5* gene is an already established model for gene regulation through chromatin remodeling of promoter structure. Therefore, characterizing the effects of antisense

transcription in this model system could be particularly useful to study the interaction between lncRNAs and chromatin. Overall, the results presented in this thesis shed light on the role of lncRNAs and the RNA exosome in the regulation of yeast stress-related genes and thus make a general contribution to the field of gene regulation in eukaryotes.

5. CONCLUSIONS

Overall, the results of this thesis demonstrate the important role of lncRNAs and the RNA exosome complex in regulation of gene expression in eukaryotic cells. Specifically, lncRNA-mediated regulatory mechanisms were demonstrated for certain yeast gene loci encoding proteins with stress-related functions. Consequently, the RNA exosome was shown to be crucial for maintenance of cell wall stability in yeast. The specific conclusions arising from this work are following:

1. The activity of the yeast RNA exosome is important for regulating cell wall stability. Its regulatory role requires the exoribonuclease activity of the catalytic subunit Dis3, a non-catalytic function of the second catalytic subunit Rrp6, and several cofactors of the nuclear exosome. In RNA exosome mutants, the cell wall is destabilized due to dysregulation of genes implicated in protein glycosylation. The temperature-sensitive phenotype of these cells can be rescued by osmotic stabilization of growth media or overexpression of the Psa1 enzyme that catalyzes synthesis of the precursor for protein mannosylation**.**

2. Expression of the *PSA1* gene is negatively regulated by the lncRNA CUT488 which is transcribed at its promoter region and inhibits the recruitment of Pol II. Degradation of CUT488 by the RNA exosome complex is important for proper protein glycosylation and therefore cell wall stability under stress conditions.

3. The temperature-sensitive phenotype of RNA exosome mutants is augmented by inactivation of the *SSD1* allele, which encodes an RNA-binding protein involved in translational regulation of cell wall-related mRNAs, showing that the exosome and Ssd1 maintain cell wall stability through parallel pathways. As W303-derived yeast genetic backgrounds carry the non-functional *ssd1-d* allele, cell wall instability phenotypes of RNA exosome mutants are more pronounced in these strains.

4. Expression of the model *PHO5* gene encoding a periplasmic acid phosphatase is negatively regulated by an antisense lncRNA transcribed over its ORF and promoter regions. Antisense transcription enhances recruitment of the Rpd3 histone deacetylase, leading in turn to decreased recruitment of the chromatin remodeling RSC complex and a more repressive chromatin conformation at the promoter region which is harder to remodel upon gene induction.

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7. *CURRICULUM VITAE*

ANA NOVAČIĆ, mag. ing., was born in Zagreb, 22nd January 1994, where she attended elementary and linguistic XVI grammar school. She graduated from the Bachelor program in Nutrition Science in 2015. In 2017, she graduated from the Master program in Bioindustrial techniques at the University of Orléans (France) and the University of Zagreb, as well as the Master program in Molecular Biotechnology at the Faculty of Food Technology and Biotechnology, University of Zagreb. In the same year, she started to work as an assistant in the Laboratory of Biochemistry and she enrolled in the Postgraduate University Doctoral Study at the Faculty of Food Technology and Biotechnology, University of Zagreb. To date, her research work has resulted in the publication of five papers in highly ranked journals. She participated in many international and national congresses where she presented her research results. During her graduate studies, she received a Rector's award for individual scientific work and was awarded the Biotechnical Foundation Scholarship. During her postgraduate studies, she was awarded the French Government Scholarship, which enabled her to undergo scientific training at Centre de Biophysique Moléculaire, CNRS in Orléans. Furthermore, she received funding from the Foundation of the Croatian Academy of Sciences and Arts for the project "Gaining insight into function and regulation of the RNA exosome complex through functional assays and quantification of non-coding transcripts" for the year 2020/2021 and in 2022 she was awarded the L'Oréal-UNESCO Scholarship "For Women in Science". As a teaching assistant, she participated in undergraduate and graduate courses and assisted in the supervision of several Bachelor and Master theses. She holds the role of the representative of postgraduate students in the Student Council of Faculty of Food Technology and Biotechnology and is a member of the Young Scientists' Forum of the Croatian Society of Biochemistry and Molecular Biology.