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SUMMARY

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 remodeled 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.

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 cells' 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 remodeled 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 signaling 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 non-coding RNAs regulates yeast gene expression and provide arguments in favor 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 are 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' region 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 level. 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 1A) (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 1B) (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, Till *et al.* 2018).

However, another interesting class of ncRNAs can also influence chromatin structure by recruiting chromatin-modifying or -remodeling 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 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 localisation 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 (protein/RNA-binding) regulatory ncRNAs are an emerging critical class of regulatory transcripts in yeast and likely also in multi-cellular 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). *S. 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).

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 signaling pathways (MAPK and cAMP/PKA pathways and Gcn4-controlled signaling) 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 (Robertson and Fink 1998). 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 2A). 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 transcriptional 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 (Grisafi *et al.* 2013). Transcription of these ncRNAs is also influenced by local chromatin structure. Curiously, the histone-deacetylase Rpd3L and 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 2A) (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 2B). 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* gene, 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 3A, 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 doesn't 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* 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 growth in rich medium (YPD) form a stable dsRNA at the *ECM3*/NUT1420 locus (Figure 4A, 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).

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.

CELL WALL-RELATED LOCI ARE ASSOCIATED WITH ANTISENSE lncRNAs

At least 201 genes encode proteins connected to the cell wall structure, its biosynthesis or remodeling 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 focused on antisense transcripts, which overlap sense ORFs and found that 88 of 201 loci (44%) exhibit antisense non-coding transcription (Fig 5A; 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).

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) (Fig 5B). Detailed classification resulted in 5 SUTs, 7 CUTs, 3 XUTs and 3 MUTs. Of note, the numbers of genes exhibiting non-coding transcription doesn't 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 favor 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 mRNA/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 remodeling, 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 remodeling, 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, remodeling 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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BIBLIOGRAPHY

Alcid, E.A. and Tsukiyama, T., 2016. Expansion of antisense lncRNA transcriptomes in budding yeast species since the loss of RNAi. *Nature Structural and Molecular Biology*, 23 (5), 450–455.

Barrales, R.R., Jimenez, J., and Ibeas, J.I., 2008. Identification of novel activation mechanisms for FLO11 regulation in Saccharomyces cerevisiae. *Genetics*, 178 (1), 145–156.

Barrales, R.R., Korber, P., Jimenez, J., and Ibeas, J.I., 2012. Chromatin modulation at the FLO11 promoter of Saccharomyces cerevisiae by HDAC and Swi/Snf complexes. *Genetics*, 191 (3), 791–803.

Becker, E., Com, E., Lavigne, R., Guilleux, M.H., Evrard, B., Pineau, C., and Primig, M., 2017. The protein expression landscape of mitosis and meiosis in diploid budding yeast. *Journal of Proteomics*, 156, 5–19.

Bourdineaud, J.P., Van Der Vaart, J.M., Donzeau, M., De Sampaïo, G., Verrips, C.T., and Lauquin, G.J.M., 1998. Pmt1 mannosyl transferase is involved in cell wall incorporation of several proteins in Saccharomyces cerevisiae. *Molecular Microbiology*, 27 (1), 85–98.

Bumgarner, S.L., Dowell, R.D., Grisafi, P., Gifford, D.K., and Fink, G.R., 2009. Toggle involving cis-interfering noncoding RNAs controls variegated gene expression in yeast. *Proceedings of the National Academy of Sciences of the United States of America*.

Bunina, D., Štefl, M., Huber, F., Khmelinskii, A., Meurer, M., Barry, J.D., Kats, I., Kirrmaier, D., Huber, W., and Knop, M., 2017. Upregulation of SPS100 gene expression by an antisense RNA via a switch of mRNA isoforms with different stabilities. *Nucleic Acids Research*, 45 (19), 11144–11158.

Catala, M., Aksouh, L., and Elela, S.A., 2012. RNA-dependent regulation of the cell wall Stress response. *Nucleic Acids Research*, 40 (15), 7507–7517.

Ceschin, R.M., 2012. Analysis of the regulation of the S. cerevisiae gene PIR3 by non-coding intergenic

transcription (Master thesis). University of Pittsburgh.
Cohen-Zontag, O., Lim, L.Q.J., Dahary, D., Olender, T., Pilpel, Y., and Gerst, J., 2019. Identification of a secretion-enhancing cis regulatory targeting element (SECReTE) involved in mRNA localization and protein synthesis. *PLoS genetics*, 15 (7), e1008248.
Cortés, J.C.G., Curto, M.-Á., Carvalho, V.S.D., Pérez, P., and Ribas, J.C., 2019. The fungal cell wall as a target for the development of new antifungal therapies. *Biotechnology Advances*, 37 (6), 107352.
Davis, C.A. and Ares, M., 2006. Accumulation of unstable promoter-associated transcripts upon loss of the nuclear exosome subunit Rrp6p in Saccharomyces cerevisiae. *Proceedings of the National Academy of Sciences*, 103 (9), 3262–3267.
Van Dijk, E.L., Chen, C.L., Daubenton-Carafa, Y., Gourvennec, S., Kwapisz, M., Roche, V., Bertrand, C., Silvain, M., Legoix-Ne, P., Loeillet, S., Nicolas, A., Thermes, C., and Morillon, A., 2011. XUTs are a class of Xrn1-sensitive antisense regulatory non-coding RNA in yeast. *Nature*, 475 (7354), 114–119.
Donaldson, M.E. and Saville, B.J., 2012. Natural antisense transcripts in fungi. *Molecular Microbiology*, 85 (3), 405–417.
Donzeau, M., Bourdineaud, J.P., and Lauquin, G.J.M., 1996. Regulation by low temperatures and anaerobiosis of a yeast gene specifying a putative GPI-anchored plasma membrane. *Molecular Microbiology*, 20 (2), 449–459.
Ecker, M., Deutzmann, R., Lehle, L., Mrsa, V., and Tanner, W., 2006. Pir proteins of Saccharomyces cerevisiae are attached to ??-1,3-glucan by a new protein-carbohydrate linkage. *Journal of Biological Chemistry*, 281 (17), 11523–11529.
Ellison, M.A., Lederer, A.R., Warner, M.H., Mavrich, T.N., Raupach, E.A., Heisler, L.E., Nislow, C., Lee, M.T., and Arndt, K.M., 2019. The Paf1 Complex Broadly Impacts the Transcriptome of *Saccharomyces cerevisiae*. *Genetics*, genetics.302262.2019.
Enyenihi, A.H. and Saunders, W.S., 2003. Large-Scale Functional Genomic Analysis of Sporulation and Meiosis in Saccharomyces cerevisiae. *Genetics*, 163 (1), 47–54.
Fink, G.R., Rnai, D.P.B., and Link, C., 2014. RNAi in budding yeast, 326 (5952), 544–550.
Friesen, H., Lunz, R., Doyle, S., and Segall, J., 1994. Mutation of the SPS1-encoded protein kinase of Saccharomyces cerevisiae leads to defects in transcription and morphology during spore formation. *Genes and Development*, 8 (18), 2162–2175.
Gagnon-Arsenault, I., Parisé, L., Tremblay, J., and Bourbonnais, Y., 2008. Activation mechanism, functional role and shedding of glycosylphosphatidylinositol-anchored Yps1p at the Saccharomyces cerevisiae cell surface. *Molecular Microbiology*, 69 (4), 982–993.
Granovskaia, M. V., Jensen, L.J., Ritchie, M.E., Toedling, J., Ning, Y., Bork, P., Huber, W., and Steinmetz, L.M., 2010. High-resolution transcription atlas of the mitotic cell cycle in budding yeast. *Genome Biology*.
Grbavac, A., Čanak, I., Stuparević, I., Teparić, R., and Mrša, V., 2017. Proteolytic processing of the Saccharomyces cerevisiae cell wall protein Scw4 regulates its activity and influences its covalent binding to glucan. *Biochimica et Biophysica Acta - Molecular Cell Research*, 1864 (3), 507–515.
Grisafi, P., Oudenaarden, A. Van, and Fink, G.R., 2013. Single-cell analysis reveals that noncoding RNAs contribute to clonal heterogeneity by modulating transcription factor recruitment. *Molecular cell*, 45 (4), 470–482.
De Groot, P.W.J., Ruiz, C., De Vázquez Aldana, C.R., Duenas, E., Cid, V.J., Rey, F. Del, Rodríquez-Peña, J.M., Pérez, P., Andel, A., Caubín, J., Arroyo, J., García, J.C., Gil, C., Molina, M., García, L.J., Nombela, C., and Klis, F.M., 2001. A genomic approach for the identification and classification of genes involved in cell wall formation and its regulation in Saccharomyces cerevisiae. *Comparative and Functional Genomics*, 2 (3), 124–142.
Hainer, S.J., Pruneski, J.A., Mitchell, R.D., Monteverde, R.M., and Martens, J.A., 2011. Intergenic transcription causes repression by directing nucleosome assembly. *Genes and Development*, 25 (1),

29–40.
Halme, A., Bumgarner, S., Styles, C., and Fink, G.R., 2004. Genetic and epigenetic regulation of the FLO gene family generates cell-surface variation in yeast. *Cell*, 116 (3), 405–415.
Ho, B., Baryshnikova, A., and Brown, G.W., 2018. Unification of Protein Abundance Datasets Yields a Quantitative Saccharomyces cerevisiae Proteome. *Cell Systems*, 6 (2), 192-205.e3.
Huber, F., Bunina, D., Gupta, I., Khmelinskii, A., Meurer, M., Theer, P., Steinmetz, L.M., and Knop, M., 2016. Protein Abundance Control by Non-coding Antisense Transcription. *Cell Reports*, 15 (12), 2625–2636.
Hurtado-Guerrero, R., Schüttelkopf, A.W., Mouyna, I., Ibrahim, A.F.M., Shepherd, S., Fontaine, T., Latgé, J.P., and van Aalten, D.M.F., 2009. Molecular mechanisms of yeast cell wall glucan remodeling. *Journal of Biological Chemistry*, 284 (13), 8461–8469.
Jung, U.S. and Levin, D.E., 1999. Genome-wide analysis of gene expression regulated by the yeast cell wall integrity signalling pathway. *Molecular Microbiology*, 34 (5), 1049–1057.
Kitagaki, H., Shimoi, H., Itoh, K., and Shimoi, H., 1997. Identification and analysis of a static culture-specific cell wall protein, Tir1p/Srp1p in Saccharomyces cerevisiae. *European Journal of Biochemistry*, 249 (1), 343–349.
Klis, F.M., Mol, P., Hellingwerf, K., and Brul, S., 2002. Dynamics of cell wall structure in Saccharomyces cerevisiae. *FEMS Microbiology Reviews*.
Lardenois, A., Liu, Y., Walther, T., Chalmel, F., Evrard, B., Granovskaia, M., Chu, A., Davis, R.W., Steinmetz, L.M., and Primig, M., 2011. Execution of the meiotic noncoding RNA expression program and the onset of gametogenesis in yeast require the conserved exosome subunit Rrp6. *Proceedings of the National Academy of Sciences of the United States of America*, 108 (3), 1058–1063.
Law, D.T. and Segall, J., 1988. The SPS100 gene of Saccharomyces cerevisiae is activated late in the sporulation process and contributes to spore wall maturation. *Molecular and Cellular Biology*, 8 (2), 912–922.
Lo, W. and Dranginis, A.M., 1998. The Cell Surface Flocculin Flo11 Is Required for Pseudohyphae Formation and Invasion by Saccharomyces cerevisiae. *Molecular Biology of the Cell*, 9 (January), 161–171.
Lozancic, M., Hossain, S.A., Mrsa, V., and Teparic, R., 2019. Surface Display — An Alternative to Classic Enzyme Immobilization. *Catalysts*, 9 (728).
Lubliner, S., Regev, I., Lotan-Pompan, M., Edelheit, S., Weinberger, A., and Segal, E., 2015. Core promoter sequence in yeast is a major determinant of expression level. *Genome Research*.
Lussier, M., White, A.M., Sheraton, J., Di Paolo, T., Treadwell, J., Southard, S.B., Horenstein, C.I., Chen-Weiner, J., Ram, A.F.J., Kapteyn, J.C., Roemer, T.W., Vo, D.H., Bondoc, D.C., Hall, J., Zhong, W.W., Sdicu, A.M., Davies, J., Klis, F.M., Robbins, P.W., and Bussey, H., 1997. Large scale identification of genes involved in cell surface biosynthesis and architecture in Saccharomyces cerevisiae. *Genetics*, 147 (2), 435–450.
Marguet, D. and Lauquin, G.J., 1986. The yeast SRP gene: positive modulation by glucose of its transcriptional expression. *Biochemical and biophysical research communications*, 138 (1), 297–303.
Mrša, V. and Tanner, W., 1999. Role of NaOH-extractable cell wall proteins Ccw5p, Ccw6p, Ccw7p and Ccw8p (Members of the Pir protein family) in stability of the Saccharomyces cerevisiae cell wall. *Yeast*, 15 (10 A), 813–820.
Nevers, A., Doyen, A., Malabat, C., Néron, B., Kergrohen, T., Jacquier, A., and Badis, G., 2018. Antisense transcriptional interference mediates condition-specific gene repression in budding yeast. *Nucleic Acids Research*.
Nguyen, T.H., Fleet, G.H., and Rogers, P.L., 1998. Composition of the cell walls of several yeast species.

Applied Microbiology and Biotechnology, 50 (2), 206–212.
Niederer, R.O., Hass, E.P., and Zappulla, D.C., 2017. Long noncoding RNAs in the yeast S. Cerevisiae. *In*: *Advances in Experimental Medicine and Biology*. Springer New York LLC, 119–132.
Orlean, P., 2012. Architecture and biosynthesis of the Saccharomyces cerevisiae cell wall. *Genetics*.
Raupach, E.A., Martens, J.A., and Arndt, K.M., 2016. Evidence for Regulation of ECM3 Expression by Methylation of Histone H3 Lysine 4 and Intergenic Transcription in Saccharomyces cerevisiae. *G3: Genes, Genomes, Genetics*, 6 (9), 2971–2981.
Robertson, L.S. and Fink, G.R., 1998. The three yeast A kinases have specific signaling functions in pseudohyphal growth. *Proceedings of the National Academy of Sciences of the United States of America*, 95 (23), 13783–13787.
Rupp, S., Summers, E., Lo, H.-J., Madhani, H., and Fink, G., 1999. MAP kinase and cAMP filamentation signaling pathways converge on the unusually large promoter of the yeast FLO11 gene. *The EMBO Journal*, 18 (5), 1257–1269.
Sanz, A.B., Garcia, R., Rodriguez-Peña, J.M., and Arroyo, J., 2017. The CWI Pathway: Regulation of the Transcriptional Adaptive Response to Cell Wall Stress in Yeast. *Journal of Fungi*, 4 (1), 1.
Sanz, A.B., García, R., Rodríguez-Peña, J.M., Nombela, C., and Arroyo, J., 2018. Slt2 MAPK association with chromatin is required for transcriptional activation of Rlm1 dependent genes upon cell wall stress. *Biochimica et Biophysica Acta - Gene Regulatory Mechanisms*, 1861 (11), 1029–1039.
Shoemaker, D.D., Schadt, E.E., Armour, C.D., He, Y.D., Garrett-Engele, P., McDonagh, P.D., Loerch, P.M., Leonardson, A., Lum, P.Y., Cavet, G., Wu, L.F., Altschuler, S.J., Edwards, S., King, J., Tsang, J.S., Schimmack, G., Schelter, J.M., Koch, J., Ziman, M., Marton, M.J., Li, B., Cundiff, P., Ward, T., Castle, J., Krolewski, M., Meyer, M.R., Mao, M., Burchard, J., Kidd, M.J., Dai, H., Phillips, J.W., Linsley, P.S., Stoughton, R., Scherer, S., and Boguski, M.S., 2001. Experimental annotation of the human genome using microarray technology. *Nature*.
Singh, V., Azad, G.K., Sariki, S.K., and Tomar, R.S., 2015. Flocculation in Saccharomyces cerevisiae is regulated by RNA/DNA helicase Sen1p. *FEBS Letters*.
Spellman, P.T., Sherlock, G., Zhang, M.Q., Iyer, V.R., Andrers, K., Eisen, M.B., Brown, P.O., Botstein, D., and Futcher, B., 1998. Comprehensive Identification of Cell Cycle–regulated Genes of the Yeast Saccharomyces cerevisiae by Microarray Hybridization, 9 (12), 3273–3297.
Tanaka, T. and Kondo, A., 2015. Cell-surface display of enzymes by the yeast Saccharomyces cerevisiae for synthetic biology. *FEMS Yeast Research*, 15 (1), 1–9.
Teparić, R. and Mrša, V., 2013. Proteins involved in building, maintaining and remodeling of yeast cell walls. *Current Genetics*, 59 (4), 171–185.
Till, P., Mach, R.L., and Mach-Aigner, A.R., 2018. A current view on long noncoding RNAs in yeast and filamentous fungi. *Applied Microbiology and Biotechnology*, 102 (17), 7319–7331.
Traven, A., Jänicke, A., Harrison, P., Swaminathan, A., Seemann, T., and Beilharz, T.H., 2012. Transcriptional Profiling of a Yeast Colony Provides New Insight into the Heterogeneity of Multicellular Fungal Communities. *PLoS ONE*, 7 (9).
Wang, L.C., Montalvo-Munoz, F., Tsai, Y.C., Liang, C.Y., Chang, C.C., and Lo, W.S., 2015. The histone acetyltransferase Gcn5 regulates ncRNA- ICR1 and FLO11 expression during pseudohyphal development in Saccharomyces cerevisiae. *BioMed Research International*.
Wery, M., Descrimes, M., Vogt, N., Dallongeville, A.S., Gautheret, D., and Morillon, A., 2016. Nonsense-Mediated Decay Restricts LncRNA Levels in Yeast Unless Blocked by Double-Stranded RNA Structure. *Molecular Cell*, 61 (3), 379–392.
Wheelan, S.J., Martínez Murillo, F., and Boeke, J.D., 2008. The incredible shrinking world of DNA microarrays. *Molecular BioSystems*.
Wilkinson, D., Váchová, L., Hlaváček, O., Maršíková, J., Gilfillan, G.D., and Palková, Z., 2018. Long Noncoding RNAs in Yeast Cells and Differentiated Subpopulations of Yeast Colonies and Biofilms.

Oxidative medicine and cellular longevity, 2018, 4950591.
Winston, F., Martens, J.A., and Wu, P.-Y.J., 2005. Regulation of an intergenic transcript controls adjacent gene transcription in Saccharomyces cerevisiae. *Genes and Development*, 19 (22), 2695–2704.
Wyers, F., Rougemaille, M., Badis, G., Rousselle, J.C., Dufour, M.E., Boulay, J., Régnault, B., Devaux, F., Namane, A., Séraphin, B., Libri, D., and Jacquier, A., 2005. Cryptic Pol II transcripts are degraded by a nuclear quality control pathway involving a new poly(A) polymerase. *Cell*, 121 (5), 725–737.
Xie, B., Becker, E., Stuparevic, I., Wery, M., Szachnowski, U., Morillon, A., and Primig, M., 2019. The anti-cancer drug 5-fluorouracil affects cell cycle regulators and potential regulatory long non-coding RNAs in yeast. *RNA Biology*, 16 (6), 727–741.
Xu, Z., Wei, W., Gagneur, J., Perocchi, F., Clauder-Münster, S., Camblong, J., Guffanti, E., Stutz, F., Huber, W., and Steinmetz, L.M., 2009. Bidirectional promoters generate pervasive transcription in yeast. *Nature*, 457 (7232), 1033–7.

FIGURE LEGENDS

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.

Figure 2. RNA and dsRNA profiling data for *FLO11* (*MUC1*). (A) A color-coded heatmap is shown 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 red lines delineate the target gene. The RNA profiling data were published by van Dijk *et al*. Nature 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 was published by Wery et al., Mol Cell 2015. 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.

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

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 region of cell wall related genes.

Figure 1.

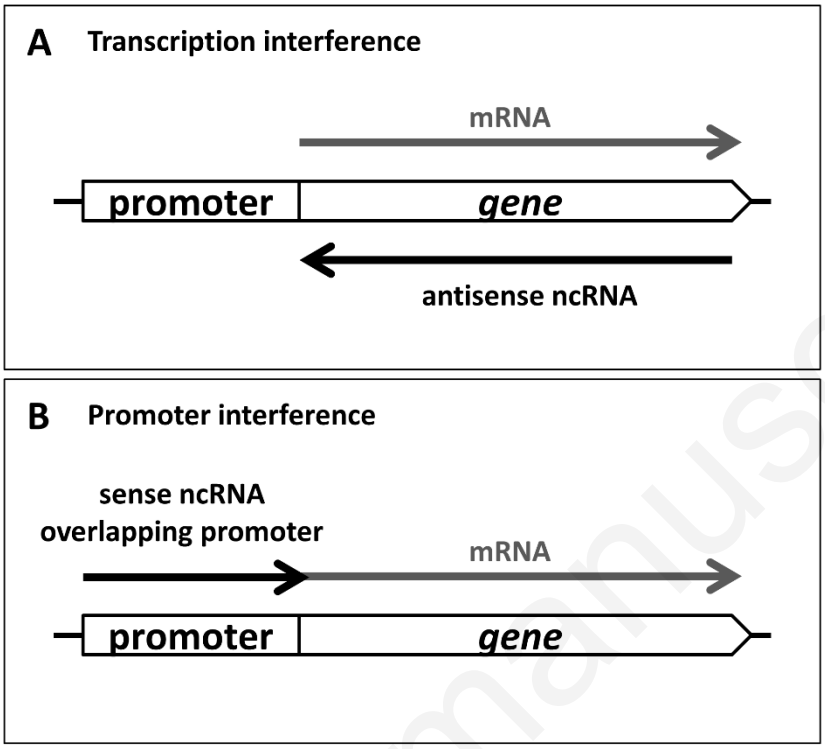

Figure 2.

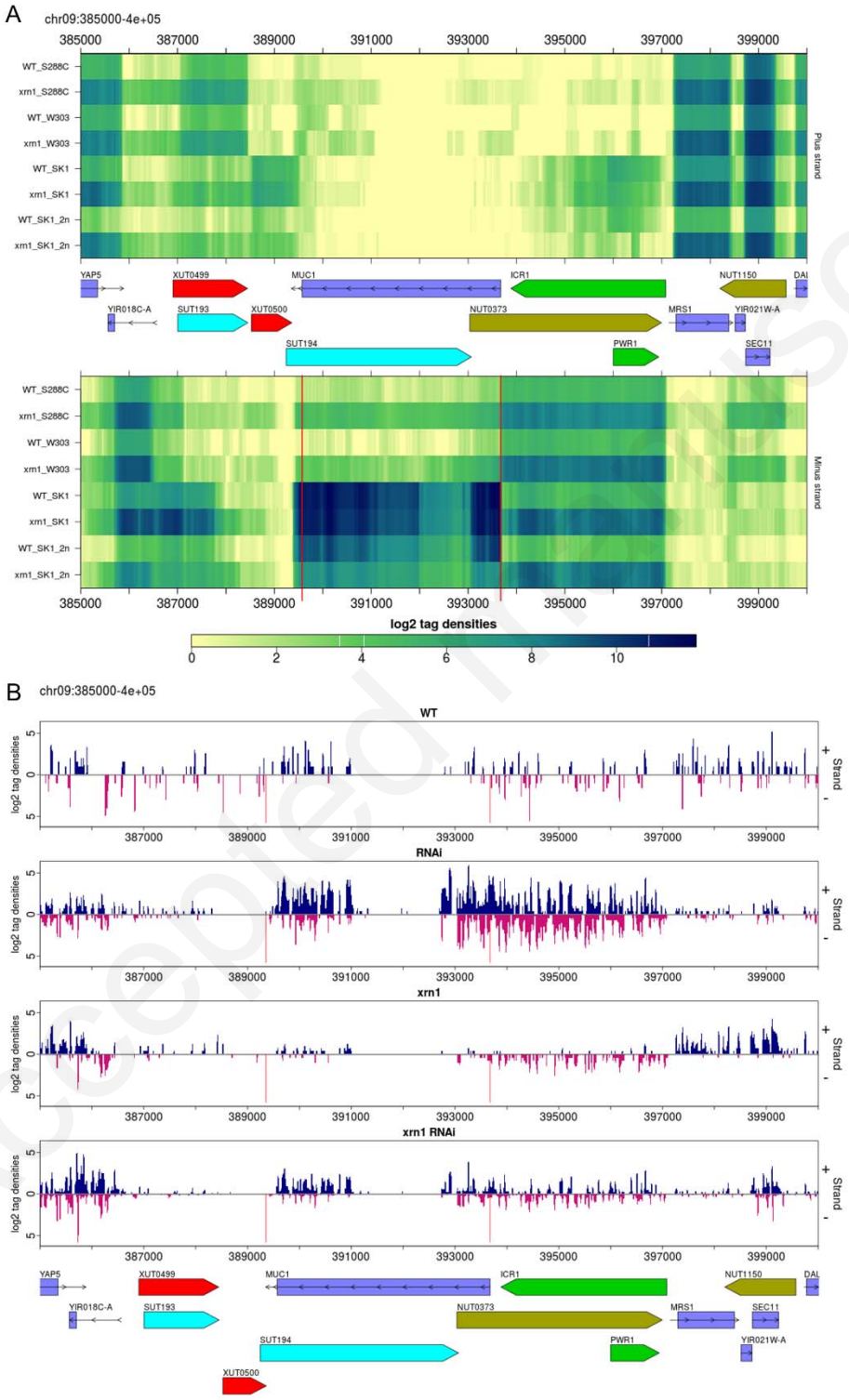

Figure 3.

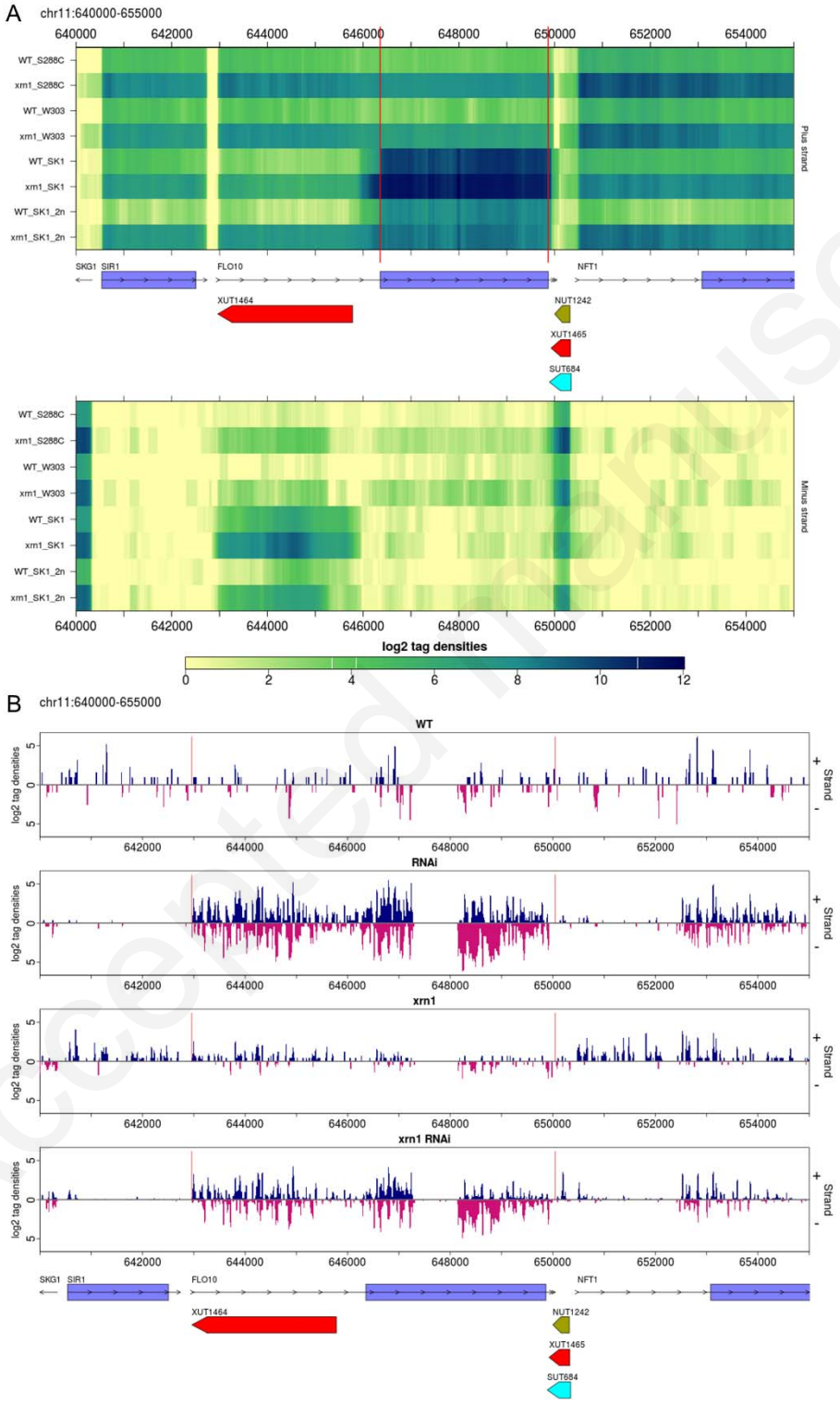

Figure 4.

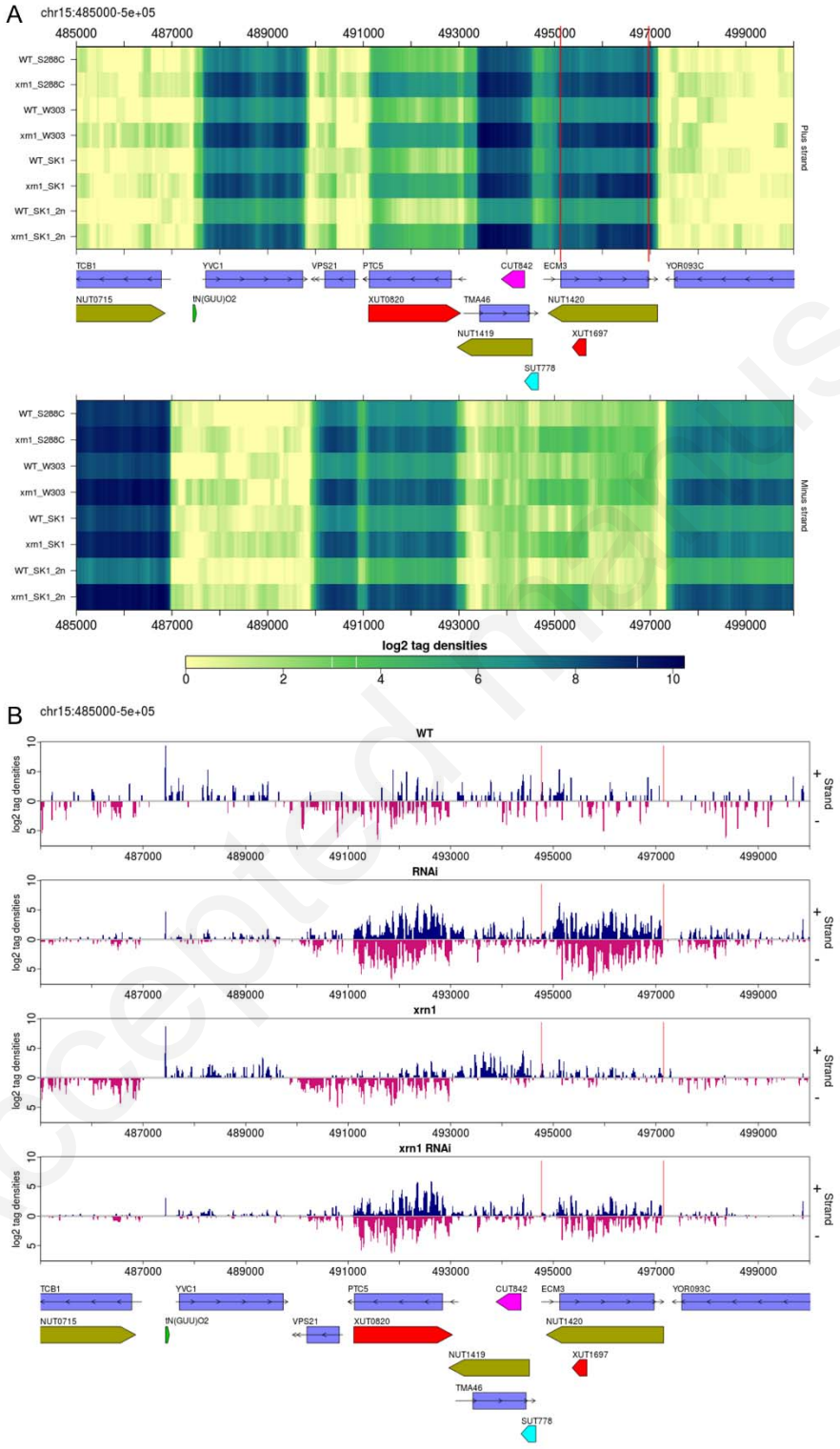

Figure 5.