Antisense IncRNAs expression correlates with attenuation of highly transcribed genes in fission yeast

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ABSTRACT

Antisense (as) lncRNAs can regulate gene expression but whether this occurs at the transcriptional or post-transcriptional level remains unclear. Furthermore, the molecular bases for aslncRNAs-mediated regulation remain incomplete. Here, we report that inactivation of the cytoplasmic exoribonuclease Exo2 results confers sensitivity to oxidative stress in fission yeast. Mechanistic investigations revealed that induction of the catalase-coding gene \textit{ctt1} is impaired in \textit{exo2} \textit{Δ} cells, correlating with the accumulation an Exo2-sensitive lncRNA (XUT), antisense to \textit{ctt1}. Interestingly, expression of the asXUT was also activated in wild-type cells upon oxydative stress, concomitant to \textit{ctt1} induction, indicating a potential attenuation feedback. This attenuation is Dicer-independent, characterized by low RNAPII-ser5 phosphorylation and requires histone deacetylase activity. Using Native Elongating Transcript sequencing in \textit{exo2} \textit{Δ} cells, we revealed asXUT-associated attenuation for a subset of highly transcribed genes displaying prominent promoter-proximal nucleosome depletion and histone acetylation, suggesting that asXUTs attenuate genes whose transcription exceeds a critical threshold. We propose that asXUT could mediate transcriptional regulation via sense-paired gene promoter features using a general conserved mechanism, independent of RNAi.
Eukaryotic genomes are pervasively transcribed (Clark et al, 2011), generating plenty of non-coding (nc) transcripts, distinct from the housekeeping rRNAs, tRNAs and sn(o)RNAs, and that are arbitrarily classified into small (< 200 nt) and long (≥ 200 nt) ncRNAs (Jarroux et al, 2017; Wery et al, 2011).

Long (l)ncRNAs are produced by RNA polymerase II (RNAPII), capped and polyadenylated, yet lack protein-coding potential (Guttman et al, 2009; Khalil et al, 2009), although this last point is subject to exceptions (de Andres-Pablo et al, 2017).

Several lines of evidence suggest that they are functionally important. First, lncRNAs show tissue-specific expression (Djebali et al, 2012) and respond to diverse stimuli, such as oxidative stress (Giannakakis et al, 2015), suggesting that their expression is precisely controlled. Second, several lncRNAs are misregulated in diseases including cancer and neurological disorders (Renganathan & Felley-Bosco, 2017; Saha et al, 2017; Schmitt & Chang, 2016). Furthermore, there is a growing repertoire of cellular processes in which lncRNAs play important roles, including X-chromosome inactivation, imprinting, maintenance of pluripotency and transcriptional regulation (Mercer et al, 2009; Rinn & Chang, 2012).

Several classes of lncRNAs have been described (Jarroux et al, 2017). Among them, large intervening non-coding (linc)RNAs, which result from transcription of intergenic regions, have attracted a lot of attention as being involved in cis- and trans-regulation, mostly at the chromatin level, of genes important for development and cancer (Rinn & Chang, 2012).

Another class of lncRNAs consists in antisense transcripts, that are produced from DNA strand antisense to genes (Pelechano & Steinmetz, 2013). Several examples of regulatory antisense (as)lncRNAs acting on sense gene expression in cis or in trans have been described in the budding yeast Saccharomyces cerevisiae (Berretta et al, 2008; Camblong et al, 2009; Camblong et al, 2007; Houseley et al, 2008; Pinskaya et al, 2009; Uhler et al, 2007; van Werven et al, 2012), in the fission
yeast *Schizosaccharomyces pombe* (Bitton et al, 2011; Leong et al, 2014), in plant (Swiezewski et al, 2009) and in mammalian cells (Lee & Lu, 1999; Yap et al, 2010).

Our previous studies in budding and fission yeasts revealed that aslncRNAs are globally unstable and are mainly targeted by the cytoplasmic 5'-3' RNA decay pathway dependent on the Xrn1 and Exo2 exoribonucleases in *S. cerevisiae* (Van Dijk et al, 2011; Wery et al, 2016) and *S. pombe* (Wery et al, 2017), respectively. Inactivation of Xrn1/Exo2 leads to the stabilization of a family of IncRNAs, referred to as Xrn1-sensititve Unstable Transcripts (XUTs), the majority of which are antisense to protein-coding genes (Van Dijk et al, 2011; Wery et al, 2016; Wery et al, 2017). Interestingly, in *S. cerevisiae*, we defined among these antisense (as)XUTs a subgroup for which the sense-paired genes (referred to as class 1) undergo antisense-mediated transcriptional silencing (Van Dijk et al, 2011). However, the molecular mechanism by which asXUTs could regulate sense gene expression remains largely unknown to date, still interrogating whether sense transcription is impaired at the initiation and/or elongation and/or termination stages, whether any post-transcriptional event is in play, and whether the epigenetic landscape contributes in the regulatory determinants. In addition, such a transcriptional asIncRNA-mediated regulation has not been documented yet in *S. pombe*, where RNAi could potentially contribute to the regulation.

Here we show that the catalase-coding gene *ctt1* in fission yeast is transcriptionally attenuated upon oxidative stress when its paired-asXUT is stabilized. Mechanistic characterization revealed that this process is RNAi-independent, characterized by low RNAPII Ser5-phosphorylation (Ser5-P) and mediated by histone deacetylase (HDAC) activity. Using Native Elongating Transcript sequencing, we identified class 1 as a subset of highly transcribed genes, with particular epigenetic marks, showing transcriptional attenuation upon stabilization of their paired-asXUTs. Our data support a model where asXUTs could modulate expression of their associated sense genes, only when expression exceeds a critical threshold, using a conserved mechanism independent of RNAi.
RESULTS

Exo2-deficient cells are sensitive to hydrogen peroxide and defective for ctt1 induction upon oxidative stress

In a preliminary phenotypical characterization, we observed that in addition to the previously described temperature sensitivity (Szankasi & Smith, 1996), exo2Δ cells were sensitive to H₂O₂ (Figure 1A).

Survival to oxidative stress upon exposure to H₂O₂ requires an enzyme known as catalase (Mutoh et al, 1999), which is encoded by the ctt1 gene in fission yeast and is strongly induced in response to H₂O₂ treatment (Nakagawa et al, 1995). We analyzed ctt1 mRNA induction in WT and exo2Δ cells. Northern-blot (Figure 1B) and RT-qPCR kinetics analyses (Supplementary Figure S1A-B) showed that exo2Δ exhibits a 3-fold reduction in induction rate, with a peak of induction reached 15 minutes after H₂O₂ addition vs 10 for the WT (Supplementary Figure S1A).

Thus, full induction of ctt1 upon addition of H₂O₂ requires Exo2.

Antisense XUT attenuates ctt1 upon stress-mediated induction independently of RNAi

In fission yeast, loss of Exo2 leads to the stabilization of 1638 Xrn1/Exo2-sensitive Unstable Transcripts (XUTs), most of which are antisense to protein-coding gene (Wery et al, 2017). Analysis of the annotation revealed that ctt1 has an antisense (as)XUT, namely XUT0794 (Figure 1C).

We hypothesized that XUT0794 could directly control ctt1 expression or provide substrate for siRNA-mediated silencing. To discriminate between these possibilities, we tested whether ctt1 attenuation requires Dicer. As shown in Figure 1D, ctt1 attenuation was not suppressed in the exo2Δ dcr1Δ double mutant. Conversely, Dicer overexpression in exo2Δ cells had no impact on ctt1 attenuation (Supplementary Figure S1C-E). These data indicate that Dicer is not necessary to the regulation, which is consistent with the observation that asXUTs are globally not targeted by RNAi in S. pombe (Wery et al, 2017).
Strikingly, XUT0794 was also activated upon oxidative stress in a WT context (Figure 1E). Furthermore, its peak of induction was reached very rapidly (5 min), before the ctt1 mRNA peak (10 min), suggesting that it might be part of a natural attenuation mechanism (feedback loop) for ctt1 expression.

In summary, our data suggest that ctt1 induction requires Exo2 activity for maintaining a low level of a XUT antisense to ctt1. Upon induction, the XUT is activated and could modulate expression of ctt1, in a similar way as shown for asXUT-associated genes in S. cerevisiae, such as GAL1-10 (Houseley et al, 2008; Pinskaya et al, 2009).

Efficiency of ctt1 attenuation correlates with antisense XUT0794 abundance

In order to test whether ctt1 attenuation directly depends on antisense XUT0794 and is not an indirect effect of exo2Δ, we overexpressed the XUT in cis, in WT cells, using a regulatable P41nmt1 promoter (Supplementary Figure 2A). Upon promoter activation, XUT0794 accumulated and ctt1 was not induced in response to H2O2 (Supplementary Figure 2A). However, in this context, ctt1 silencing might result from transcriptional interference. Note that expression of the XUT in trans had no effect on ctt1 induction (Supplementary Figure 2B).

We also tried to disrupt XUT0794 promoter using a kanR marker. Unexpectedly, this failed to repress XUT0794. Instead, ctt1-kanR cells accumulated XUT0794 in absence of H2O2 more than exo2Δ cells, and ctt1 mRNA was attenuated upon oxidative stress (Supplementary Figure 2C).

As a third strategy, we inserted a self-cleaving hammerhead ribozyme (RZ) at position 254/815 of XUT0794 (Figure 2A) and integrated the construct at the ctt1 locus in WT and exo2Δ strains, without any manipulation of XUT0794 promoter. In the WT + RZ context, neither the 5’ nor the 3’ fragment of XUT0794 accumulated (Figure 2B), and ctt1 induction was similar to WT cells (Figure 2C). In the exo2Δ + RZ context, the 5’ fragment was not detected, but the 3’ fragment accumulated 5x more than in exo2Δ without RZ (Figure 2B). This imbalance between the two RNA parts indicates that RZ was efficiently cleaved, the 5’ fragment being presumably degraded (Khvorova
et al, 2003) while the 3’ fragment accumulated. Importantly, the higher abundance of the 255-815 fragment of XUT0794 in exo2Δ + RZ cells compared to exo2Δ without RZ correlated with a significantly stronger attenuation of ctt1 (Figure 2C).

We conclude that the 255-815 fragment of XUT0794 is sufficient to attenuate ctt1 and that the efficiency of attenuation depends on the abundance of the antisense XUT, which is consistent with our hypothesis that the regulation is mediated by the RNA itself and is not an indirect effect of Exo2 inactivation.

XUT-mediated transcriptional attenuation of ctt1 is characterized by partial RNAPII Ser5 phosphorylation

To determine whether ctt1 attenuation occurs at the transcriptional level, we performed RNAPII ChIP experiments in WT and exo2Δ cells. In oxidative stress conditions, RNAPII occupancy in the mutant showed a significant 2- to 4-fold decrease along the ctt1 locus (Figure 3A-B), indicating that the attenuation is transcriptional.

Analysis of the distribution of differentially phosphorylated forms of RNAPII largest subunit CTD provided further insights into the mechanism of transcriptional attenuation. Ser5-P is associated to initiation of transcription and predominates in the promoter-proximal region of the gene, while Ser2-P is associated to transcription elongation and increases along the gene core (Drogat & Hermand, 2012). Upon oxidative stress, we observed a 30% decrease of Ser5-P RNAPII in the 5’ and core regions of ctt1, in the exo2Δ mutant (Figure 3C). In contrast, Ser2-P RNAPII occupancy was similar in the two strains (Figure 3D).

In summary, stabilization of XUT0794 impairs the early stage of ctt1 transcription, with less RNAPII loaded on the gene in response to oxidative stress and an additional reduced level of Ser5-P.

XUT-mediated attenuation of ctt1 depends on histone deacetylation
Several studies in budding yeast have pointed out the role of HDAC, including the class II HDAC Hda1, in antisense lncRNA-mediated gene silencing (Berretta et al., 2008; Camblong et al., 2007; Houseley et al., 2008). To test whether asXUT-mediated attenuation of ctt1 depends on HDAC activity, WT and exo2Δ cells were treated with trichostatin A (TSA), an inhibitor of class I-II HDAC. When exposed to oxidative stress, TSA-treated exo2Δ cells accumulated ctt1 mRNA to the same level as the control (DMSO-treated) WT strain (Figure 4A). We also noted that the uninduced level of ctt1 mRNA was increased in the TSA-treated WT and exo2Δ cells, indicating that ctt1 repression requires an HDAC activity. Furthermore, both ctt1 mRNA and XUT0794 levels in TSA-treated WT cells showed a 2-fold increase compared to the DMSO-treated control after H2O2 addition (Figure 4A-B). This indicates that the XUT0794-depending feedback loop that should modulate ctt1 expression in WT cells upon exposure to H2O2 is impaired when HDAC activity is inhibited.

Based on this observation, we predicted histone acetylation along ctt1 to be affected upon XUT0794 stabilization. ChIP experiments in ctt1 induction conditions revealed a significant 50% and 30% reduction of histone H4K5/8/12/16 acetylation and H3K14 acetylation, respectively, in the exo2Δ mutant, in the region where ctt1 gene and XUT0794 overlap (Figure 4C-D, probe C). These data support the idea that XUT-mediated gene attenuation depends on HDAC, resulting in reduced levels of histone acetylation.

In an attempt to identify the HDAC involved, we tested the effect of Clr3 (the ortholog of Hda1), the class I HDAC Hos2 and the ING family protein Png2, a non-essential subunit of the Clr6 complex I (Nicolas et al., 2007). Attenuation of ctt1 was not suppressed in the exo2Δ clr3Δ, exo2Δ hos2Δ and exo2Δ png2Δ mutants (Supplementary Figure 4A-C), indicating that none of the three tested factors is involved in the attenuation mechanism. The png2Δ single mutant exhibited a strong defect of ctt1 induction and was synergic with exo2Δ (Supplementary Figure 4C), suggesting that both Exo2 and Png2 are required for efficient ctt1 induction but act independently.

In conclusion, XUT-mediated attenuation of ctt1 requires a HDAC activity, suggesting that mechanisms of regulation of gene expression by lncRNAs have been conserved across the yeast
clade, cryptic antisense lncRNAs attenuating expression of their paired-sense gene in a HDAC-dependent manner.

**Antisense XUT-associated transcriptional gene attenuation in fission yeast**

The data above show that *ctt1* is attenuated when its paired-asXUT is stabilized in *exo2Δ* cells, and the attenuation occurs at the level of transcription. We ask whether other genes are transcriptionally attenuated upon stabilization of their paired-asXUT. In *S. cerevisiae*, such genes have been previously identified using RNAPII ChIP-Seq, constituting the so-called ‘class 1’ (Van Dijk et al, 2011).

To identify class 1 genes in *S. pombe*, here we performed NET-Seq analysis in WT and *exo2Δ* cells. Although global mRNA synthesis was found to be unchanged upon *exo2* inactivation (Supplementary Figure S5A), differential expression analysis discriminated genes for which transcription in *exo2Δ* was significantly reduced (classes 1 & 2, n=723) or not (classes 3 & 4, n=4405). Within each category, we distinguished genes with (classes 1 & 3) or without (classes 2 & 4) asXUTs (Figure 5A-B; lists in Tables S1-4).

175 of the 723 genes transcriptionally attenuated in *exo2Δ* have asXUTs (class 1). Despite the proportion of class 1 genes among the attenuated genes is limited (24.2%), it is significantly higher than expected if presence of asXUT and sense gene attenuation were independent (Chi-square test, \( P = 0.03 \)), suggesting that the attenuation depends on the stabilized asXUTs, at least in some cases. On the other hand, the transcriptional down-regulation of class 2 (no asXUT) is likely to be an indirect effect reflecting the slow growth phenotype of the *exo2Δ* mutant (Szankasi & Smith, 1996). This hypothesis is supported by the observation that class 2 is significantly enriched for GO terms “ribosome biogenesis” (\( P=1.36e^{-08} \)) and “cellular component biogenesis (\( P= 1.04e^{-02} \)), the expression levels of genes involved such biological processes directly depending on the growth rate (Kief & Warner, 1981). Altogether, these observations suggest that for a subgroup of genes, stabilization of the asXUT might contribute to attenuate transcription of the paired-sense gene.
Both classes 1 and 3 have asXUTs, but only class 1 is transcriptionally attenuated upon asXUTs stabilization. This suggests the existence of specificities discriminating the two classes. Indeed, in WT cells, class 1 is transcribed to higher levels than class 3 (Figure 5C), the latter actually showing the lowest transcription levels among the four classes (Supplementary Figure SSB). In exo2Δ, transcription of class 1 falls to the low, basal level of class 3 (Figure 5D). Notably, transcription of XUTs antisense to class 1 and 3 genes is similar in the WT and exo2Δ conditions (Supplementary Figure SSC), indicating that XUTs accumulation in exo2Δ is due to the inactivation of their decay and not to a global increase of their synthesis (Figure 5E).

Remarkably, in WT cells, the nascent antisense transcription signal surrounding the TSS of class 1 genes is higher than for class 3 (Figure 5F; see also Supplementary Figure 5D). This suggests that sense TSS overlap also constitutes a key factor for the potential regulatory activity of the XUTs antisense to class 1 genes.

At the chromatin level, class 1 shows a more pronounced nucleosome depletion in the TSS-proximal region than class 3 (Figure 6A), higher H3K14 (Figure 6B) and H4K5/8/12/16 acetylation (Figure 6C). Notably, when compared to the four classes, levels of histone acetylation at the TSS-proximal region were similar in classes 1 and 2 (Figure 6B-C).

Together, these results show that transcriptional attenuation could be associated with asXUT stabilization in fission yeast, suggesting that asXUT-mediated gene regulation is functionally conserved. We defined class 1 as highly transcribed genes, characterized by high promoter-proximal antisense nascent transcription and high histone acetylation levels. Upon asXUT stabilization, class 1 transcription decreases to basal levels, suggesting that asXUTs might be involved in the modulation of sense genes expression.
DISCUSSION

In this report, we show that induction of the catalase-coding gene ctt1 in response to oxidative stress is impaired in cells inactivated for the Exo2-dependent 5’-3’ cytoplasmic RNA decay pathway. This attenuation of ctt1 correlates with the accumulation of an unstable Exo2-sensitive aslncRNA (XUT0794). Interestingly, antisense XUT0794 also rapidly accumulated in WT cells after H$_2$O$_2$ addition, suggesting that it could participate in the modulation of ctt1 induction. In this respect, a recent study in human fibroblasts has identified a class of stress-induced asIncRNAs, which are activated upon oxidative stress (Giannakakis et al, 2015), suggesting that aslncRNAs induction might be part of a conserved response to oxidative stress in Eukaryotes.

Our data indicate that asXUT-associated regulation of ctt1 (i) is independent of RNAi, which is consistent with the observations that asXUTs are not targeted by Dicer in fission yeast (Wery et al, 2017), (ii) occurs at the early transcriptional level, (iii) and is mediated by HDAC activity. Our attempts to identify the HDAC involved were unsuccessful, probably due to redundancy of HDAC activities. On the other hand, we could not test the role of the Set2 histone-methyltransferases due to lethality of the exo2Δ set2Δ mutant in our hands. Further mutational strategies would be required to address this question, as alternative models propose that it is antisense transcription itself, rather than the antisense IncRNA, that promotes histone deacetylation via Set2-mediated H3K36 methylation, which is coupled to RNAPII transcription and recruits HDAC complexes (Venkatesh & Workman, 2013). However, the observation at the genome-wide level that class 1 genes are attenuated upon accumulation of the associated antisense IncRNAs, while antisense transcription is unchanged, suggests that in this case, this is the RNA itself, rather than antisense transcription, that is important for the HDAC-mediated gene attenuation. Future mechanistic studies will be required to confirm this hypothesis. This will necessitate the implementation in fission yeast of techniques developed in S. cerevisiae to strand-specifically block aslncRNA synthesis (Huber et al, 2016; Lenstra et al, 2015), which remains technically challenging to date. For instance, a recent report revealed that
at some loci, the CRISPR interference approach is not strand-specific and results in the production of novel isoforms of the targeted aslncRNA (Howe et al, 2017).

Using NET-Seq in exo2Δ cells, we defined class 1 in S. pombe, ie those genes showing transcriptional attenuation upon stabilization of their paired-asXUT. Importantly, asXUT presence and sense gene attenuation in exo2Δ are not independent, supporting the idea that the regulation is mediated by the stabilized asXUTs and is not a side effect of Exo2 inactivation. Additional mechanistic analyses are required to confirm this hypothesis.

In our recent report, we showed show that genes with asXUT are globally less transcribed that genes without asXUT and that histone acetylation was reduced at their promoter but increased along the gene body (Wery et al, 2017). Here we show that genes with asXUT can be separated into two distinct classes, namely class 1 and class 3, according to their transcriptional attenuation or not upon stabilization of their paired-asXUT, respectively. Class 1 is highly transcribed and shows strong nucleosome depletion and high levels of histone acetylation at the promoter. In addition, class 1 displays high TSS-proximal antisense transcription, pointing the TSS region as a possible determinant for aslncRNA-mediated regulation. In contrast, class 3 is weakly transcribed, with poor promoter-proximal nucleosome depletion and low histone acetylation. Upon stabilization of asXUTs, transcription of class 1 drops down to the basal levels of class 3. This suggests the existence of a regulatory threshold, ie asXUTs would modulate expression of their associated sense genes, only if expression is above this threshold (Figure 7). This contrasts with a previous model based on the analysis of sense-antisense RNA levels in budding yeast, which proposed that antisense-mediated repression would be restricted to low sense expression, with no effect on highly expressed genes (Xu et al, 2011).

Our model suggests that at least a subset of asXUTs could regulate gene expression at the transcriptional level, reducing sense transcription, as shown previously in S. cerevisiae (Berretta et al, 2008; Van Dijk et al, 2011). In addition, XUTs could also act at other steps of the gene expression process, especially at the post-transcriptional level. In this regard, aslncRNAs have been shown to
modulate protein production in response to osmotic stress in *S. pombe* (Leong et al, 2014). In *S. cerevisiae*, a recent study showed that disruption of several aslncRNAs results into increased protein synthesis from their paired-sense mRNAs, indicating a role of these aslncRNAs in the control of protein abundance (Huber et al, 2016). Future investigations will be required to demonstrate the regulatory potential of asXUTs and to determine the step(s) of the gene expression process they act on.

In conclusion, our works in budding and fission yeasts show that the cytoplasmic 5′-end RNA decay plays a key role in controlling aslncRNAs endowed with a regulatory potential. Given the high conservation of Xrn1 in Eukaryotes, it is tempting to speculate that asXUTs and their regulatory activity are conserved in higher eukaryotes, contributing in buffering genome expression, and adding another layer to the complexity of gene regulation.
MATERIALS & METHODS

Yeast strains, plasmids and media

All the strains used in this study are listed in Supplementary Table S5. Mutant strains were constructed by meiotic cross or transformation, and verified by PCR on genomic DNA. Plasmid pAM353 for expression of XUT0794 in trans was constructed by cloning XUT0794 in the Sall site of pREP41 (ars1 LEU2 P41nmt1). Sanger sequencing confirmed the correct orientation of the insert and the absence of mutation. Hammerhead ribozyme (Libri et al, 2002) was inserted in XUT0794 by two-step PCR, giving a 3.2 Kb final product corresponding to ctt1 mRNA coordinates +/- 500 bp that was cloned in pREP41. After verification of absence of additional mutations by Sanger sequencing, the ribozyme-containing construct was excised and transformed in the YAM2534 strain (ctt1::ura4). Transformants were selected on 5-FOA plates and analyzed by PCR on genomic DNA. Deletion of exo2 was performed subsequently.

Strains were grown at 32°C to mid-log phase (OD_{595} 0.5) in YES or EMM-L medium. For ctt1 induction, 1 mM H$_2$O$_2$ was added for 15 minutes (Calvo et al, 2012), or different time points for analysis of kinetics of induction. Expression from P41nmt1 was repressed by growing cells in EMM-L + 15 µM thiamine for 24 hours.

NET-Seq

NET-Seq libraries were constructed from biological duplicates of YAM2507 (exo2Δ rpb3-flag) cells and sequenced as previously described (Wery et al, 2017). Libraries for the WT strain YAM2492 (rpb3-flag) were described in the same previous report (Wery et al, 2017).

After removal of the 5'-adapter sequence, reads were uniquely mapped to the reference genome (ASM294v2.30) using version 0.12.8 of Bowtie (Langmead et al, 2009), with a tolerance of 2 mismatches.
Differential analysis was performed between the IP samples from WT and \textit{exo2Δ} using DESeq (Anders & Huber, 2010). Genes showing significant decrease (\(P\)-value <0.05, adjusted for multiple testing with the Benjamini-Hochberg procedure) in the mutant were defined as class 1 & 2.

\textit{Total RNA extraction}

Total RNA was extracted from exponentially growing cells using standard hot phenol procedure, resuspended in nuclease-free H\textsubscript{2}O (Ambion) and quantified using a NanoDrop 2000c spectrophotometer.

\textit{Northern blot}

10 µg of total RNA were loaded on denaturing 1.2% agarose gel and transferred to Hybond\textsuperscript{TM}-XL nylon membrane (GE Healthcare). \textit{ctt1} mRNA and \textit{U3B} were detected using AMO2063 and AMO2081 oligonucleotides, respectively (see Supplementary Table S6). \(^{32}\text{P}\)-labelled probes were hybridized overnight at 42°C in ULTRAhyb\textsuperscript{®}-Oligo hybridization buffer (Ambion). Quantitation used a Typhoon Trio PhosphorImager and the ImageQuant TL v5.2 sofware (GE Healthcare).

\textit{Strand-specific RT-qPCR}

Strand-specific reverse transcription (RT) reactions were performed from at least three biological replicates (unless specified), using 1 µg of total RNA and the SuperScript\textsuperscript{®}II Reverse Transcriptase kit (Invitrogen), in the presence of 6.25 µg/ml actinomycin D. For each sample, a control without RT was included. Subsequent quantitative real-time PCR were performed on technical duplicates, using a LightCycler\textsuperscript{®} 480 instrument (Roche). Oligonucleotides used are listed in Supplementary Table S6.

\textit{ChIP}
ChIP analysis was performed from three biological replicates of strains YAM2400 (WT) and YAM2402 (exo2Δ) of *S. pombe*. Exponentially growing (OD₅₉₅ 0.5) cells were fixed for 10 minutes at room temperature using formaldehyde (1% final concentration), then glycine was added (0.4 M final concentration). Antibodies used were 8WG16 (Covance) for RNAPII, H14 (Covance) for RNAPII S5-Pho, 3E10 (Millipore) for RNAPII S2-Pho, ab1791 (Abcam) for histone H3, 05-1355 (Millipore) for acetyl-H4 (Lys5/8/12/16) and 07-353 (Millipore) for acetyl-H3 (Lys14). Quantitative real-time PCR were performed in technical duplicates on a StepOnePlus™ machine (Applied Biosystems) and a LightCycler® 480 instrument (Roche). Oligonucleotides used are listed in Supplementary Table 6.
**ACCESSION NUMBERS**

Raw sequences have been deposited to the NCBI Gene Expression Omnibus (accession number GEO: GSE106649).

A genome browser for visualization of NET-Seq processed data is accessible at [http://vm-gb.curie.fr/mw3](http://vm-gb.curie.fr/mw3).

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**AUTHOR CONTRIBUTIONS**

MW & AM conceived and designed the study. MW, MY, DH and VM performed experiments. MW, CG, MD & AM designed NGS data analysis. CG and MD performed bioinformatics analysis. MW & AM wrote the article. AM supervised the project.

**CONFLICT OF INTEREST**

The authors declare that they have no competing interests.
FIGURE LEGENDS

Figure 1. Attenuation of ctt1 induction in exo2Δ cells upon oxidative stress correlates with antisense XUT0794 accumulation.

A. Loss of Exo2 confers sensitivity to hydrogen peroxide. Serial 1:10 dilutions of YAM2400 (WT) and YAM2402 (exo2Δ) cells were dropped on solid rich medium (YES) containing or not 1 mM or 2 mM H₂O₂. Plates were incubated at the indicated temperature for 3-4 days.

B. Northern blot analysis of ctt1 mRNA induction in WT and exo2Δ cells. YAM2400 (WT) and YAM2402 (exo2Δ) cells were grown to mid-log phase in rich medium and collected before or after addition of 1 mM H₂O₂ for 15 min. ctt1 mRNA and U3B snoRNA were detected from total RNA using ³²P-labelled oligonucleotides. Numbers represent the ctt1 mRNA/U3B ratio (ND: not determined).

C. Snapshot of RNA-Seq signals along ctt1 in WT and exo2Δ. RNA-Seq data were previously published (Wery et al, 2017). Signal for the + and - strands are visualized as heatmaps in the upper and lower panels, respectively, using VING (Descrimes et al, 2015). ctt1 mRNA and antisense XUT0794 are represented by blue and red arrows, respectively.

D. Strand-specific RT-qPCR analysis of ctt1 mRNA induction in WT, exo2Δ, dcr1Δ and exo2Δ dcr1Δ cells. Strains YAM2400 (WT), YAM2402 (exo2Δ), YAM2406 (dcr1Δ) and YAM2404 (exo2Δ dcr1Δ) were grown as described above. ctt1 mRNA level was determined by strand-specific RT-qPCR from total RNA and normalized on the level of U3B snoRNA. Data are presented as mean +/- standard deviation (SD), calculated from three biological replicates. * p < 0.05; ** p < 0.01; *** p < 0.001 upon t-test.

E. Strand-specific RT-qPCR analysis of ctt1 mRNA and XUT0794 induction in WT cells upon H₂O₂ treatment. YAM2400 cells grown in rich medium to mid-log phase were collected 0, 2, 5, 10, 15, 30, 45 and 60 min after addition of H₂O₂. ctt1 mRNA and XUT0794 were quantified as above. Relative level of each transcript in the non-induced condition (T0) was set to 1.

Figure 2. Level of ctt1 attenuation correlates with XUT0794 abundance.
A. Schematic representation of hammerhead ribozyme (RZ) inserted within \textit{XUT0794}. Self-cleaving RZ was inserted at position 254 of \textit{XUT0794}. Position of qPCR amplicons C and D is indicated.

B. Analysis of \textit{XUT0794} upstream from and downstream to RZ insertion site. YAM2400 (WT), YAM2402 (exo2Δ), YAM2565 (WT + RZ) and YAM2567 (exo2Δ + RZ) were grown as described in Figure 1B. Strand-specific RT on \textit{XUT0794} was performed from total RNA using oligonucleotide AMO2069. Oligonucleotides AMO2535-6 (amplicon D) and AMO2069-70 (amplicon C) were used for qPCR detection of \textit{XUT0794} 5’ and 3’ fragment, respectively. Data were normalized on U3B snoRNA. For each amplicon, the normalized level of \textit{XUT0794} in exo2Δ was then set to 1. Results are presented as mean +/- SD, from four biological replicates.

C. Analysis of \textit{ctt1} mRNA levels. Strains and cultures were as above; strand-specific RT on \textit{ctt1} mRNA was performed using oligonucleotide AMO2535; qPCR detection was performed using AMO2535-6 (amplicon D). Data were normalized on U3B levels and are presented as mean +/- SD, from four biological replicates. **p<0.01; ***p<0.001; ns, not significant upon t-test.

**Figure 3. asXUT-mediated attenuation of \textit{ctt1} is transcriptional.**

A. Schematic map of the \textit{ctt1} locus, with positions of the qPCR oligonucleotide pairs.

B. ChIP analysis of RNAPII occupancy along \textit{ctt1}. Strains YAM2400 (WT) and YAM2402 (exo2Δ) were grown as in Figure 1B. After cross-linking, chromatin extraction and sonication, RNAPII was immunoprecipitated using antibody against the CTD of its largest subunit Rpb1. Co-precipitated DNA was purified and quantified by qPCR. Data were normalized on the \textit{act1} signal, which is not controlled to an asXUT (see Supplementary Figure 3). The dashed line indicates the background signal observed on an intergenic region of chromosome I used as negative control. Data are presented as mean +/- SD, from three biological replicates. *p<0.05; ***p<0.001; ns, not significant upon t-test.

C-D. ChIP analysis of Ser5-P and Ser2-P occupancy along \textit{ctt1}. RNAPII was immunoprecipitated from the same chromatin extracts as above, using antibody against the Ser5-P (C) or the Ser2-P (D) form of
Rpb1 CTD. Data normalization was as above. For each position of ctt1, the ratio between Ser5-P or Ser2-P and total RNAPII is shown. Data are presented as above. *p<0.05; **p<0.01; ***p<0.001; ns, not significant upon t-test.

Figure 4. asXUT-mediated attenuation of ctt1 depends on HDAC.

A-B. Strand-specific RT-qPCR analysis of asXUT-mediated attenuation of ctt1 mRNA in the presence of HDAC inhibitor. Strains YAM2400 (WT) and YAM2402 (exo2Δ) were grown in rich medium to mid-log phase before addition of 40 µg/ml TSA or equivalent volume of DMSO for 2 hours. TSA-treated and control cells were then collected prior or after addition of H2O2 for 15 min. ctt1 mRNA (A) and XUT0794 (B) were quantified as described above. Data are presented as mean +/- SD, from three biological replicates.

C-D. ChIP analysis of H4K5/8/12/16 (C) and H3K14 (D) acetylation along ctt1. Culture, cross-linking and chromatin extraction were as described in Figure 3B. For each position, data were first normalized on act1, then on the level of histone H3, immunoprecipitated from the same chromatin. Probe dg is specific for the centromeric dg elements. Data are presented as mean +/- SD, from three biological replicates. *p<0.05; **p<0.01; ***p<0.001; ns, not significant upon t-test.

Figure 5. Antisense XUT stabilization induces transcriptional attenuation of a class of highly expressed genes.

A. Transcriptional attenuation in exo2Δ cells. NET-Seq analysis was performed in biological duplicates of WT and exo2Δ cells. Data for the WT strain were previously described (Wery et al, 2017). After sequencing, differential analysis discriminated genes showing significant (P<0.05) reduction of transcription (classes 1-2) or not (classes 3-4). Among them, classes 1 and 3 have asXUT. The number of genes for each class is indicated.

B. Box-plot of nascent transcription (NET-Seq) signal for class 1-4 genes in WT (dark grey boxes) and exo2Δ (Δ; light grey boxes).
C. Metagene view of NET-Seq signals along class 1 and 3 genes in WT cells. For each class, normalized coverage (tag/nt, log₂) along mRNA transcription start site (TSS) +/- 1000 nt (+ strand) and the antisense (as) strand were piled up, in a strand-specific manner. Average signal for the sense and antisense strands was plotted for class 1 (red) and class 3 (green). The shading surrounding each line denotes the 95% confidence interval.

D. Same as above in exo2Δ cells.

E. Density-plot showing the global NET-Seq (dashed lines) and total RNA-Seq (solid lines) signals for XUTs in the WT (black) and exo2Δ (pink) strains. Total RNA-Seq data were previously described (Wery et al, 2017).

F. Metagene view of nascent antisense transcription (NET-Seq) signal around the sense gene TSS of class 1 (red) and 3 (green) genes, in WT cells. The shading surrounding each line denotes the 95% confidence interval.

Figure 6. Class 1 genes show prominent promoter-proximal nucleosome depletion and histone acetylation in WT cells.

A. Metagene of H3 levels for class 1-4 genes in WT cells. The analysis was performed using previously published ChIP-Seq data (Wery et al, 2017). Metagene representation of signal for class 1 (red), class 2 (blue), class 3 (green) and class 4 (black) was performed as above, in a strand-unspecific manner. The shading surrounding each line denotes the 95% confidence interval.

B. Metagene view of H3K14 acetylation for class 1-4 genes in WT cells. ChIP-Seq libraries construction and sequencing were previously described (Wery et al, 2017). Metagene representation of signal for each class of genes was performed as above, using ratio of coverage (log₂) for H3K14ac and H3.

C. Same as above for H4K5/8/12/16ac (H4ac).

Figure 7. Regulatory threshold model.
Antisense XUT can attenuate highly transcribed genes displaying prominent promoter-proximal nucleosome depletion and high levels of histone acetylation (class 1), but not genes showing low/basal transcription, with poor nucleosome depletion and low histone acetylation (class 3). See main text for details.
SUPPLEMENTARY FIGURE LEGENDS

Supplementary Figure S1. ctt1 mRNA induction in WT and exo2Δ cells does not depend on Dicer.

A. YAM2400 (WT) and YAM2402 (exo2Δ) cells grown in rich YES medium to mid-log phase were collected 0, 2, 5, 10, 15, 30 and 45 minutes after addition of 1 mM H2O2. ctt1 mRNA was quantified from total RNA using strand-specific RT-qPCR and normalized on the level of the U3B snoRNA. Average values and standard deviations were calculated from two biological replicates.

B. T0, 2, 5 10 time points from the figure above were plotted separately and used to calculate linear trend lines. Equation and R-squared coefficient are indicated for each trend line.

C-E. WT cells with pREP-nmt1/LEU2 empty vector (pDM829, vector), and exo2Δ cells with pREP-nmt1/LEU2, pREPFLAG-Dcr1 (pDM914, Dcr1) or pREPFLAG-Dcr1-D837A,D1127A (pDM941, Dcr1*) plasmids (Yu et al, 2014) were grown to mid-log phase in EMM-Leu medium, before addition of H2O2 for 15 min. ctt1 mRNA (C), XUT0794 (D) and dcr1 mRNA (E) were quantified from total RNA using strand-specific RT-qPCR and normalized on the level of the U3B snoRNA. Average values and standard deviations were calculated from two biological replicates.

Supplementary Figure S2. Effect of XUT0794 expression in cis and in trans on ctt1 attenuation.

A. Attenuation of ctt1 mRNA upon overexpression of XUT0794 in cis. Strains YAM2400 (WT) and YAM2474 (P41nmt1-XUT0794) were grown for 24 hours to mid-log phase in EMM medium +/- 15 µM thiamine, before addition of H2O2 for 15 min. Levels of XUT0794 (red) and ctt1 mRNA (blue) were quantified from total RNA using strand-specific RT-qPCR and normalized on the level of the U3B snoRNA. Data are presented as mean +/- SD from three biological replicates.

B. Induction of ctt1 mRNA upon XUT0794 overexpression in trans. YAM2475 (empty vector) and YAM2476 (pAM353; P41nmt1-XUT0794) cells were grown for 24 hours to mid-log phase in EMM-L medium +/- 15 µM thiamine, before addition of H2O2 for 15 min. Determination of XUT0794 and ctt1 mRNA levels and data presentation are as above.
C. Strains YAM2400 (WT), YAM2402 (exo2Δ) and YAM2533 (ctt1-kanℓ; kanMX6 inserted 113 bp upstream from XUT0794) were grown to mid-log phase in YES medium and collected prior (white bars) or after addition of 1 mM H₂O₂ for 15 min (black bars). Levels of XUT0794 (left panel) and ctt1 mRNA (right panel) were determined as described above. Mean values +/- SD were calculated from three biological replicates.

Supplementary Figure S3. Transcription of act1 is not controlled by an asXUT.

Snapshot of total (input) and nascent (IP) NET-Seq signals along the act1 gene in WT (upper panels) and exo2Δ (lower panels) cells. In each panel, the signal corresponding to the sense (+) and antisense (-) strand is shown in blue and pink, respectively. Blue arrows and boxes represent the mRNAs and coding sequences, respectively. NET-Seq data for the WT strain were previously described (Wery et al, 2017). The snapshot was produced using VING (Descrimes et al, 2015).

Supplementary Figure 4. Analysis of asXUT-mediated attenuation of ctt1 in HDAC mutants.

A. Effect of Clr3 class II HDAC on XUT-mediated ctt1 mRNA attenuation. YAM2400 (WT), YAM2402 (exo2Δ), YAM2407 (clr3Δ) and YAM2444 (exo2Δ clr3Δ) cells were grown in rich medium before (white) or after addition of 1 mM H₂O₂ for 15 minutes (black). ctt1 mRNA levels were determined by strand-specific RT-qPCR from total RNA. Normalization was as above. Data are presented as mean +/- SD from three biological replicates. *p<0.05; **p<0.01; ns, not significant upon t-test.

B. Effect of Hos2 class I HDAC on XUT-mediated ctt1 mRNA attenuation. Same as above using YAM2400 (WT), YAM2402 (exo2Δ), YAM2471 (hos2Δ) and YAM2472 (exo2Δ hos2Δ). *p<0.05; ***p<0.001; ns, not significant upon t-test.

C. Effect of the Png2 subunit of the Clr6 HDAC complex I on XUT-mediated ctt1 mRNA attenuation. Same as above using YAM2400 (WT), YAM2402 (exo2Δ), YAM2561 (png2Δ) and YAM2562 (exo2Δ png2Δ). *p<0.05; **p<0.01; ***p<0.001; ns, not significant upon t-test.
Supplementary Figure S5. Antisense XUT stabilization induces transcriptional attenuation of a class of highly expressed genes.

A. Global RNAPII transcription in WT and exo2△ cells. Density plot of exo2△/WT NET-Seq signal ratio for mRNAs (blue), sn(o)RNAs (black) and XUTs (red).

B. Metagene view of NET-Seq signals along class 1-4 genes in WT cells. For each class, normalized signal (tag/nt, log₂) along mRNA transcription start site (TSS) +/- 1000 nt (sense strand) and the antisense (as) strand were piled up, in a strand-specific manner. Average signal for each strand was plotted for class 1 (red), 2 (blue), 3 (green) and 4 (black). The shading surrounding each line denotes the 95% confidence interval.

C. Box-plot of NET-Seq signal (tag/nt, log₂) for XUTs antisense to class 1 and class 3 genes in WT and exo2△ (∆) cells.

D. Metagene view of nascent antisense transcription (NET-Seq) signal around the sense gene TSS of class 1-4 genes, in WT cells. The shading surrounding each line denotes the 95% confidence interval.
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B

H₂O₂ - - WT exo2Δ + +

ctt1 mRNA

U3B snoRNA

c tt1/U3B ND ND 1 0.3

C

XUT0794

WT exo2Δ

Densities (log2)

WT exo2Δ dcr1Δ ctt1 mRNA

Chr.III 57000 59000 + - Strand

Densities (log2)

WT exo2Δ ctt1

E

Fold of induction in WT

Time after H₂O₂ addition (min)

No H₂O₂ 1 mM H₂O₂
Figure 2: Gene expression analysis.

A) Diagram showing ribozyme targeting sites on the XUT0794 mRNA. Ribozyme interaction sites are indicated by arrows.

B) Graph showing relative XUT0794 expression levels. The x-axis represents different conditions: WT, exo2Δ, WT + RZ, exo2Δ + RZ. The y-axis represents the relative expression level. The amplicons D and C are indicated.

C) Graph showing ctt1 mRNA expression levels. The x-axis represents different conditions: WT, exo2Δ, WT + RZ, exo2Δ + RZ. The y-axis represents the ctt1 mRNA level. The conditions are either no H2O2 or 1 mM H2O2. Statistical significance is indicated by *** for p < 0.001, ** for p < 0.01, and ns for non-significant differences.
Wery_Figure 3
A) ctt1 mRNA

B) XUT0794

C) H4-acetyl ChIP

D) H3K14-acetyl ChIP

Wery_Figure 4
| Class | asXUT | # of genes | Nascent RNA levels in exo2Δ |
|-------|-------|------------|------------------------------|
| 1     | +     | 175        | Down                         |
| 2     | -     | 548        | Down                         |
| 3     | +     | 910        | Unchanged                    |
| 4     | -     | 3495       | Unchanged                    |

**NET-Seq, WT & exo2Δ**

**C**

**NET-Seq, WT**

**D**

**NET-Seq, exo2Δ**

**E**

Signal for XUTs

**Antisense NET-Seq signal, WT**

Wery_Figure 5
Wery Figure 6
Class 1 (expression > regulation threshold)

Class 3 (expression < regulation threshold)