Promoter-dependent nuclear RNA degradation ensures cell cycle-specific gene expression

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Cell cycle progression depends on phase-specific gene expression. Here we show that the nuclear RNA degradation machinery plays a lead role in promoting cell cycle-dependent gene expression by triggering promoter-dependent co-transcriptional RNA degradation. Single molecule quantification of RNA abundance in different phases of the cell cycle indicates that relative curtailment of gene expression in certain phases is attained even when transcription is not completely inhibited. When nuclear ribonucleases are deleted, transcription of the Saccharomyces cerevisiae G1-specific axial budding gene AXL2 is detected throughout the cell cycle and its phase-specific expression is lost. Promoter replacement abolished cell cycle-dependent RNA degradation and rendered the RNA insensitive to the deletion of nuclear ribonucleases. Together the data reveal a model of gene regulation whereby RNA abundance is controlled by promoter-dependent induction of RNA degradation.
Cells proliferate through a well-coordinated cycle ensuring the replication and redistribution of all cellular components. This cell cycle requires the synthesis of a panel of proteins required for replication, many of which are expressed only when they are needed. Consequently, progression through the cell cycle is accompanied by dramatic reorganization of gene expression that is often referred to as cell cycle-regulated transcription. 20% of yeast genes are expressed in a cell cycle-dependent manner, guided by the activation of transcription factors and cycling genes. These waves of expression are often divided into four events that take place in G1, S, G2/M, and in the transition between the M and G1 phases. Genes associated with these waves are classified into clusters based on expression patterns and promoter motifs that promote cell cycle-regulated transcription. Furthermore, shared common promoter elements are co-regulated by specific subsets of transcription factors, like the G1/S SCB-binding factor and MCB-binding factor. These promoters can be used experimentally to induce phase-specific expression of reporter proteins.

More recently, cell cycle changes have been associated with changes in RNA stability. Metabolic RNA labeling and dynamic transcriptome analysis identified hundreds of genes in budding yeast with periodic changes in transcription and mRNA degradation, rates. In general RNA degradation follows peaks of mRNA synthesis at defined times during the cell cycle. Strikingly, it was shown that the promoters of two mitotic genes (SWI5 and CLB2) trigger cell cycle-dependent RNA degradation in the cytoplasm. In these two cases, it was proposed that RNA is marked for decay by factors loaded on the RNA during transcription, leading to cytoplasmic degradation at the end of the cell cycle. However, it is not clear how the factors are recruited to the promoter, nor how the RNA is degraded in the cytoplasm. It is also unclear if co-transcriptional RNA degradation in the nucleus contributes to cell cycle repression. A few examples of targeted nuclear RNA degradation have been linked to nutritional changes and other stresses. It remains unclear generally how nuclear RNA degradation contributes to transcriptional regulation or how it regulates specific cell cycle genes.

Most biochemical assays provide estimates of average RNA amounts in a population of cells but cannot distinguish between nuclear and cytoplasmic RNA degradation. In such assays it is difficult to determine the location and timing of gene expression and the resolution of the expression cycle is often blurred by stochastic differences between cells. In this study, we monitored the synthesis and degradation of the phase-specific axial budding gene AXL2 during the cell cycle using single molecule analysis to determine the location, timing, and mechanism of phase-specific gene expression. Like many cell cycle genes AXL2 is thought to be expressed in the G1 phase of the cell cycle in a promoter-dependent manner. However, in vitro studies suggested that this gene might also be regulated post-transcriptionally. Here, we demonstrate that despite the sharp decline of AXL2 transcripts at the end of G1, it is transcribed albeit at a lower level throughout the cell cycle; live cell analysis and chromatin immunoprecipitation of RNA polymerase II confirmed that transcription of AXL2 continues in the S and G2/M phases of the cell cycle. Strikingly, deletion of nuclear ribonucleases completely blocked the characteristic periodicity of mature AXL2 mRNA levels. Ribonucleases were associated with the chromatin linking the RNA degradation machinery to transcription. Consistently, substitution of the AXL2 promoter impaired the cycling of gene expression. Together our data reveal a model of gene expression, in which cell cycle expression is achieved through promoter-dependent RNA degradation.

**Results**

RNA decay drives the cell cycle expression of AXL2. To determine the regulatory events controlling the expression of cell cycle-associated genes, we monitored the life cycle of the axial budding gene AXL2 using single-molecule fluorescence in situ hybridization (smFISH). AXL2 expression is required in the G1 phase of the cell cycle, and defects in its expression are easily scored by changes in budding patterns. The Axl2 mRNA was detected using two independent sets of single labeled oligonucleotides probes (Fig. 1a). The first probe set hybridizes to the 5′ end of the mRNA, while the other hybridizes to the 3′ end. Signals generated by the 5′ end probe at the transcription site in the nucleus identify nascent RNA, while complete or mature RNA, which is mostly cytoplasmic is identified by signals generated from both 5′ and 3′ end probes. To determine the number of probes sufficient for accurate RNA detection we compared the signal intensity and the number of RNAs detected by 12- and 24-probe sets. The results indicated that while a set of 24 probes resulted in higher signal intensity indicating a higher number of RNAs detected, the use of 12 probes provided a narrower peak and less inter-experiment variation without changing the overall conclusion (Supplementary Fig. 1). Accordingly, we used the 12-probe set for all subsequent experiments comparing either nascent (nuclear) or complete (mostly cytoplasmic) RNA in different conditions and phases of the cell cycle. We also confirmed the accuracy of nuclear targeting of the transcripts by comparing compressed 2D- with uncompressed 3D images, as 2D stacking of the images, which simplify quantification, do not result in more than 10% inaccuracies (Supplementary Fig. 2).

Examining the expression pattern of Axl2 during the cell cycle using either the 5′ or the 3′ end probe reproduced the established expression pattern of Axl2 mRNA, which peaks in G1 and is repressed in later phases (Fig. 1b). We next analyzed the Axl2 expression dynamics qualitatively as our technique allows quantification of the number of nascent RNAs (hybridizing to the 5′ end probe only in the nucleus) and completed RNAs (hybridizing to both 5′ and 3′ end probes in the cytoplasm) per cell. Both nascent and full-length RNA decreased as the cells exited from the G1 phase of the cell cycle (Fig. 1c and Supplementary Fig. 3). On average 4 RNA molecules per cell were detected in G1, while only 1 molecule per cell was detected in S phase. Together these data illustrate the overall accuracy of the Axl2 mRNA targeted probes. We also found the amount of Axl2 mRNA varies depending on the cell size (Supplementary Fig. 3c). In general, more Axl2 mRNA was detected in larger G1 cells. In all cases RNA peaked in G1 and gradually dipped over S phase to a minimum in G2/M. However, cytoplasmic and nascent RNA did not decrease at the same rate; full length RNA decreased by 6-fold, whereas nascent RNA decreased only 3-fold (Fig. 1c). Notably, RNA was detected in the nucleus in all phases of the cell cycle suggesting that transcription continues throughout the cell cycle (Fig. 1d). To verify the continuing transcription of the AXL2 gene beyond the G1 phase of the cell cycle, we synchronized cultures and compared the association of RNA polymerase (RNAPII) at the AXL2 locus to accumulation of its RNA.

As indicated in Fig. 1e, the association of RNAPII with the promoter of AXL2, did not decrease but instead, increased in the S and G2/M phases. On the other hand, the association of RNAPII with the AXL2 open reading-frame was modestly reduced but continued to be detected in the S and G2/M phases. This data clearly indicate that Axl2 mRNA transcription continues beyond G1. Consistently, monitoring the accumulation of Axl2 mRNA in the same synchronized culture by RT-qPCR once again indicated that Axl2 mRNA abundance decreases but remains detectable in S and G2/M (Fig. 1f). Together these results
clearly indicate that the cycling of AXL2 expression is not produced by strict repression and induction of transcription.

To monitor the dynamics of AXL2 expression, we examined the accumulation of Axl2 mRNA in real-time (Fig. 2 and Supplementary Figs. 4 and 5). We did this by inserting repeats of the MS2 coat protein binding site into the 5′ UTR of the gene (Fig. 2a), which then acted as a reporter of the mRNA by attracting a coat protein-EGFP fusion in live cells22,23. We have used the tag version and location previously certified for the detection of transcription and nascent RNA 22. Tagging Axl2 mRNA slightly increased its abundance but did not affect its expression cycle (Fig. 2b, c). Indeed, the overall number of spots detected by the tagged version was similar to those detected by the untagged version using FISH (compare, Figs. 1b and 2c). We monitored Axl2 mRNA every 10 min across the cell cycle and every minute across the G1/S transition. Interestingly, de novo transcription was detected in the nucleus at all phases of the cell cycle and was not completely repressed in S and G2/M (Fig. 2c). On the other hand, cytoplasmic RNA was clearly reduced in S and G2/M. Interestingly, while there were only 1 and 0.6 Axl2 RNAs per cell in S and G2/M phases respectively on average, transient pulses of RNA expression were detected in live cells in these phases (Fig. 2d). This indicates that RNA is both made and degraded during S and G2/M. It should be noted that since the pictures were taken every 10 min and the transcription pulses take ~2 min followed by the export or degradation of the RNA.
many RNA transcription events may escape detection. Indeed, acquiring images every minute reveals similar number of transcription pulses in the G1 and S phases (Fig. 2e). This clearly indicates once again that the rapid decrease in Axl2 mRNA amounts is not only due to transcription repression. We conclude that the phase-specific expression of Axl2 is not only created by peak transcription in G1 but through accelerated RNA decay in S phase.

**Nuclear ribonucleases promote AXL2 cell cycle-dependent repression.** The data above indicate that at the G1/S transition there is a discrepancy between the decrease in number of Axl2 mRNAs in the nucleus and its decrease in transcription, suggesting increased nuclear RNA decay. To evaluate the contribution of nuclear RNA degradation to cell cycle-dependent expression, we examined the effect of abolishing the activity of different nuclear ribonucleases on AXL2 expression cycle. We deleted or inactivated 4 ribonucleases: three exoribonucleases (Rrp6p, Rat1p, and Xrn1p) and one endoribonuclease (Rnt1p). Rrp6p is a component of the nuclear exosome implicated in the 3′–5′ end degradation of non-polyadenylated RNA with free 3′-OH. On the other hand, Rat1p is a 5′–3′ exoribonuclease that degrades uncapped RNA with 5′ monophosphate involved in transcription termination. Unlike Rrp6p and Rat1p that are exclusively nuclear, Xrn1p is a mostly cytoplasmic paralog of Rat1p, but it has also been linked to nuclear RNA degrada- tion. Finally, Rnt1p is a nuclear endoribonuclease that exits from the nucleolus to the nucleoplasm at the end of the G1 phase of the cell cycle and cleaves RNA with conserved NGNN stem loop structures including one in Axl2 mRNA. Mutating each of the 4 ribonucleases individually increased the abundance of both nascent and complete RNA in S phases when compared to that detected in G1 or when compared to the wild type RNA in the same phase (Fig. 3a). Similarly, certain ribonuclease mutations (e.g., rat1-1 and rnt1a) increased the ratio of the RNA in G2/M phase compared to G1 (Fig. 3b). However, only RNT1 deletion increased cytoplasmic Axl2 mRNA in the G2/M phase compared to wild type while the detected RNA remained stable at around 0.2–0.5 copies per cell in the other ribonuclease deletions (Supplementary Fig. 6a). Surprisingly, XRN1 deletion not only altered the amount of complete Axl2 mRNA in the cytoplasm but it also increased nascent RNA in the nucleus in G1 and S (Fig. 3b, c). This suggests that Xrn1p...
directly or indirectly contributes to Axl2 degradation in the nucleus. Double and triple deletions or mutations of exoribonucleases (e.g., RRP6 and XRN1) increased the effect of the endoribonuclease RNT1 in G2/M but not in S phase (Supplementary Fig. 6b). Therefore, RNA that is not cleaved by Rnt1p in S phase can be degraded by the other exoribonucleases later in the cell cycle. The other nuclear ribonucleases may also complete the degradation of RNA fragments generated by Rnt1p cleavage. Together these observations suggest that nuclear ribonucleases work together to ensure the rapid degradation of Axl2 mRNA in S phase. Strikingly, double deletion of RNT1 and XRN1 completely abolished the periodic expression of AXL2 resulting in the production of increasing amounts of RNA as the cells grow (Supplementary Fig. 6a, b). We conclude that nuclear ribonucleases are required for the cell cycle-dependent repression of AXL2.
Rnt1p is required for AXL2 cell cycle-dependent repression.

We next focused on Rnt1p as it is the only ribonuclease of the four tested with well-defined substrate selectivity\(^27\), a deletion phenotype of net increase in the Axl2 mRNA (Supplementary Fig. 6a), and previously shown Axl2 mRNA cleavage activity in vitro\(^20\). We thus studied if and how Rnt1p endoribonuclease represses AXL2 during the cell cycle. To test this, we first re-evaluated the impact of RNT1 deletion on Axl2 mRNA in vivo and the specificity of Rnt1p’s cleavage of Axl2 mRNA in vitro. Total RNA was extracted from wild-type cells, cells lacking RNT1 (rnt1Δ) or cells expressing a mutation in the Axl2 mRNA stem-loop recognized by Rnt1p (rnt1Δ LoopM, Fig. 4a). We then incubated the RNAs with, or without, recombinant Rnt1p. As expected, deletion of RNT1 increased the overall abundance of Axl2 mRNA, confirming the results obtained by smFISH (Fig. 4b, c). Recombinant enzyme generated the previously seen cleavage product (P) 14–16 nucleotides from the loop\(^20\). The cleavage was dependent on both the presence of the site that Rnt1p cleaves (rnt1Δ LoopM+) and the catalytic activity of Rnt1p (rnt1Δ c) (Fig. 4c). This clearly illustrates the accuracy and specificity of Rnt1p’s cleavage of Axl2 mRNA.

The stem-loop mutation (LoopM) that blocks Axl2 mRNA cleavage in vitro (Fig. 4c) also increased the abundance of Axl2 mRNA, but only in the absence of the exoribonucleases Xrn1p and Rrp6p (Supplementary Fig. 7a). Deleting RNT1 in the strains carrying the stem-loop and exoribonucleosome mutations (rnt1Δ rrp6Δ xrn1Δ LoopM) did not increase the abundance of nascent or full-length RNA in S phase (Supplementary Fig. 7b). In contrast, deletion of RNT1 increases the abundance of Axl2 mRNA in the G2/M phase even when the loop is mutated. Therefore the site that Rnt1p cleaves is required for Axl2 repression in S phase and Rnt1p is required for maintaining the repression during G2/M. The reason why Rnt1p loop mutation by itself did not increase RNA abundance is likely because of
degradation by other exoribonucleases that are otherwise inactivated by RNT1 deletion, as previously shown for other substrates of Rnt1p\(^{15}\). Notably, the catalytically inactive Rnt1p mutant that nevertheless preserves RNA binding, increased AXL2 mRNA abundance and partially inhibited its cycling, clearly indicating Axl2 cleavage is required for its repression (Supplementary Fig. 7c).

To differentiate between direct and secondary effects of RNT1 deletion, we examined the impact of short-term inactivation of Rnt1p activity on the abundance of Axl2 mRNA and compared it to the effect of RNT1 deletion by qRT-PCR. As expected, the deletion of RNT1 (rnt1Δ) had the most impact on Axl2, increasing its mRNA 4-fold (Fig. 4d). Strikingly, the increase in Axl2 RNA was also observed shortly after the inactivation of a temperature-sensitive allele of RNT1 (rnt1-1)\(^{28}\), consistent with direct cleavage by Rnt1p (Fig. 4d). Indeed, the increase in Axl2 mRNA was observed after only 1 h of shift to the restrictive temperature, at the same time as change in the abundance of the known substrate Mig2 mRNA\(^{16}\) observed and before any increase in an unrelated mRNA, Toa1 (Fig. 4e). Together these data confirm the direct link between Rnt1p catalytic activity and the increase in Axl2 mRNA abundance. Since Rnt1p accumulates exclusively in the nucleus its deletion would be expected to increase the number of RNAs leaving the nucleus without affecting the overall half-life of the majority of the RNA that accumulates in the cytoplasm. Accordingly, there was no significant difference between the half-life of Axl2 RNA extracted from wild-type and rnt1Δ cells (Supplementary Fig. 8). This further confirms the specificity of Rnt1p, on the nascent Axl2 transcripts, observed above by smFISH (Supplementary Fig. 8). In contrast, deletion of RNT1 (rnt1Δ) cells (Supplementary Fig. 8). This further confirms the specificity of Rnt1p on the nascent Axl2 transcripts, observed above by smFISH (Supplementary Fig. 8). Further, blocking this exit of Rnt1p from the nucleolus should prevent the cleavage of Axl2 and increase it incrementally in S and G/2M. As indicated in Fig. 4f, deletion of the N-terminal domain of Rnt1p, which blocks the cell cycle-dependent exit of Rnt1p from the nucleolus\(^{29}\), increased Axl2 mRNA. We conclude that repression of AXL2 requires Rnt1p to leave the nucleolus in S phase.

Live-cell analysis also indicated that RNT1 deletion perturbs the cycling of Axl2 mRNA (Fig. 5a and Supplementary Fig. 5). The presence of the tag did not inhibit the effect of RNT1 deletion as an increase in MS2 tagged Axl2 mRNA was still observed albeit to a lower extent upon deletion of RNT1 in the tagged strain (Fig. 5a). Both nuclear and cytoplasmic RNA continued to accumulate before and after budding (Fig. 5b). However, it was not clear whether Rnt1p induced degradation of Axl2 mRNA led to changes in the abundance of Axl2 protein or affected Axl2 function. Therefore, we examined the impact of RNT1 deletion on Axl2-dependent axial budding pattern. Cells were stained using the bud scar stain calcofluor and number and position of bud scars were compared between wild type and rnt1Δ cells. As indicated in Fig. 5c, deletion of RNT1 increased the number of random budding pattern normally associated with AXL2 overexpression\(^{18}\). As expected, the defect in budding pattern was not exaggerated by multiple nuclease deletion confirming that the different ribonucleases affect budding through the same pathway (Supplementary Fig. 9). We conclude that nuclear mRNA degradation inhibits the production of Axl2 protein to maintain budding patterns.

Rnt1p cotranscriptionally degrades Axl2 mRNA. It was previously shown that Rnt1p cleaves its substrates cotranscriptionally\(^{16,30,31}\) explaining the rapid decrease in nascent AXL2 transcripts upon the exit from G1. To evaluate this possibility, we examined the association of Rnt1p with the AXL2 locus using chromatin immunoprecipitation (ChIP). As indicated in Fig. 6a, b, Rnt1p was modestly enriched in the promoter region and Rnt1p binding site but the enrichment was noticeably increased when the turn over of the enzyme is blocked (rnt1Δ D245R). Indeed, impairing the catalytic activity of Rnt1p increased by 3 folds the enzyme association with AXL2 chromatin (Fig. 6b probes 1 and 2). Active enzyme rapidly turnovers decreasing the stability of its association with chromatin while the catalytically impaired version does not\(^{32–34}\). Replacement of the stem loop structure (LoopR) inhibited Rnt1p association with AXL2 chromatin near the cleavage site, while increasing the interaction with promoter region (Fig. 6b probes 1, 3, and 4).

To evaluate the contribution of Rnt1p to the transcription of AXL2, we monitored the impact of RNT1 deletion on RNAP II occupancy by ChIP. As indicated in Fig. 6c, deletion of RNT1 does not affect the overall level of RNAP II occupancy near the promoter and the 5’ end of the AXL2 open reading-frame (Fig. 6c probes 1 and 2). Instead, the deletion increased RNAP II occupancy with the region surrounding the cleavage site in AXL2 RNA, suggesting that cleavage may result in RNAP II release (Fig. 6c probes 3 and 4). This is consistent with the established role of Rnt1p in inducing transcription termination\(^{30}\).

Discussion

It is believed that modulation of gene expression is always linked to changes in transcription rates. For example, the cell cycle is
when transcription is not repressed. Gene expression whereby the promoter regulates RNA stability observations reveal a model of cell cycle-dependent regulation of Axl2 mRNA depends on the specific promoter sequence. Rnt1p to the transcription start site and renders the Axl2 insensitive to nuclear ribonucleases. These observations reveal a model of cell cycle-dependent regulation of gene expression whereby the promoter regulates RNA stability when transcription is not repressed.

Previous studies indicated that promoter sequence may control RNA stability in the cytoplasm. In all these examples, accelerated RNA decay is coupled to transcription repression and the degradation events occur in the cytoplasm after transcription is completed. Therefore, in these examples accelerated RNA decay functions to accentuate transcriptional repression. In the case of Axl2, the role of RNA decay takes center stage as the promoter is directly responsible for the recruitment and/or activation of the RNA decay machinery to the transcription site.

It is unlikely that this direct feedback between synthesis and degradation is restricted to Axl2. Rnt1p RNase has hundreds of potential cleavage targets in the yeast transcriptome, including in...
cell cycle genes. Equally, there are at least two other mRNAs coding for protein required for cell polarity and bud site selection (REM2 and BNI1) that were identified using high-throughput assay as targets for nuclear RNA degradation. However, it remains to be seen if these degradation events act as the primary mechanism of gene regulation or function as fail-safe mechanisms to ensure clean transcription repression. Distinguishing between transcriptional repression and cotranscriptional RNA degradation of transcribed genes is challenging since both mechanisms reduce the amount of RNA and, as we show, may both depend on the promoter sequence. In addition, bulk measurements of total RNA decay cannot easily differentiate between these two nuclear mechanisms since most mRNA is cytoplasmic. Live cell analysis coupled with single molecule counting and RNAPII ChIP assay, as we performed here, will be required to clearly establish the regulatory mechanism of these candidate genes. Comparing both untagged RNA FISH assay and live cells assay of tagged RNA increases confidence in the data obtained and reduces the chance of technique specific artefacts. In addition, comparing between conditions or ribonuclease deletion using the same probe set or tag reduces the chance of errors of this techniques. In any case, the findings in this study caution against the automatic interpretation of the dependency of gene expression on promoter sequence as evidence for transcription regulation.

RNA stability is regulated by a large network of ribonucleases and blocking the mutation by one may activate the degradation by another. This explains why mutation of the RNA site that Rnt1p cleaves in the presence of exoribonucleases does not increase RNA expression (Supplementary Fig. 7b). RNA accumulating in the nucleus upon the mutation of Rnt1p loop are targets of the DRN or the nuclear RNA degradation mechanism that include a component of the nuclear cap binding complex (Cbc1p) and nuclear ribonucleases like Rrp6p and Rat1p. Indeed, deletion of exoribonucleases including Rrp6p increases RNA abundance in a Rnt1p cleavage site-dependent manner (Supplementary Fig. 7b).

The data presented in this study suggest a model of cell cycle-dependent gene expression (Fig. 8). In this model, in the absence of Rnt1p, which is mostly nucleolar in the G1 phase of the cell cycle, Axl2 mRNA accumulates and is translated in the cytoplasm. Upon the exit of Rnt1p from the nucleolus to the nucleoplasm in the S phase of the cell cycle, it is recruited to the promoter region of AXL2 to survey RNA synthesis and cleaves it upon the transcription of the cleavage signal. This cleavage leads to a sharp decrease in the number of complete RNA exported to the cytoplasm and releases RNAPII. It is not clear how Rnt1p is recruited to the promoter. We have shown that the interaction between Rnt1p and the promoter does not depend on the RNA sequence of the open reading-frame. Indeed, disrupting the RNA site that Rnt1p cleaves increases the interaction with the promoter region (Fig. 6). This reinforces a model whereby Rnt1p binds to the promoter region before translocating to the AXL2 stem loop when it is synthesized. Rnt1p may be recruited by cell cycle-specific transcription factors or directly by interaction with RNAPII itself. Protein interaction assay identified binding of Rnt1p with several transcription factors and interaction with RNAPII CTD was also proposed. Unlike standard, transcription-based models of cell cycle-dependent regulation, this RNA decay-dependent mechanism provides faster response time permitting rapid cycling of gene expression.

**Methods**

**Yeast strains.** Yeast strains were generated in the BY background starting from strain LLY36 using standard procedures. 24xMS2 tags were introduced in the 5′ UTR of AXL2 by transforming a PCR fragment amplified from plasmid pMS2-AXL2 synthesized by BioBasic. Likewise, 24xPP7 tags were introduced in the 3′ UTR of AXL2 by transforming a PCR fragment amplified from plasmid pAXL2-PP7, which was synthesized by BioBasic. Strains expressing RNA tags were crossed to BY4730 to remove TRP1 to use plasmid pNoel-NLSred or pNoel. The LoopR mutant was produced by replacing the cleavage site of Rnt1p in AXL2 with a GFP cassette. The LoopM mutant was produced by introducing five silent point mutations in the sequence of the cleavage site of Rnt1p in AXL2 to disrupt its predicted secondary structure without changing the coding sequence. The AXL2 promoter replacement by the promoter of TOA1 was performed using a synthesized plasmid as template to amplify a replacement cassette.
Fig. 7 The cell cycle-dependent degradation of Axl2 mRNA is induced by its promoter. a The promoter of AXL2 is required for Rnt1p-dependent repression of gene expression. The abundance of Axl2 mRNA was monitored in wild type or mt1Δ cells expressing AXL2 from its own promoter (WT and mt1Δ) or a heterologous promoter (TOA1p-AXL2 and TOA1p-AXL2 mt1Δ). The mRNA was quantified as described in Fig. 1c and is shown on the left while examples of cells captured in S and G2/M phases are shown on the right. White bar equals 2 μm. b Schematic representation of the AXL2 locus depicting the position of the different fragments amplified by qPCR after chromatin immunoprecipitation. The position of the RNA target Rnt1p cleavage site (RCS) is shown at the top and the TOA1 promoter is indicated by a gray box. The probe specific to the substituted sequence is indicated by ′. c The promoter substitution increases the association of RNApolII with the AXL2 locus. The RNAPII association pattern was examined in wild type cells (WT) or cells expressing AXL2 from a heterologous promoter (TOA1p-AXL2) as described in Fig. 6c. d Substitution of the AXL2 promoter inhibits the recruitment of Rnt1p to the AXL2 locus. Rnt1p association with the AXL2 locus, expressed from a wild-type or heterologous promoter, was examined as described in Fig. 6b with primers for amplicon 1 specific to the AXL2 or TOA1 promoter as required. Shown is the enrichment of Rnt1p relative to RNAPII. The bar graphs represent the average values obtained from 3 independent biological replicates shown as dots. Asterisks indicate significant difference between strains (*p < 0.05, **p < 0.01, ***p < 0.001 by two-tailed unpaired t test)

Yeast cultures. All yeast strains were grown in yeast complete (YC) media lacking the appropriate nucleotide or amino acid when needed for plasmid maintenance (1.7 g/L yeast nitrogen base without amino acids and ammonium sulfate, 1 g/L sodium glutamate, 100 mg/L cysteine, 100 mg/L threonine, 85 mg/L tryptophan, 80 mg/L leucine, 60 mg/L lysine, 50 mg/L aspartic acid, 50 mg/L isoleucine, 50 mg/L methionine, 50 mg/L phenylalanine, 50 mg/L proline, 50 mg/L serine, 50 mg/L tyrosine, 50 mg/L uracil, 50 mg/L valine, 20 mg/L adenine, 20 mg/L arginine, 20 mg/L histidine, pH 6) with 2 g/L dextrose. Cultures were grown at 26 °C to accommodate thermosensitive strains such as mt1Δ and rat1-1 unless restrictive conditions were required in which case pre-cultures grown at the permissive temperature were incubated at the restrictive temperature of 37 °C for 4 h prior to cell collection. Cell cycle arrest was performed with cultures grown in YEPD (10 g/L yeast extract, 20 g/L bacto-peptone, 100 mg/L adenine, 2 g/L dextrose) at 30 °C. G1 arrest was performed by adding 5 μg/ml α-Factor to the culture and monitoring the arrest by looking at the budding index of sonicated culture samples. After 3 h of treatment, >95% were un-budded and cells were collected. S phase arrest was performed by the addition of 200 mM hydroxyurea powder to the cultures and monitoring the arrest as for G1. After 3 h of treatment, >90% were budded and cells were collected.

The G2/M arrest was performed by the addition of 15 μg/ml nocodazole from 1.5 mg/ml stock in DMSO to the cultures and monitoring the arrest as above. After 3 h of treatment, >90% of the cells were arrested with large buds and the cells were collected. Transcription arrests were performed in cells grown in YC media at 26 °C. The cultures were shifted to 37 °C for 20 min prior to the addition of 150 μM
Fig. 8 Model for cell cycle-dependent repression of the budding gene AXL2. In this model, AXL2 transcription is induced in the G1 phase of the cell cycle and repressed in S phase when Rnt1p is released from the nucleolus. Association of Rnt1p to the promoter stalls transcription and initiates RNA degradation. Exoribonucleases degrade the RNA cleaved by Rnt1p and function as a fail-safe to remove the RNA escaping cleavage by Rnt1p. In the G2/M phase AXL2 repression is maintained by a combination of reduced transcription initiation and increased RNA degradation. After mitosis, Rnt1p is re-localized to the nucleolus releasing the RNAPII for rapid transcription of AXL2.
compared to the DyLight 550 and DyLight 650 channels of FISH we only counted DNA by re-suspending the beads in 100 µl TE containing 1% SDS and incubating overnight at 65°C. Input DNA was de-crosslinked at the same time by incubation of samples of the cleared lysate diluted in TE containing 1% SDS overnight at 65°C. The DNA was purified by RNase A and proteinase K treatments followed by 2 phenolchloroformisooamyl alcohol (25:24:1) extractions, chloroformisoamyl alcohol (24:1) extraction, and ethanol precipitation with glycogen. The purified DNA pellets were resuspended in 10 mM Tris pH 8 and further diluted in water at 1:200 for the input DNA, 1:20 for the RNAPII ChiP, 1:10 for the Rnt1p ChiP.

Quantitative and statistical analysis. smFISH analysis: smFISH experiments were performed on 3 independent cultures and a target of 50 cells per phase were scored coming from each culture. In the figures, all data points are presented in the form of violin plots unless stated otherwise in the legends and Supplementary Data 2. A Supplementary Figure S1 shows the dataset. The RNA molecule number per spot was calculated by dividing the spot intensity (corrected to local background) by the value corresponding to 1 RNA molecule and rounding to the closest integer. As the primary object detection was based on nuclei identification, many G2/M cells had to be reconstructed from two cells identified from dividing nuclei. Nascent RNA per cell numbers were calculated from the brightest 5 probe spots in the nucleus when more than 1 nuclear spot was detected. Chromatin RNAs were scored when a spot with a 5 probe spot also scored positive for the 3 probe signal.

RT-qPCR and chromatin immunoprecipitation analysis: Relative mRNA quantification was achieved through normalization to the Act1 mRNA using the formula: RQ = 2^[ΔCTmutant−ΔCTWT (CTWT−CTinput)]ACT, where CTs are the cycle threshold values determined by the Mastercycler software. PCR reactions were performed in technical duplicates or triplicates for data collected produced from 3 independent cultures for both the wild-type (WT) and mutant strains. Relative enrichment of RNAPII or Rnt1p at the AXL2 locus was achieved through normalizing to a region of the chromosome V using the formula: RE = 2^[(ΔCTWT−ΔCTmutant)ACT]/2(ΔCTWT−ΔCTmutant)]ACT. PCR reactions were performed in technical duplicates or triplicates for each independent cultures.

Statistics and reproducibility: Statistical analyses of mean values of measurements were conducted using GraphPad Prism version 7.03 for Windows using Student’s t-test if so stated in the figure legends with Welch’s correction for FISH data comparison, where variance between strains was unequal. The number of cells analyzed per FISH experiment, means and fold changes to wild-type with its dataset. The RNA molecule number per spot was calculated by dividing the spot intensity (corrected to local background) and localization data was exported in a table format. After analysis of the 2D images, cells were manually assigned to G1, S, or G2/M phases of the cell cycle according to the bud size and nucleus position. The value of fluorescence intensity corresponding to 1 RNA molecule was established for each strain by taking the median value of the cytoplasmic RNAs of all its dataset. The RNA molecule number per spot was calculated by dividing the spot intensity (corrected to local background) by the value corresponding to 1 RNA molecule and rounding to the closest integer. As the primary object detection was based on nuclei identification, many G2/M cells had to be reconstructed from two cells identified from dividing nuclei. Nascent RNA per cell numbers were calculated from the brightest 5 probe spots in the nucleus when more than 1 nuclear spot was detected. Chromatin RNAs were scored when a spot with a 5 probe spot also scored positive for the 3 probe signal.

RT-qPCR and chromatin immunoprecipitation analysis: Relative mRNA quantification was achieved through normalization to the Act1 mRNA using the formula: RQ = 2^([ΔCTWT−ΔCTinput]ACT)/2(ΔCTWT−ΔCTmutant)ACT, where CTs are the cycle threshold values determined by the Mastercycler software. PCR reactions were performed in technical duplicates or triplicates for data collected produced from 3 independent cultures for both the wild-type (WT) and mutant strains. Relative enrichment of RNAPII or Rnt1p at the AXL2 locus was achieved through normalizing to a region of the chromosome V using the formula: RE = 2^[(ΔCTWT−ΔCTmutant)ACT]/2(ΔCTWT−ΔCTmutant)]ACT. PCR reactions were performed in technical duplicates or triplicates for each independent cultures.

Data availability

All gels and microscopy images are available at Mendeley https://doi.org/10.17632/ds4g825jdw1. Statistical analysis of the smFISH dataset, genotypes of strains, and primer sequences used in this study are presented in Supplementary Data 1. The source data underlying all figures are provided in Supplementary Data 2. The source data collected from smFISH experiments is available in Supplementary Data 3.

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