Long Noncoding RNAs Promote Transcriptional Poising of Inducible Genes

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Abstract

Long noncoding RNAs (lncRNAs) are a class of molecules that impinge on the expression of protein-coding genes. Previous studies have suggested that the GAL cluster-associated lncRNAs of Saccharomyces cerevisiae repress expression of the protein-coding GAL genes. Herein, we demonstrate a previously unrecognized role for the GAL lncRNAs in activating gene expression. In yeast strains lacking the RNA helicase, DBP2, or the RNA decay enzyme, XRN1, we find that the GAL lncRNAs specifically accelerate gene expression from a prior repressive state. Furthermore, we provide evidence that the previously suggested repressive role is a result of specific mutant phenotypes, rather than a reflection of the normal, wild-type function of these noncoding RNAs. To shed light on the mechanism for lncRNA-dependent gene activation, we show that rapid induction of the protein-coding GAL genes is associated with faster recruitment of RNA polymerase II and reduced association of transcriptional repressors with GAL gene promoters. This suggests that the GAL lncRNAs enhance expression by derepressing the GAL genes. Consistently, the GAL lncRNAs enhance the kinetics of transcriptional induction, promoting faster expression of the protein-coding GAL genes upon the switch in carbon source. We suggest that the GAL lncRNAs poise inducible genes for rapid activation, enabling cells to more effectively trigger new transcriptional programs in response to cellular cues.

Introduction

Essential cellular processes, such as growth, organ development, and differentiation, require precise spatial and temporal control of gene expression. Eukaryotes have developed intricate pathways for regulating gene expression at the transcriptional level in both global and gene-specific manners [1,2]. Recent studies have provided evidence that lncRNA molecules facilitate transcriptional control of protein-coding genes [3,4]. Thus far, the most well-characterized lncRNA is Xist, which facilitates X chromosome inactivation in mammalian cells [3,6]. Similar to a transcription factor, Xist functions by directing corepressor complexes to the targeted DNA loci [7]. Other examples of repressive lncRNAs include HOTAIR, a 2.1 kilobase transcript that directs repression of developmental gene loci, and PANDA, which regulates cell-cycle–dependent gene expression [8,9]. Recruitment of transcription factors may also be a primary mechanism for lncRNAs associated with transcriptional activation [10–13], suggesting that these molecules may recruit both activators and repressors. Other lncRNAs, however, appear to function solely through their synthesis, whereby the act of transcription alters the chromatin structure of a targeted gene promoter [14–16]. This diversity of action may account for the fact that individual lncRNAs are more conserved in their position than in their nucleotide sequence [17]. Interestingly, many mammalian lncRNAs are associated with genes that require precise temporal control of initiation to facilitate proper cell growth and differentiation [9,13,18–23]. This suggests that these molecules may control the timing of gene expression in response to specific signals.

The GAL10 lncRNA in the S. cerevisiae budding yeast model system is encoded within the GAL gene cluster, which is composed of the GAL7, GAL10, and GAL1 metabolic genes required for utilization of galactose as a carbon source [24–26]. Budding yeast are able to utilize glucose and this catabolite is the preferential carbon source for energy production. However, yeast also has the capacity to utilize galactose when it is the sole carbon source in the media [27,28]. The transition from glucose to galactose metabolism requires an intricate switch in transcriptional programs, whereby genes repressed in the presence of glucose must be activated for production of galactose metabolizing enzymes [29–31]. The highly orchestrated series of events required to facilitate this GAL gene metabolic switch is well understood and involves modulation of carbon source-dependent transcriptional activators and repressors [1,29,32–34]. Interestingly, the GAL10 lncRNA has been proposed to act additively with transcriptional repressors, to provide tighter control of this gene expression network.
Long noncoding RNAs (lncRNAs) are a recently identified class of molecules that regulate the expression of protein-coding genes through a number of mechanisms, some of them poorly characterized. The GAL gene cluster of the yeast *Saccharomyces cerevisiae* encodes a series of three inducible genes that are turned on or off by the presence or absence of specific carbon sources in the environment. Previous studies have documented the presence of two lncRNAs—GAL10 and GAL10s—encoded by genes that overlap the GAL cluster. We have now uncovered a role for both these lncRNAs in promoting the activation of the GAL genes when they are released from repressive conditions. This activation occurs at the kinetic level, through more rapid recruitment of RNA polymerase II and decreased association of the co-repressor, Cyc8. Under normal conditions, but also especially when they are stabilized and their levels are up-regulated, these GAL lncRNAs promote faster GAL gene activation. We suggest that these lncRNA molecules poise inducible genes for quick response to extracellular cues, triggering a faster switch in transcriptional programs.

Previous studies from our laboratory demonstrated that loss of the RNA helicase DBP2 results in accumulation of a 3’ extended GAL10s lncRNA under conditions when the protein-coding GAL genes are transcriptionally repressed (+glucose) [37]. Based on previous evidence that up-regulation of the GAL10 lncRNA impairs transcriptional activation of the GAL1 and GAL10 genes [25], we anticipated that loss of DBP2 would similarly delay transcriptional activation of GAL7. To this end, we analyzed the transcriptional induction profile of GAL7 in wild-type and dbp2A cells following a media shift from repressed to activated conditions (+glucose to galactose) by isolating RNA fractions over time at 30 min intervals from three, independent biological replicates per strain. We then conducted northern blotting of isolated RNAs and then obtained a semiquantitative estimate of the degree of repression by calculating the average lag time or time to the first appearance of GAL7 transcripts after normalization to the *SCR1* loading control (Figure 1B). In contrast to wild-type cells, which exhibited a normal, ∼2-h lag time to induction [40,41], dbp2A cells displayed detectable GAL7 transcripts within an average of 40 min (Figure 1B). This was unexpected and suggested that loss of DBP2 results in a rapid induction of GAL7 expression from repressive conditions. To determine if the requirement for DBP2 is specific to GAL7, we then assayed GAL10 induction (Figure 1B, bottom). This revealed that GAL10 is also rapidly induced in dbp2Δ cells (Figure 1C). In addition to full-length GAL10 transcripts, we also observed the appearance of shorter GAL10 products, which are likely the result of previously noted cryptic initiation defects in dbp2Δ cells (Figure 1C, bottom) [37]. Regardless, this reveals that the loss of DBP2 results in rapid induction of both the GAL7 and GAL10 genes from repressed (+glucose) conditions.

Loss of the GAL lncRNAs Restores Repression in dbp2Δ Cells

The results above suggest that DBP2 is required to maintain glucose-dependent repression of the protein-coding GAL genes. To determine if this requirement is dependent on the presence of the GAL lncRNAs, we constructed a dbp2Δ lncRNA strain that lacks expression of both of the GAL10 and GAL10s lncRNA molecules. Expression of the GAL10 lncRNA is dependent on the Reb1 transcription factor, which has four putative binding sites within the 3’ end of the GAL10 coding region [24,26]. Although it is not known which Reb1 site(s) is necessary for expression of the GAL10 lncRNA, previous studies have shown that the lncRNA strain, which harbors silent mutations of all four sites, abolishes synthesis of this lncRNA (Figure 1D) [24]. Because the GAL10 and GAL10s lncRNAs arise from juxtaposed sites within the protein-coding GAL10 gene, we speculated that the lncRNA Δ mutation would also abolish synthesis of the GAL10s lncRNA. To test this, we conducted reverse transcription-quantitative PCR (RT-qPCR) analysis to measure lncRNA abundance in isogenic wild-type, dbp2Δ, lncRNAΔ, and dbp2Δ lncRNAΔ cells grown in the presence of glucose, using primers positioned within the 5’ ends of the respective lncRNAs (nc10 and nc10s in Figure 1D). This revealed a slight increase in the GAL10 lncRNA and greater overabundance of the GAL10s lncRNA in the dbp2Δ strain similar to previous studies [37]. More importantly, neither the GAL10 nor the GAL10s lncRNA were detectable in strains harboring the lncRNAΔ (Figure 1D). This suggests that the lncRNAΔ mutation abolishes expression of both lncRNAs, consistent with our prediction.

Next, we conducted transcriptional induction analysis as above using isogenic *dbp2Δ* and *dbp2Δ lncRNAΔ* cells to determine if the rapid induction phenotype is linked to the presence of the GAL lncRNAs. Strikingly, incorporation of the lncRNAΔ mutation in the *dbp2Δ*-deficient strain restored the induction kinetics of both GAL7

### Results

The GAL7 and GAL10 Genes Are Rapidly Induced from Repressed Conditions in *dbp2Δ* Cells as Compared to Wild Type

The GAL cluster is a group of gene loci that have been extensively utilized to define the mechanism and order of events in transcriptional regulation [1,27–29,39]. The cluster encodes three genes, GAL1, GAL7, and GAL10, which exist in three distinct transcriptional states in response to carbon sources: repressed (+glucose), derepressed (+raffinose), and activated (+galactose) (Figure 1A). This cluster also encodes the GAL10 lncRNA, which is a 4.0 kb antisense transcript that overlaps GAL10 and GAL1, and the GAL10s lncRNA, a 0.5 kb sense-oriented transcript upstream of GAL7 (Figure 1A) [24,26,30]. The protein-coding GAL genes are regulated by carbon source-responsive repressors and activators (Figure 1A) [27,29,32]. In contrast, the GAL lncRNAs are expressed when the protein-coding GAL genes are transcriptionally inactive (+glucose or raffinose) [24–26] and are dependent on the transcription factor, Reb1 (Figure 1D) [24,26].
Derepression occurs under nonrepressing, noninducing conditions when the repressors are no longer present and the transcription factors (not shown), which then recruit other proteins such as the Tup1–Cyc8 co-repressor complex to promote repression of the target genes. Previous studies have utilized mutant strains with impaired RNA decay pathways to demonstrate the roles of lncRNAs at targeted gene loci [25,38]. The 5′-3′ exonuclease, Xrn1, is required for degradation of both the GAL10 and GAL10s lncRNAs [26,30,42,43]. DCP2-deficient cells also accumulate lncRNAs but through a defect in RNA decapping [25]. Interestingly, up-regulation of the GAL lncRNAs, via loss of Dcp2, has been linked to delayed transcriptional activation of the GAL genes from derepressed conditions (+raffinose) [25]. This was also observed for xrn1Δ cells, but to a lesser extent [25]. Recent studies have shown that both Dcp2 and Xrn1 are present in the nucleus and associate with transcribed chromatin, indicative of a direct link between decay and gene expression [44,45]. However, contribution of RNA decay pathways to induction from repressed conditions (+glucose) has not been addressed.

Figure 1. Loss of DBP2 results in rapid, lncRNA-dependent induction of GAL10 and GAL7 from repressed conditions. (A) Simplified model for carbon-source-dependent regulation of GAL1, GAL7, and GAL10 genes within the GAL cluster. Glucose-dependent repression is mediated by transcription factors (not shown), which then recruit other proteins such as the Tup1–Cyc8 co-repressor complex to promote repression [28,32,40,46,47,51]. Derepression occurs under nonrepressing, noninducing conditions when the repressors are no longer present and the GAL genes are not transcriptionally active [29]. Activation only occurs in the presence of galactose [1,29]. Synthesis of the GAL10 lncRNA, and likely the GAL10s lncRNA, is mutually exclusive with activated expression of the GAL genes [24,25]. (B–C) GAL7 (B) and GAL10 (C) genes are rapidly induced in dbp2Δ cells following a switch from repressed to activated conditions. Transcriptional induction of wild-type (BY4741) and dbp2Δ strains was conducted by isolating RNA from cells at 30 min intervals prior to and immediately following a nutritional shift from repressive (+YPD) to activated (+galactose) conditions. Transcripts were detected by northern blotting using 32P-labeled, double-stranded (ds)DNA probes corresponding to GAL7, GAL10, or SCR1 RNA as indicated. Each time course was conducted in triplicate. Average lag times to induction are shown with the standard deviation (s.d.) for the mean (SEM). (D) Top) Schematic diagram of the IncRNAΔ strain with GAL10 and GAL10s IncRNAs and primer sets for RT-qPCR. The four previously identified binding sites for the Reb1 transcription factor are present within the 3′ end of the GAL10 coding region [24]. The IncRNAΔ harbors silent mutations that disrupt all binding sites for the Reb1 transcription factor [24]. (D, Bottom) The IncRNAΔ mutation abolishes expression of both the GAL10 and GAL10s lncRNAs in wild-type and dbp2Δ cells. GAL10 and GAL10s lncRNAs were detected in the indicated strains following growth in glucose using RT-qPCR as previously described with primers nc10 and nc10s [37]. Transcript levels were normalized to ACT1, which does not vary between these strains, and is the average of three biological replicates with respect to wild type and standard error from the mean (SEM). (E–F) Loss of GAL10 and GAL10s lncRNAs restores repression at GAL7 (E) and GAL10 (F) loci in DBP2-deficient cells. Transcriptional induction assays from repressive conditions were conducted with isogenic dbp2Δ and dbp2Δ IncRNAΔ strains as in Figure 1B–C. doi:10.1371/journal.pbio.1001715.g001
To determine if the up-regulation of lncRNAs, via loss of RNA decay and/or decapping pathways, impacts the expression of the GAL genes from repressed conditions, we analyzed the transcriptional induction of GAL7 and GAL10 in xrn1Δ and dcp2Δ strains (Figure 2A–B). We also included dbp2Δ cells in this analysis for comparison to studies above. Surprisingly, and in contrast to defective expression, this revealed that GAL7 and GAL10 are rapidly induced in both xrn1Δ and dcp2Δ strains with overabundant lncRNAs. In fact, detectible transcripts appear 2- to 3-fold faster in these strains than in wild type, similar to the rapid induction kinetics of dbp2Δ cells (Figure 2A–B). Note that the GAL10 lncRNA is also readily detectable in these RNA decay-deficient strains due to the use of a double-stranded DNA probe and consistent with the role of Xrn1 and Dcp2 in lncRNA decay (Figure 2B, asterisks) [25, 26, 38]. Thus, loss of genes encoding either the RNA helicase DBP2 or the RNA decay factors XRN1 or DCP2 results in faster activation of the protein-coding GAL genes from repressive conditions. This suggests that the GAL lncRNAs may actually promote gene expression.

GAL1 Is Also Rapidly Activated from Repressed Conditions

In contrast to our results above, prior studies have proposed a repressive role for the GAL10 lncRNA [24–26]. However, a major difference between our studies and past reports is that prior experiments were primarily focused on GAL1 induction from derepressive conditions (+raffinose), rather than GAL10 and GAL7 from a repressive state (+glucose) [24–26]. To determine if GAL1 exhibits a different induction profile than GAL7 and GAL10, we analyzed the induction of this gene as above (Figure 2C). Northern blotting analysis of RNAs from wild-type, dbp2Δ, xrn1Δ, and dcp2Δ strains revealed that GAL1 is also rapidly induced from repressive conditions in all three mutant strains with lag times of ~50 min (Figure 2C). This suggests a common mechanism for the GAL lncRNAs at all three GAL cluster genes.

Induction of the GAL Cluster Genes from Derepressive Conditions Occurs with Wild-Type Kinetics for dbp2Δ, xrn1Δ, and dcp2Δ Strains

In the presence of glucose, the GAL genes are repressed through several mechanisms, including the action of glucose-dependent transcriptional repressors (Figure 1A) [28, 31, 46–48]. However, when cells use raffinose as a carbon source, the GAL genes become derepressed due to environmentally induced loss of repressors (Figure 1A). To determine if the rapid induction of the GAL genes is specific for activation from repressive conditions (+glucose), we conducted induction analysis from the derepressed state (+raffinose). Interestingly, wild-type, dbp2Δ, xrn1Δ, and dcp2Δ strains all...
exhibited similar induction kinetics from derepressed to activated conditions with the appearance of transcripts within ~30 min for all three GAL cluster genes (Figure 2D–F). This is consistent with a recent study showing that xrn1Δ cells accumulate GAL7 and GAL10 transcripts at the same rate as wild-type cells when induced from raffinose [45]. DCP2-deficient cells also displayed detectible transcripts at 30 min postinduction for all three GAL genes, albeit with an apparent reduction of transcript levels for GAL1 as compared to wild type (Figure 2D–F, bottom). This demonstrates that the rapid induction of GAL7, GAL10, and GAL1 is specific for the environmental switch from repressive (+galactose) to activating (+glucose) conditions. Moreover, it suggests that the loss of the RNA decay machinery does not necessarily result in lncRNA-dependent repression [25,36].

RNA Decapping Deficiencies Impair GAL1 Transcript Accumulation

Prior studies suggested that GAL lncRNAs are repressive based on defective induction of the GAL genes in RNA decapping and decay-deficient strains [25]. However, our results suggest that this is not the case for xrn1Δ cells with defective RNA decay. To determine if the apparent reduction in mRNA levels in dcp2Δ cells above indicates a specific requirement for decapping in GAL gene induction, we conducted longer induction analyses from derepressive conditions for three, independent biological replicates. We then graphed the resulting transcript levels over time as the average transcript levels with respect to a fully induced wild-type RNA sample (‘‘Control’’) then graphed the resulting transcript levels over time as the percentage of the average transcript levels with respect to a fully induced, wild-type ‘‘control’’ RNA for normalization between replicates. The ‘‘control’’ corresponds to total RNA isolated from wild-type cells after 5 h in galactose media following initial growth in raffinose for maximal expression. Error bars indicate the SEM. Statistical significance was calculated using a two-tailed t test. Time points with significantly different transcript levels (p<0.05) between wild-type and dcp2Δ cells for each gene are as follows: GAL10, 60–120 min time points; GAL1, 90–150, 240, 300 min time points. The 210 and 270 min time points for GAL7 correspond to p<0.10, whereas no other time points in the GAL7 analysis displayed significantly different transcript levels between wild-type and dcp2Δ cells.

Thus, the previously described lncRNA-dependent repression at the GAL cluster in RNA decay-deficient strains may reflect a requirement for decapping in the accumulation of GAL1 transcripts, and especially GAL1, rather than a repressive role for the GAL lncRNAs.

DBP2- and XRN1-Deficient Cells Display Faster Recruitment of RNA Polymerase II to GAL7 and GAL10 Genes

Our results above provide evidence that the GAL lncRNAs may act in a positive manner by stimulating induction of the protein-coding GAL genes from repressed conditions. However, it is also possible that the increase in transcript abundance over time is due to a decrease in mRNA decay rather than an increase in transcriptional activity. To determine if the rapid induction correlates with an increased rate of transcriptional induction in dcp2Δ and xrn1Δ cells as compared to wild type, we asked if RNA polymerase II (RNAPII) is recruited to the GAL7 and GAL10 gene promoters [39,49]. RNAPII recruitment was measured by conducting chromatin immunoprecipitation (ChIP) over a 300-min time course following induction from repressed conditions with an antibody to a RNAPII core subunit (anti-Rph3) (Figure 4A). Suggestive of a transcriptional effect, this revealed that RNAPII is recruited to the GAL7 and GAL10 promoters more rapidly in both dcp2Δ and xrn1Δ cells (Figure 4A). This faster recruitment was most evident at 120 min postinduction, with ~4-fold and ~6- to 9-fold higher levels of RNAPII at GAL7 and GAL10, respectively (Figure 4A). This suggests that loss of DBP2 or XRN1, and the resulting accumulation of the GAL lncRNAs, results in a direct effect on transcription initiation. In contrast, analysis of the galactose-inducible GAL6 gene revealed similar RNAPII recruitment rates for all three strains with a slightly lower RNAPII signal for xrn1Δ and dcp2Δ cells at the 300 min time point (Figure 4B) [50]. The latter is consistent with recent studies showing that xrn1Δ cells have reduced steady-state transcription levels [45]. Furthermore, it demonstrates that the rapid recruitment of RNAPII is specific for the GAL lncRNA-targeted genes within the GAL cluster.

Figure 3. Loss of DCP2 impairs GAL1 transcript accumulation when induced from derepressive conditions. (A–C) Extended time course for analysis of GAL7 (A), GAL10 (B), and GAL1 (C) induction from derepressed conditions in dcp2Δ cells. Wild-type and dcp2Δ cells were grown in raffinose as above and were shifted to galactose to induce transcription of the GAL cluster genes. RNA fractions were isolated at 30 min intervals over a 300 min time frame. Resulting transcript profiles from three biological replicates were normalized to scr1 and plotted over time as a percentage of the average transcript levels with respect to a fully induced, wild-type ‘‘control’’ RNA for normalization between replicates. The ‘‘control’’ corresponds to total RNA isolated from wild-type cells after 5 h in galactose media following initial growth in raffinose for maximal expression. Error bars indicate the SEM. Statistical significance was calculated using a two-tailed t test. Time points with significantly different transcript levels (p<0.05) between wild-type and dcp2Δ cells for each gene are as follows: GAL10, 60–120 min time points; GAL1, 90–150, 240, 300 min time points. The 210 and 270 min time points for GAL7 correspond to p<0.10, whereas no other time points in the GAL7 analysis displayed significantly different transcript levels between wild-type and dcp2Δ cells.

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Figure 4. Rapid induction of the GAL genes correlates with faster recruitment of RNAPII and reduced corepressor binding to chromatin. (A) RNAPII is recruited faster to GAL7 (left) and GAL10 (right) promoters following a shift from repressive to activating conditions in XRN1- and DBP2-deficient cells. Wild-type, dbp2Δ, and xrn1Δ cells were shifted from transcriptionally repressive conditions (+glucose) to transcriptionally active conditions (+galactose). Cells were collected before (0 min) and at 30 min, 60 min, 120 min, and 300 min time points following a shift to galactose media. ChIP was conducted using an anti-Rpb3 antibody followed by qPCR. Results are presented as the relative Rpb3 occupancy at the GAL10 or GAL7 promoter with respect to the constitutively activated ACT1 gene. Numbers above each bar represent the fold above wild type at the same time point postinduction for both dbp2Δ and xrn1Δ cells. (B) The galactose-dependent GAL6 gene does not show increased RNAPII recruitment in dbp2Δ or xrn1Δ cells. ChIP was conducted as above followed by qPCR at GAL6 promoter. Results are represented as the relative Rpb3 occupancy at the GAL6 promoter with respect to the ACT1 gene. (C) Both dbp2Δ and xrn1Δ cells display reduced association of the Cyc8 component of the Tup1–Cyc8 co-repressor complex at GAL genes under repressive conditions. Briefly, wild-type, dbp2Δ, and xrn1Δ cells harboring a 3x-FLAG-tagged CYC8 at the endogenous locus as well as a wild-type strain with untagged CYC8 were grown under transcriptionally repressive conditions (+glucose).
representing the 0 min time point for the induction time courses above, and were then subjected to ChIP with anti-FLAG antibodies. Cyc8-3×FLAG occupancy is presented as the percentage of isolated DNA over input. Numbers above each bar represent the fraction of bound DNA in each strain versus that in the wild-type strain harboring the 3×FLAG-tagged CYC8. (D) Cyc8-3×FLAG is expressed at similar levels in wild-type, dbp2Δ, and xen1Δ strains. Western blotting was conducted with whole cell lysates from the indicated strains and Cyc8–3×Flag was detected with polyclonal anti-FLAG antibodies. Pgk1 serves as a loading control, whereas wild type with an untagged Cyc8 (lane 4) demonstrates antibody specificity.
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Derepression of GAL7 and GAL10 Correlates with Reduced Binding of the Cyc8 Corepressor

Glucose-dependent repression is accomplished by transcription factors Mig1, Mig2, and Nrg1, which recognize specific DNA sequences and subsequently recruit co-repressor complexes like the Tup1–Cyc8 complex [28,40,46–48,51,52]. To determine why dbp2Δ and xen1Δ cells exhibit faster recruitment of RNAPII, we asked if these strains display lower levels of bound co-repressors. To test this, we conducted ChIP assays to measure the association of Cyc8 at GAL7 and GAL10 at the 0 min time point when the GAL genes are repressed. We tested both the promoter and 5′ end of GAL7 and GAL10 as Tup1 has been shown to associate with the ORF and the promoter of specific gene loci [53]. Consistent with the more rapid recruitment of RNAPII, both DBP2- and XRN1-deficient cells exhibited severely reduced Cyc8 binding at both the promoter and 5′-end of the open reading frame (ORF), with levels equivalent to background ChIP signal (Figure 4C). Western blotting revealed that CYC8–3×FLAG is expressed at similar levels in all three strains, indicating that reduced binding is not due to different protein levels (Figure 4D). Thus, the rapid induction of GAL7 and GAL10 in xen1Δ and dbp2Δ cells correlates with reduced association of Cyc8 corepressor. This provides an explanation for the rapid induction of GAL gene expression from the repressed (+glucose) but not derepressed (+raffinose) conditions (Figures 1 and 2); the GAL genes are derepressed in dbp2Δ and xen1Δ strains.

The GAL lncRNAs Do Not Alter the Transcriptional Induction Profiles of GAL7 or GAL10 from Derepressed Conditions in XRN1-Deficient Cells

If derepression is caused by the GAL lncRNAs, then deletion of these noncoding RNA molecules should have no effect on the induction or final levels of GAL7 and GAL10. To determine if this is the case, we constructed xen1Δ and xen1Δ lncRNAΔ cells, as xen1Δ and dbp2Δ cells exhibit similar induction profiles. We then conducted extended time courses of wild-type, xen1Δ, lncRNAΔ, and xen1Δ lncRNAΔ strains to measure both the induction kinetics and steady-state transcript levels of the GAL genes from the derepressed (+raffinose) condition (Figure S1, representative northern blot). Resulting induction profiles were then graphed for each condition, lag times were determined as above, and the velocity of transcript accumulation and final steady-state levels were determined after normalization to SCR1 and with respect to a fully induced, wild-type strain (“control”) (Figure 5).

Consistent with our shorter time course analysis (Figure 2), both wild-type and xen1Δ cells displayed similar lag times for induction and final steady-state transcript levels for both GAL7 and GAL10 when induced from derepressive conditions (+raffinose)

Figure 5. The GAL lncRNAs do not alter the GAL7 or GAL10 transcription profile in xen1Δ cells when induced from derepressed conditions. (A–B) The xen1Δ and xen1Δ lncRNAΔ strains display superimposable transcriptional induction profiles of GAL7 (A) and GAL10 (B) from derepressed conditions. Isogenic wild-type (closed black circle), lncRNAΔ (closed red square), xen1Δ (open blue square), and xen1Δ lncRNAΔ (open green triangle) strains were analyzed for both rapid induction from derepressive conditions (+raffinose) and final, steady-state transcript levels by conducting time courses as above to 300 min. Resulting induction profiles were plotted as in Figure 3 following normalization to a fully induced GAL “control” and to SCR1. Representative northern blots are shown in Figure S1. (C–D) GAL7 (C) and GAL10 (D) transcriptional induction kinetic profiles are similar between xen1Δ and xen1Δ lncRNAΔ cells. The lag times were calculated as above for each individual biological replicate following normalization to SCR1 and are reported as the average with s.d. The Tlag and T½ correspond to the time point when transcript levels plateau and the half-time to Tmax, respectively. Initial velocities were calculated as the slope of the straight line from the lag time to Tmax with increases most likely reflecting greater transcript production in a given cell population over time. All kinetic parameters were calculated independently for each biological replicate after graphical analysis, after normalization to SCR1 and the control RNA, and are the average of the three replicates with the s.d. doi:10.1371/journal.pbio.1001715.g005
This is in line with other studies demonstrating identical induction profiles from derepressive conditions between wild-type and xrn1D cells [26,45]. Moreover, this further illustrates that GAL lncRNA-dependent repression is not a general phenotype of RNA decay-deficient strains. XRN1-deficient cells, however, showed increased transcript levels at early time points within the induction profile of both genes, as evidenced by the higher “shoulder” in the graphical analysis (Figure 5A–B) and increased initial velocities of transcript accumulation (Figure 5C–D). These increases are not due to the GAL lncRNAs though, as the induction profiles of GAL7 and GAL10 in the xrn1D strain are superimposable with the xrn1D lncRNA strain. This also demonstrates that the lncRNA mutation itself, and resulting loss of Rcb1 binding, does not impair the transcriptional activity of GAL7 or GAL10. Consistently, both the xrn1Δ and xrn1Δ lncRNA strains have similar kinetic parameters for transcriptional induction. This includes identical initial velocities as well as half time (T1/2) and time to maximum transcript levels (Tmax) between xrn1Δ strains regardless of the presence or absence of the GAL lncRNAs (Figure 5C–D). Thus, the GAL lncRNAs do not alter the transcriptional induction of the GAL genes in XRN1-deficient cells from derepressive conditions.

The GAL lncRNAs Alter the Kinetics of Induction from Repressed Conditions in xrn1Δ Cells

We then analyzed the transcriptional induction kinetics of GAL7 (E) and GAL10 (F) induction in wild-type cells (closed black circle) from derepressed to activated conditions as compared to xrn1Δ cells (open blue square) from repressive conditions. DOI:10.1371/journal.pbio.1001715.g006
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transcriptional profiles between xrn1Δ and wild-type cells (Figure 6). In fact, xrn1Δ cells showed shorter lag times as well as ~3-fold higher levels of GAL7 and GAL10 transcripts as compared to wild type (Figure 6A–B). Kinetic analysis revealed that xrn1Δ cells have a more rapid approach to steady state than wild-type cells when induced from repressive conditions, as evidenced by the reduced lag time and 3- to 6-fold increase in the initial rate (V₀) of transcript accumulation for both GAL7 and GAL10 (Figure 6C–D). This is also illustrated by the fact that xrn1Δ cells reach 100% of the fully induced “control” within the 300 min time frame, while wild-type cells do not (Figure 6A–B). This rapid, high-level induction in xrn1Δ cells during the switch from repressed to activated conditions is consistent with the reduced association of Cyc8 and faster recruitment of RNAPII (see Figure 4).

Strikingly, removal of the GAL lncRNAs abolished both the rapid induction and high transcript levels in the xrn1Δ strain, resulting in profiles more similar to wild type (Figure 6A–B). In fact, the GAL10 induction profile of xrn1Δ lncRNAΔ cells is superimposable with that of wild-type cells, demonstrating that the rapid induction of this gene in xrn1Δ cells is fully dependent on the GAL lncRNAs (Figure 6B,D). The induction profile of GAL7 was also restored by incorporation of the lncRNAΔ mutation into the xrn1Δ strain, but to a lesser extent (Figure 6A–C). This partial reduction may be due to the contribution of another lncRNA that overlaps GAL7, as prior studies have indicated the presence of a GAL7 antisense transcript that originates outside of the lncRNAΔ mutation [24]. Interestingly, removal of the GAL lncRNAs resulted in both a longer lag time as well as decreased initial velocity in XRV1-deficient cells (Figure 6C–D). This suggests that the GAL

Figure 7. The GAL lncRNAs kinetically enhance GAL gene induction from repressed conditions in wild-type cells. (A–B) The GAL lncRNAs increase the rate of GAL7 and GAL10 activation in wild-type cells. Graphical representation of transcriptional induction of GAL7 (A) and GAL10 (B) in wild-type (closed black circle) and lncRNAΔ (closed red square) strains from repressed to activated conditions. High-resolution transcriptional analysis was conducted with wild-type or lncRNAΔ cells from repressed conditions from 0 to 300 min by including 10 additional 10-min time points between 90 and 150 min. Transcript abundance is reported as a percentage of the control as previously described. The differences in final transcript levels at the 300 min time point are not statistically significant (p value > 0.2). Representative northern blots are shown in Figure S3. (C–D) The GAL lncRNAs increase the kinetics of transcriptional activation from repressive conditions. Transcription induction parameters for the wild-type and lncRNAΔ strains were determined as above for three independent biological replicates. Calculated lag times were determined using curve-fitting analysis (DM Fit v. 2.0) [54], which facilitates quantitative assessment of lag from the curve fit (Figure S4). Lag times assessed from the data points as in prior figures are denoted as “estimated” lag times for differentiation from the curve fitting values. The estimated lag times result in p values from a two-tailed t test of 0.12 and 0.09 for GAL7 and GAL10, respectively, whereas calculated lag times are significantly different between wild-type and lncRNAΔ strains (GAL7 lag p value = 0.01; GAL10 lag p value = 0.07). The initial velocities of transcript accumulation are not significantly different. (E–F) The presence of GAL lncRNAs does not alter the final levels of GAL7 and GAL10 transcripts at longer time points postactivation. GAL7 (E) and GAL10 (F) transcript levels were measured by RT-qPCR under repressed conditions (0 min time point) and after a 12-h shift to activated conditions (12-h time point) from repressed to activating conditions. GAL7 and GAL10 transcripts were measured from three biological replicates and normalized to ACT1. Normalized expression is presented as the average fold change from the first wild-type biological replicate with error bars representing the SEM. Note that the GAL10 lncRNA, which is also recognized by the GAL10 primers, is not evident at the 0 min time point due to the high expression levels of GAL7 and GAL10 after 12 h and necessary scaling of the bar graph.
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lncRNAs function at the kinetic level by enhancing the approach to steady state. It also indicates that the GAL lncRNA molecules have the largest impact at the point of induction of the protein-coding GAL genes.

Next, we asked if the induction of xrn1Δ cells from repressed conditions (+glucose) is similar to that of wild-type cells from derepressed conditions (+raffinose), with the idea that lncRNA-dependent derepression in XRN1-deficient cells should mimic the derepressed transcriptional state in wild-type cells. Overlaying the GAL7 and GAL10 induction profiles revealed that xrn1Δ cells exhibit a similar induction trend from repressed conditions as wild-type cells induced from derepressed conditions (Figure 6E–F). This is consistent with the fact that xrn1Δ cells have reduced association of Cyc8 (Figure 4) and the idea that the GAL lncRNAs promote derepression of the protein-coding GAL genes in xrn1Δ cells. The difference in shape of the two curves between wild type and xrn1Δ may reflect the activity of other, glucose-dependent repression mechanisms (see Discussion) or the presence of low levels of Cyc8 at the GAL gene promoters that are below our detection by ChIP. Regardless, this is consistent with a model whereby the GAL lncRNAs activate gene expression by promoting derepression.

The GAL LncRNAs Promote Induction of GAL7 and GAL10 Genes from Repressed Conditions in Wild-Type Cells

Our results above demonstrate a positive role for the GAL lncRNAs in promoting gene expression. Furthermore, our studies suggest that these noncoding RNAs impact the timing of transcriptional activation by stimulating the kinetics of induction. Given this knowledge, we then asked if the GAL lncRNAs have any effect in wild-type cells, which were not initially evident due to the analysis of induction with 30 min time points. To this end, we conducted a higher time-resolved analysis of GAL7 and GAL10 induction from repressed conditions in wild-type and lncRNA Δ strains (Figure 7A–B; Figure S3). More specifically, wild-type cells expressing the GAL lncRNAs induced both GAL7 and GAL10 faster than the lncRNA Δ cells, resulting in a clear separation of the transcriptional profiles between the two strains along the x-axis (Figure 7A–B). Lag time calculation revealed that lncRNA Δ cells lacking the GAL lncRNAs exhibit transcriptional lags of ~125–137 min, and wild-type cells induced both GAL7 and GAL10 ~30 min faster (Figure 7C–D, estimated lag time). This suggests that the GAL lncRNAs promote induction in wild-type cells. To more quantitatively assess lag times between wild-type and lncRNA Δ strains, we then utilized a curve fitting method for mathematical assignment of lag times (DM fit v2.0 Excel Macro [54], which was only possible with higher time-resolved analysis (Figure S4). The calculated lag times, although similar in magnitude to the estimates, resulted in more statistically significant
differences between wild-type and IncRNAA strains (Figure 7C–D; p values<0.1 for both genes). This suggests that curve fitting may be a more accurate assessment of lag times from biological data sets. More importantly, however, this demonstrates that the GAL IncRNAs promote a subtle but reproducible acceleration of induction in wild-type cells. In contrast to the IncRNA-dependent reduction of lag times, we did not observe a significant difference in the initial velocity of transcript accumulation between strains, however (Figure 7C–D). This indicates that either the GAL IncRNAs do not alter transcript accumulation rates in wild-type cells or that this effect is not evident by analysis across a cell population when the IncRNA levels are low (est. 1 in 14 cells in [24]). Regardless, the statistically significant shift in lag times suggests that the GAL IncRNAs enhance the induction of the GAL7 and GAL10 genes in wild-type cells, consistent with an effect on induction kinetics rather than steady-state levels. Moreover, the final levels of GAL7 and GAL10 within the 5-h time course (Figure 7A–B) or after 12 h postinduction were not significantly different between wild-type and IncRNAA strains (Figure 7E–F). This indicates that the GAL IncRNAs promote transcriptional induction in wild-type cells without altering the final transcript abundance of the targeted protein-coding genes. We propose that the GAL IncRNAs poise the protein-coding GAL genes for rapid induction, thereby enhancing the transcriptional switch from repressed to activated conditions.

Taken together, our studies demonstrate that the GAL IncRNAs enhance the activation kinetics of the inducible GAL genes from repressed conditions. Based on these observations, we present a model whereby the GAL IncRNAs displace glucose-dependent repressors from the GAL gene promoters under typically repressive conditions (Figure 8). Because this role does not result in full derepression in wild-type cells, we suggest that this displacement is transient due to the action of Dhp2 and Xrn1, which promote IncRNA release and decay, respectively [37,38,43,55]. If this is the case, this suggests that the GAL IncRNAs complement the roles of proteinaceous factors to increase the efficiency of the GAL gene transcriptional switch [29,39,56]. Moreover, these studies indicate that the GAL IncRNAs promote formation of a dynamic chromatin template. These dynamics facilitate faster activation by poising the GAL genes for induction in response to galactose, which may provide a selective advantage for cells responding to changing environmental conditions. This indicates that the GAL IncRNAs temporally regulate gene expression by influencing the rate of transcriptional responses to extracellular stimuli.

Discussion

In an effort to define the role of the GAL10b IncRNA at the GAL cluster, our studies uncovered an important new role for both this IncRNA and the previously characterized GAL10 IncRNA in activating gene expression from repressed conditions [24–26]. Glucose-dependent repression of the GAL genes is accomplished through several, mechanistically distinct processes including inhibition of the Gal4 activator, reduction of intracellular galactose uptake, and transcriptional repression of GAL promoters [28,31,34,40,46,48,51,56]. Our studies suggest that the GAL IncRNAs act on the latter mechanism by transiently displacing repressors from bound promoters, eliciting a dynamic equilibrium between derepressed and repressed states (Figure 8). We predict that this equilibrium poises the GAL genes for rapid induction, enhancing the transcriptional switch in response to extracellular signals.

The role of the GAL IncRNAs in enhancing induction is distinctly different from a true role in transcriptional activation, as has been documented for the roX RNAs in Drosophila or the activating ncRNAs (ncRNA-a) in mammalian cells [10,57]. Instead, our studies are more consistent with an interference-based model, whereby the GAL IncRNAs prevent the association of transcription factors with targeted gene promoters. This is supported by our observation that the GAL IncRNAs prevent derepression by reducing the association of Cyc8 with the GAL genes. It is also in line with the fact that the GAL genes are not activated by the GAL IncRNAs per se but that the rate of induction is faster. It is also important to note that the kinetics reported here reflect the average transcriptional profile across a cell population and not the profile of individual cells. Because the abundance of the GAL mRNAs varies widely across single cells during early induction [58], it is possible that the IncRNA-dependent derepression proposed here facilitates a more robust mRNA accumulation in individual cells. Alternatively, the GAL IncRNAs may allow a larger population of cells to rapidly “switch” from repression to activation. Recent studies of the antisense PHO084 IncRNA have proposed such a model whereby synthesis of this IncRNA results in cellular heterogeneity within a culture, with a fraction of cells exhibiting IncRNA-dependent repression [24,59].

One of the most surprising aspects of our findings is that the GAL10 IncRNA was thought to be exclusively repressive, [24,25]. Although our studies show that both the GAL10 and GAL10b IncRNAs promote gene expression, this is not necessarily mutually exclusive with a repressive role under specific conditions. However, it should be noted that the mechanism by which GAL IncRNAs induce transcriptional repression is still unknown. Early analysis of the GAL10 IncRNA reported a delay of induction in a mixed glucose/galactose carbon source, making mechanistic insight difficult due to simultaneous presence of repressors and activators [24]. Subsequent studies then suggested that the GAL IncRNAs are repressive based on defective induction of the GAL genes in RNA decapping and decay-deficient strains [25]. While our studies corroborate the requirement for decapping for normal expression of the GAL1, and to a lesser extent GAL10, the fact that xrn1Δ cells do not show expression deficiencies and that both xrn1Δ and dcp2Δ cells show enhanced induction from repressed conditions argues against a repressive model. Instead, it is more likely that both the apparent expression defects in dcp2Δ cells and enhanced transcriptional induction occur through a common mechanism, whereby the GAL IncRNAs simply occlude transcription-factor binding sites at the targeted promoters. These transcription factors include glucose-dependent repressors when the GAL genes are induced from repressive conditions. However, the high level of the GAL IncRNAs in dcp2Δ cells may also cause interference with Gal4 or transcriptional coactivators such as SAGA and/or Mediator. This model would account for both the decreased transcriptional activity and histone acetylation at targeted chromatin (Figure 3) [25]. It is not clear, however, why GAL1 is more sensitive to loss of decapping than other genes within the GAL cluster. Alternatively, the decreased transcriptional activity in dcp2Δ cells may be due to the recently proposed, and as-of-yet uncharacterized, role for decapping and decay factors in transcription [45]. Nevertheless, the fact that ablation of the GAL10 IncRNA rescues GAL1 transcriptional delays indicates that at least some part of the expression defect in dcp2Δ cells is dependent on the IncRNA [25]. Interestingly, the Set3C histone deactylase complex has also been shown to influence the kinetics of inducible genes [60], suggesting that IncRNA-dependent gene expression involves a complex interplay between histone modifications, IncRNAs, and metabolic genes.
One mechanism for promoter occlusion by lncRNAs is the formation of transient lncRNA-DNA hybrids at the GAL gene promoters. RNA-DNA hybrids, or R loops, are found in all organisms from yeast to humans and have been recently linked to regulation of chromatin architecture [61–63]. These structures form during transcription and have historically been associated with defects in termination and/or mRNA assembly (for review, see [63]). However, recent studies have found widespread formation of RNA-DNA hybrids at multiple gene loci in normal cells, with roles linked to transcriptional regulation, termination, replication, and recombination [63–65]. Interestingly, the mammalian DHFR lncRNA forms an RNA-DNA triplex at the DHFR promoter [23]. This lncRNA represses transcription of the DHFR gene by interfering with the association of the TFIIB basal transcription factor, demonstrating that formation of this RNA-DNA hybrid occludes the binding site for the transcriptional apparatus. Although not an R loop, this study is consistent with the idea that lncRNAs may act through base pairing with target DNA. Recent studies implicating Dhp2 in both co-transcriptional mRNP assembly and in termination of coding and noncoding RNAs [37,55], two processes that prevent RNA-DNA hybrid formation, is also suggestive of a role for these nucleic acid structures in GAL gene induction. This model may even account for transcriptional interference of GAL7, and reduced association of the Gal4 activator, in strains with defects in GAL10 transcriptional termination [66,67]. Moreover, recent work from the Tollervey lab has revealed striking differences in the termination/3′-end formation pathways and assembly of mRNA export factors between the majority of lncRNAs as compared to mRNAs, suggesting that the function of a transcript may be largely determined at late maturation steps [68]. The fact that p68, the human ortholog of Dhp2, also functions in lncRNA-dependent gene regulation suggests that the role for Dhp2 in RNA-mediated transcriptional control may be conserved between yeast and multicellular eukaryotes [69,70].

Due to predominantly cytoplasmic localization [71–73], both Xrn1 and Dcp2 were long thought to function solely in cytoplasmic RNA decay. However, studies of noncoding RNAs implicated both of these factors in nuclear RNA decay, as loss of either gene product elicited transcriptional defects [25,38,74]. The Choder laboratory has now provided evidence that both of these RNA decay factors are present in the nucleus and associate with chromatin [45]. Although it was suggested that these RNA decay factors promote transcription through an as-of-yet uncharacterized mechanism, it is possible that Xrn1 and Dcp2 function in co-transcriptional RNA decay. If this is the case, RNA-DNA hybrids may accumulate in rRNA and dRNA2a strains as a result of failure to “clear” aberrant transcriptional products. This would be consistent with prior studies showing that the GAL10 lncRNA functions in cis by suggesting that these decay enzymes also function at the site of synthesis [24].

Given that the GAL lncRNAs promote induction, one might ask why we do not observe a net increase in steady-state transcript levels. This is consistent with studies of the Set3C complex, whose loss results in altered GAL gene induction kinetics with no effect on the final transcript levels [60]. Moreover, this is a well-known phenomenon in pre–steady state enzyme kinetics, which depends on different mechanisms than steady state [75]. In the case of GAL7 and GAL10 expression, steady state is the period when the rate of RNA synthesis and decay are matched. Pre–steady state, however, is governed by release of transcriptional repressors and recruitment of RnapII. Our data strongly suggest that it is these latter two processes that are likely accelerated by the GAL lncRNAs.

The idea that lncRNAs play a kinetic role was initially put forth by studies of the PHO5 lncRNA, which promotes transcriptional activation of the PHO5 gene by altering the rate of chromatin remodeling [15]. It is well established that the protein-coding genes within the GAL cluster are highly regulated through carbon-source-specific transcription factors [27,29,32,40]. Upon a switch to galactose, glucose-dependent transcription factors are shunted to the cytoplasm, and the transcriptional activator Gal4 is released from the Gal80 inhibitor (Figure 7) [28,32,56,76]. Our studies now show that the GAL lncRNAs add to this mechanism by promoting this transcriptional switch. This suggests that lncRNAs promote “kinetic synergism,” which is a model stating that kinetic alterations can have greater, combined effects on transcription than thermodynamics alone [77]. Kinetic synergism describes how the combination of multiple slow steps in transcriptional induction results in a more rapid and effective transcriptional activation. The GAL lncRNAs would function by promoting a more dynamic chromatin template, which synergistically enhances the activity of transcription factors by allowing transient access to DNA.

Our studies now complement the current knowledge regarding the function of lncRNAs by demonstrating that lncRNAs can influence the rate of transcriptional responses to extracellular cues. This is an exciting possibility because it suggests that the presence of lncRNAs may confer a selective advantage for a given organism to rapidly adapt to changing conditions. For example, wild-type cells could begin utilizing galactose as a carbon source at least 30 min earlier than cells without the GAL lncRNAs (Figure 7). This ability to influence the timing of a transcriptional switch would provide a rationale for the presence of lncRNAs in all eukaryotes and the conservation of these molecules near developmentally regulated genes in multicellular organisms [13,17,18,21,22]. Moreover, the ability of lncRNAs to alter chromatin dynamics may provide a more universal, functional role for widespread transcription of these noncoding molecules. Analysis of temporal effects of lncRNAs in multicellular organisms represents a future challenge in deciphering the role of these multifunctional regulators of the eukaryotic genome.

Materials and Methods

Plasmids and Strains

All plasmids were constructed by standard molecular biology techniques and are listed in Table 1. Yeast strains were constructed using classical yeast genetic techniques and are listed in Table 2. Oligos for PCR-mediated homologous recombination are listed in Table 3.

| Table 1. Template plasmids for northern blot probes and strain construction. |
|----------------------------------------|-------------------|----------------------|
| Name | Description | Source/Reference |
| pGAL1-GAL10-GAL7 | pYGPM111H4 | Open Biosystems |
| pSCHR | pYGPM29b01 | Open Biosystems |
| pUG6 | KanMX disruption cassette plasmid | (78) |
| pAG32 | HygB disruption cassette plasmid | (79) |
| p3×FLAG | p3×FLAG/KanMX | (80) |

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GAL Induction Analyses

Time courses were performed by growing strains at 30°C to an OD₆₀₀ of 0.4 in YP 2% glucose or raffinose when indicated and shifting to 2% galactose media. 3OD units were harvested at OD₆₀₀ of 0.4 in YP 2% glucose or raffinose when indicated and OD₆₀₀ = 100%, whereby GAL mRNA signal above background after normalization to SCR1. Lag time error between biological replicates is reported as the standard deviation to illustrate the range of variation. Transcript levels were determined as the percentage of a wild-type control using the following equation: (GAL Transcript Signal/SCR1 signal) ÷ (GAL Control/SCR1 Control)×100%, whereby GAL positive corresponds to total RNA from a wild-type culture following a 300-min induction from derepressive (+raffinose) conditions. Transcriptional profiles were fitted to a dose response curve with variable slope in GraphPad Prism using the following equation: Y = lowest level + (highest level − lowest level)×(1+10⁻X×HillSlope). Tₘₐₓ corresponds to the first time point in a series when the GAL mRNA signal reaches a steady-state plateau, whereas initial velocities were determined by calculating the slope of a straight line with increasing GAL mRNA signal above background after normalization to SCR1. Lag time error between biological replicates is reported as the standard deviation to illustrate the range of variation. Transcript levels were determined as the percentage of a wild-type control using the following equation: (GAL Transcript Signal/SCR1 signal) ÷ (GAL Control/SCR1 Control)×100%, whereby GAL positive corresponds to total RNA from a wild-type culture following a 300-min induction from derepressive (+raffinose) conditions. Transcriptional profiles were fitted to a dose response curve with variable slope in GraphPad Prism using the following equation: Y = lowest level + (highest level − lowest level)×(1+10⁻X×HillSlope). Tₘₐₓ corresponds to the first time point in a series when the GAL mRNA signal reaches a steady-state plateau, whereas initial velocities were determined by calculating the slope of a straight line with increasing GAL mRNA signal above background after normalization to SCR1. Lag time error between biological replicates is reported as the standard deviation to illustrate the range of variation. Transcript levels were determined as the percentage of a wild-type control using the following equation: (GAL Transcript Signal/SCR1 signal) ÷ (GAL Control/SCR1 Control)×100%, whereby GAL positive corresponds to total RNA from a wild-type culture following a 300-min induction from derepressive (+raffinose) conditions. Transcriptional profiles were fitted to a dose response curve with variable slope in GraphPad Prism using the following equation: Y = lowest level + (highest level − lowest level)×(1+10⁻X×HillSlope). Tₘₐₓ corresponds to the first time point in a series when the GAL mRNA signal reaches a steady-state plateau, whereas initial velocities were determined by calculating the slope of a straight
error between transcript levels as SEM.

experiments were conducted with three biological replicates with 2.0) [54] and are reported as the average with the s.d. All replicate to a multivariable, exponential growth curve (DM Fit v.

calculated lag times in Figure 7 were determined average time to reach 50% maximum transcript levels within the RNA Isolation and Quantitation

line from the lag time to the Tmax. T 1/2 times correspond to the are listed in Table 5.

primers are listed in Table 4. Primers for Northern blotting probes using the DNA plasmid templates listed in Table 1. RT-qPCR performed as in [37]. Probes were made from PCR products

Table 4. RT-qPCR oligos.

| Name     | Forward               | Reverse               | Probe               |
|----------|-----------------------|-----------------------|---------------------|
| nc10 F   | GAGGTCTTGACCAAGGATCGCA | GCTCATGTGCTATATGAGCTCGG | CGGTGAGACGGAGGACGGAC |
| nc10 R   | TTCCAGACCTTTTGCTGACA  | AGGGAATGTGGATCTTGTCG  | TGACTGTGGTTCGTGTAACCTG|
| nc7 F    | TGAACCAAGGCTATAGGAGCA | TCCGAGCATATCGGCTGACA  | CCGTAGAACGAGGACGGAC |
| nc7 R    | CGAGGATATAGCCGGATGAGA | CAAAATGCGTCGGCAGAACACT | |
| GAL10 5’ F | GGAGTTGCTACACACGATGAC | TTTCCGACCTTCTTGACGAC  | |
| GAL10 5’ R | TCCGAGACCTTTTGCTGACA  | AGGGAATGTGGATCTTGTCG  | |
| GAL7 5’ F | CAAAATGCGTCGGCAGAACACT | GCTTTATTTCCTGGAGTGCAG | |
| GAL7 5’ R | GCATTGCAGATTTTGACGACG | CTTTTATTTCCTGGAGTGCAG | |
| ACT1 F   | TGATCCCGTGTAGGTGT    | CGGTGAGACGGAGGACGGAC | |
| ACT1 R   | TCAAATGCGTCGGGAGTACAG | CCGTAGAACGAGGACGGAC | |

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Table 5. Oligos for northern blotting (dsDNA probes).

| Name     | Forward               | Reverse               |
|----------|-----------------------|-----------------------|
| SCR1 F   | GGATACGTTGAGAATCTGGCCGGAGG | |
| SCR1 R   | AATTGCGGAGAATAATGATGAGCATGTCGACC | |
| GAL7 F   | CCTTTAGCTGTCAGCAGAGG | |
| GAL7 R   | AGTCGAGATCAACAGGACGC | |
| GAL10 F  | GCATCAATGCTCTACTTGAG | |
| GAL10 R  | ACATTAGGCTACATGCGG | |
| GAL1 F   | TGGACGCTTCTATGTCAC | |
| GAL1 R   | GAGACTGTTCATCAAGACGC | |
| SCR1 F   | GGATACGTTGAGAATCTGGCCGGAGG | |
| SCR1 R   | AATTGCGGAGAATAATGATGAGCATGTCGACC | |
| GAL7 F   | CCTTTAGCTGTCAGCAGAGG | |
| GAL7 R   | AGTCGAGATCAACAGGACGC | |
| GAL10 F  | GCATCAATGCTCTACTTGAG | |
| GAL10 R  | ACATTAGGCTACATGCGG | |
| GAL1 F   | TGGACGCTTCTATGTCAC | |
| GAL1 R   | GAGACTGTTCATCAAGACGC | |

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line from the lag time to the T max. T 1/2 times correspond to the average time to reach 50% maximum transcript levels within the cell population. Calculated lag times in Figure 7 were determined by fitting transcriptional induction data points for each biological replicate to a multivariable, exponential growth curve (DM Fit v. 2.0) [54] and are reported as the average with the s.d. All experiments were conducted with three biological replicates with error between transcript levels as SEM.

RNA Isolation and Quantitation

RNA extraction, northern blotting, and RT-qPCR were performed as in [37]. Probes were made from PCR products using the DNA plasmid templates listed in Table 1. RT-qPCR primers are listed in Table 4. Primers for Northern blotting probes are listed in Table 5.

ChIP Analysis

ChIP was performed as described previously [37], with the following modifications. After formaldehyde fixation, cells were pelleted and washed twice with cold wash buffer (50 mM HEPEs-KOH, 140 mM NaCl, and 1 mM EDTA) and frozen in liquid nitrogen. Cells were then lysed cryogenically using a Retsch Oscillating Mill MM400. Cell lysates were then resuspended in cold Lysis Buffer (50 mM HEPEs-KOH, 140 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.1% sodium deoxycholate, 1 mM PMSF and 1 x protease inhibitor (complete, ET-Dera-free, Roche)), and chromatin was sheared by sonication. For Cyc8–3flag ChIP, chromatin from ~1.4 x 10 6 cells was immunoprecipitated with 1 μL of FLAG M2 monoconal antibody (F3163, Sigma) and 12 μL of Protein G Dynabeads (30 mg/mL, Invitrogen) at 4°C for 2 h. For PolII ChIP, chromatin from 2–3 x 10 6 cells was immunoprecipitated with 1 μL of Rpb3 monoclonal antibody (WP012, Neocline) and 12 μL of Protein G Dynabeads (30 mg/mL, Invitrogen) at 4°C for 2 h. Immunoprecipitated DNA was isolated as described previously [37]. Quantitative PCR was performed using Bio-Rad CFX96 Real-time system using PrimeTime Assay primers purchased from IDT (Table 6). All ChIP experiments were performed with three biological replicates and three technical repeats. Error bars represent the SEM of three biological replicates.

Yeast Cell Lysate Preparation and Western Blotting

Yeast cells were grown in YP 2% glucose to an O.D. of 0.4–0.6. We harvested 30 mg of yeast cells and lysed them with 1.85 M NaOH and 7.4% β-mercaptoethanol on ice for 10 min. Proteins were precipitated with 50% TCA on ice for 10 min and resuspended into 300 μL 1x SDS-PAGE loading dye. We then resolved 1–1.5 mg proteins by SDS-PAGE, and transferred them to a nitrocellulose membrane. FLAG-tagged Cyc8 and Pgk1 were detected by rabbit anti-3×FLAGS (F7425, Sigma) and monoclonal mouse anti-yeast Pgk1 (459250, Invitrogen), respectively. Proteins were visualized by alkaline phosphatase-based detection using AP-conjugated anti-rabbit secondary antibody and AP-conjugated anti-mouse secondary antibody, respectively, followed by a BCIP/NBT chemistry (S3771, Promega).

Supporting Information

Figure S1 Representative northern blots for GAL7 and GAL10 induction from derepressed conditions in XRN1-deficient cells. (A–B) GAL7 (A) and GAL10 (B) induction profile of one biological replicate for wild-type, lncRNA, xrn1A, and lncRNA xrn1A strains from derepressed conditions. Transcriptional induction assays were conducted from cells grown in derepressive (+raffinose) to activated (+galactose) conditions. GAL7 and GAL10 transcripts were detected by northern blotting using a

Table 6. Primetime assays for ChIP.

| Name       | Forward               | Reverse               | Probe               |
|------------|-----------------------|-----------------------|---------------------|
| GAL10 promoter | CTTTATGGTCTGGAGCAGTGC  | GCTCATGTGCTATATTGAGCTACG | CGGTGAGACGGAGGACGGAC |
| GAL10 5’   | TGGTCTGGCTGATACATTGTTC | AGGGAATGTGGATCTTGTCG  | TGACTGTGGTTCGTGTAACCTG |
| GAL7 promoter | GCGGCTCGGACAATGCTTGT  | TTTGCCAGCCTCTTTTATATCCTTG | CCGTAGATCGGACTGTCCTACA |
| GAL7 5’    | ATCATACATGGGACGGTGGGG | ATGAGGACGGCTGCTGATACCC | |
| GAL6 promoter | CGGAGAAGTACCCGCTCTC  | GCGTAGAAACAAAAGAGCAGGAGG | CCGGAGGCGGACCCCATATA |
| ACT1 middle | ATGGAGGATGGCCTCCAGAAGG | ATGAGGATGGCCTCCAGAAGG | |

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POM 32P-labeled double-stranded DNA probe as in Figure 1. SCR1 was detected similarly and serves as a loading control. Lag times correspond to the average time to detection of GAL transcripts for the three independent biological replicates shown in Figure 5 following normalization to SCR1 and the control RNA (see Figure S4). RONAPII bands are detectible in wild-type and lncRNA strains in (B) at the 30 min time point (yielding similar lag times for all strains), but appear weaker than in wild-type strains due to loading differences between blots.

Figure S2 Representative northern blots for GAL7 and GAL10 induction from repressed conditions in XRN1-deficient cells. (A–B) High-resolution analysis of transcriptional induction of one biological replicate for wild-type, lncRNA, and wild-type lncRNA strains from repressed conditions. Transcriptional induction assays were conducted as above during the switch from repressed (r-glucose) to activated (r-galactose) conditions. Lag times correspond to the average time to detection of GAL transcripts for the three independent biological replicates shown in Figure 6 and are calculated following normalization to SCR1 and the GAL control. (TIF)

Figure S3 Transcriptional induction assays for wild-type and lncRNA strains from repressed to activated conditions. (A–B) High-resolution analysis of transcriptional induction in wild-type and lncRNA cells. Transcription induction was measured in wild-type or lncRNA cells from repressed conditions as above with the inclusion of additional 10 min time points from 90–150 min immediately prior to recruitment of RNAIVP [see Figure 4]. Lag times are not determined visually from the blots but were calculated as the average across three biological replicates after normalization to the SCR1 loading control. (TIF)

Figure S4 Individual transcriptional induction profiles following curve fitting analysis. Individual biological replicates of induction profiles of wild-type and lncRNA strains from repressed to activated conditions. Transcript levels were normalized to SCR1 and the GAL “control” RNA as above. Resulting data points were then fit to a dynamic exponential growth curve (DM fit v. 2.0) [54]. R2 values and lag times are shown for each individual profile. Calculated lag times are reported in Figure 7 (C and D) and correspond to the average lag time and s.d. for induction of GAL7 and GAL10 after curve fitting for wild-type and lncRNA strains.

(TIF)

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Author Contributions

The author(s) have made the following declarations about their contributions: Conceived and designed the experiments: WKM SCJ EJT. Performed the experiments: WKM SW SCC CJP EJT. Analyzed the data: WKM SW SCC JCP. Contributed reagents/materials/analysis tools: WKM SW SCC. Wrote the paper: EJT.

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