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## Regulated Formation of LncRNA-DNA Hybrids Enables Faster Transcriptional Induction and Environmental Adaptation

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### Summary

Long non-coding (lnc)RNAs, once thought to merely represent noise from imprecise transcription initiation, have now emerged as major regulatory entities in all eukaryotes. In contrast to the rapidly expanding identification of individual lncRNAs, mechanistic characterization has lagged behind. Here we provide evidence that the GAL lncRNAs in the budding yeast *S. cerevisiae* promote transcriptional induction in *trans* by formation of lncRNA-DNA hybrids or R-loops. The evolutionarily conserved RNA helicase Dbp2 regulates formation of these R-loops as genomic deletion or nuclear depletion results in accumulation of these structures across the GAL cluster gene promoters and coding regions. Enhanced transcriptional induction is manifested by lncRNA-dependent displacement of the Cyc8 co-repressor and subsequent gene looping, suggesting that these lncRNAs promote induction by altering chromatin architecture. Moreover, the GAL lncRNAs confer a competitive fitness advantage to yeast cells as expression of these non-coding molecules correlates with faster adaptation in response to an environmental switch.

### Introduction

Eukaryotic genomes are pervasively transcribed, but only a small fraction of these transcripts are translated into proteins. Instead, most of this activity corresponds to non-coding RNAs that encompass a wide variety of functional classes, including ribosomal

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#### Author Contributions

S. C. C. conducted transcriptional analysis, DRIP, genetic manipulations, and growth assays. W.K.M. performed ss-RT-qPCR. S. W. and P.E.P. conducted bioinformatic and statistical analysis. N. A. H. and Z. D. conducted 3C. This manuscript was written by S. C. C. and E. J. T. with input from A. Z., P. E. P.



fact that Dbp2 is exported from the nucleus during the shift from glucose to galactose-containing media (Beck et al., 2014), suggests that loss of nuclear Dbp2 may prime the *GAL* genes for rapid, lncRNA-dependent induction. Here we provide the mechanism and physiological role for the *GAL* lncRNAs in transcriptional induction from a transcriptionally repressed state in *S. cerevisiae* and demonstrate that Dbp2 regulates this process by controlling formation of *GAL* lncRNA-dependent R-loops. These studies underscore an emerging role for R-loops in lncRNA-dependent gene regulation and provide evidence that lncRNAs enable rapid adaptation by temporally modulating transcriptional activation.

## Results

### GAL lncRNAs Function in Trans to Enhance Activation of the GAL Cluster Genes

The *GAL* lncRNAs promote transcriptional induction during a transition from repressive to transcriptionally active conditions (Cloutier et al., 2013). To determine if lncRNA-dependent induction occurs *in cis*, we conducted transcriptional induction assays of the *GAL* genes when the *GAL* lncRNAs were encoded *in cis* or *in trans* (Figure 1A). These studies were conducted in the context of the *dbp2* strain, because loss of *DBP2* exacerbates *GAL* lncRNA-dependent induction. To construct the *dbp2 trans-lncRNA* strain, we designed a *GAL10-GAL7* construct with mutations in the Gal4-binding sites within the bidirectional *GAL1-GAL10* and *GAL7* promoter (MacIsaac et al., 2006; Rhee and Pugh, 2011) to prevent transcription of the sense *GAL10* and *GAL7* genes without impacting synthesis of the *GAL* lncRNAs. This *trans-lncRNA* construct was subsequently integrated into the *dbp2 lncRNA* strain, which harbors silent mutations of the Reb1 binding sites, preventing synthesis of the *GAL10* and *GAL10s* lncRNA (Cloutier et al., 2013; Houseley et al., 2008). Strand-specific RT-qPCR (ssRT-qPCR) confirmed expression of the *trans-lncRNAs* in both wild type and *dbp2* cells and showed that the expression levels are similar to the *cis*-encoded transcripts (Figures 1B and 1C). Moreover, both the *cis* and *trans-lncRNA* constructs exhibited overabundance of the *GAL* lncRNAs in *dbp2* cells consistent with prior studies (Cloutier et al., 2012; Cloutier et al., 2013).

We then conducted transcriptional induction assays by analyzing *GAL1*, *GAL10* and *GAL7* transcript levels in wild type, *dbp2*, *dbp2 trans-lncRNA*, and *dbp2 lncRNA* strains by northern blotting RNAs isolated at time points during a 300 min glucose to galactose (repressed to activated) shift. Consistent with previous studies (Cloutier et al., 2013), *dbp2* cells with the *GAL* lncRNAs *in cis* exhibited rapid induction of *GAL1*, *GAL10* and *GAL7* genes, which was ~5 fold faster than either wild type or the *dbp2 lncRNA* cells (Figures 1D-1F and S1). Expression of the *GAL* lncRNAs *in trans* in *dbp2* cells also promoted rapid induction of all three protein-coding *GAL* genes (Figures 1D-1F and S1). In fact, induction of *GAL1*, *GAL10* and *GAL7* in *dbp2* cells was identical regardless of whether the *GAL* lncRNA was encoded *in cis* or *in trans* (Figures 1D-1F and S1). This suggests that the lncRNA molecule itself, rather than the act of transcription, promotes transcriptional induction.

## Ectopic Expression of RNase H1 Represses Rapid Induction in *dbp2* Cells

RNA-DNA hybrids, termed R-loops, have recently been implicated in lncRNA-dependent gene activation in mammalian cells (Boque-Sastre et al., 2015; Pefanis et al., 2015). To determine if the rapid, lncRNA-dependent induction in *dbp2* cells is due to formation of R-loops, we conducted transcription induction assays with strains expressing plasmid-encoded human RNase H1 (pRNH1), an enzyme that degrades the RNA within an RNA-DNA hybrid, or empty vector (Wahba et al., 2011). Unexpectedly, use of selective growth media resulted in a reduced transcriptional induction of the *GAL* genes in both wild type and *dbp2* strains lacking the *GAL* lncRNAs (*dbp2 lncRNA*) (Figures 2A-2C and S2A-S2C). However, when comparing the profiles of RNase H1-expressing *dbp2* cells to vector alone, an RNase H1-dependent reduction in the induction kinetics of *GAL1* and *GAL10* was clearly seen (Figures 2A, 2B, S2A and S2B) with a more modest reduction in *GAL7* (Figures 2C and S2C). Reduced transcriptional induction was most evident when comparing later time points in the time course (180-300 min), as RNase H1 did not appreciably alter the lag time to induction or repress the *GAL* genes in *dbp2* cells to levels seen in *dbp2 lncRNA* strain. Induction of the *INO1* gene by inositol depletion revealed that RNase H1 expression has no effect on transcriptional induction in *dbp2* cells of a gene lacking overlapping, annotated lncRNAs (Figures 2D and S2D), suggesting that the effect of RNase H1 is specific for lncRNA-targeted genes. RNase H1 expression also reduced induction of wild type cells to that of *lncRNA* cells, as revealed by usage of a longer induction time course (Figure S3).

To determine if ectopic expression of RNase H1 reduces the levels of the *GAL* lncRNAs, suggesting that these molecules are degraded *in vivo*, we conducted ssRT-qPCR (Figures 2E and 2F). This revealed a slight but statistically significant decrease in the *GAL10* lncRNA with a much larger decrease in the *GAL10s* lncRNA in *dbp2* cells expressing RNase H1 as compared to empty vector (Figures 2E and 2F). We speculate that this differential sensitivity is due to the distinct processing mechanisms for these two lncRNAs (Tuck and Tollervy, 2013; van Dijk et al., 2011; Xu et al., 2009). In contrast to *dbp2* cells, an RNase H1-dependent decrease in *GAL* lncRNA levels was not evident in wild type cells, most likely due to technical limitations from the very low levels of the *GAL* lncRNAs across a cell population (Houseley et al., 2008) and the fact that only a fraction is likely to be chromatin-bound at any one time. Regardless, this suggests that the reduced, lncRNA-dependent induction is manifested by removal of lncRNA R-loops upon ectopic expression of RNase H1.

## DBP2-deficient Cells Accumulate lncRNA-dependent R-loops Across the *GAL* Cluster

To determine if the *GAL* lncRNAs form R-loops at the *GAL* cluster, we performed a DNA-RNA immunoprecipitation (DRIP) assay using the S9.6 RNA-DNA hybrid antibody (Boguslawski et al., 1986; El Hage et al., 2010; Hu et al., 2006). Briefly, genomic DNA was isolated from wild type, *lncRNA*, *dbp2* and *dbp2 lncRNA* cells grown under repressive (glucose) conditions and subjected to DRIP. Immunoprecipitated DNA was then measured by qPCR using primer sets across the *GAL* cluster promoters and coding regions (Figure 3A). A recent genome-wide analysis reported detection of R-loops at the 5' end of the *GALI* ORF in *S. cerevisiae* (Chan et al., 2014). Consistently, we observed R-loops at the 5' end of

the *GAL1* ORF (*GAL1* 5') in both wild type and also in *dbp2* cells, as evidenced by a qPCR signal 3-fold above RNase H-treated samples (Figure 3B). In *dbp2* cells, we also detected accumulation of R-loops across distal regions of the *GAL* cluster, including the bidirectional *GAL1/10* promoter (*GAL1/10* P) and the *GAL7* promoter (*GAL7* P), but not at the *KAP104* promoter region immediately downstream of *GAL7* (Figures 3A and 3B). This accumulation was abolished upon deletion of the *GAL* lncRNAs (Figure 3C), indicating that R-loop formation across the *GAL* cluster in *dbp2* cells is lncRNA-dependent. This suggests that the R-loops are formed by the *GAL* lncRNAs themselves and is consistent with expression of a 3' extended *GAL10s* lncRNA in *dbp2* cells, which extends to the 3' end of *GAL7* (Cloutier et al., 2012). In contrast, the 5' region of *GAL1* exhibited a more modest reduction in R-loop accumulation in *lncRNA* and *dbp2 lncRNA* cells (Figure 3C).

The predominantly nuclear Dbp2 protein is rapidly exported to the cytoplasm upon a switch from glucose to galactose in the media (Beck et al., 2014; Cloutier et al., 2012). To shed light on the kinetics of R-loop accumulation and determine if cytoplasmic re-localization of Dbp2 is sufficient, we used an “anchor away” strategy so Dbp2 could be depleted from the nucleus without the confounding transcriptional and physiological changes that occur upon glucose removal. The anchor away method exploits the inducible interaction between FK506-binding protein (FKBP12) and the FKBP12-rapamycin-binding (FRB) domain, which causes rapid, rapamycin-dependent localization of FRB-tagged proteins due to fusion of FKBP12 (via RNP13A) to the ribosome (Haruki et al., 2008). To deplete nuclear Dbp2, we constructed a DBP2-FRB-GFP at the endogenous DBP2 locus and subsequently confirmed rapamycin-dependent relocalization of the Dbp2-FRB-GFP chimera by fluorescent microscopy (Figure 3D). We then conducted DRIP to analyze accumulation of R loops at 0, 30, and 60 min following rapamycin treatment (Figures 3E-3G). Strikingly, this revealed that nuclear depletion of Dbp2 is sufficient to induce R-loop accumulation, forming initially at the 5' end of *GAL1* (Figures 3E and 3F) followed by accumulation and spreading downstream (Figure 3G) upon redistribution of Dbp2 to the cytoplasm. This suggests that cytoplasmic redistribution of Dbp2 results in a time-dependent increase in R-loops at the *GAL* cluster.

### Comparison of *dbp2* RNA-Seq and Genome-wide R-loop Detection Reveals Enrichment in Nutrient Utilization Genes

To determine the degree of overlap between genes with a propensity to form R-loops and genes whose expression is affected by loss of *DBP2*, we compared published genome-wide data sets of DRIP in wild-type cells to the RNA sequencing results of *dbp2* cells (Beck et al., 2014; Chan et al., 2014). This revealed that differentially expressed, protein-coding, sense transcripts in *dbp2* have a tendency to be associated with R-loops (two-sided Fisher's test,  $p = 0.033$ ) (Figure 4A). Moreover, 220 out of 322 (68.3%) of these genes were up-regulated in *dbp2* ( $X^2=14.6$ ,  $p=1.31 \times 10^{-4}$ ) (Table S8), correlating *DBP2*-dependent repression with the propensity to form R-loops. We also found co-occurrence of R-loops at regions associated with *DBP2*-dependent antisense transcripts (two-sided Fisher's test,  $p = 6.0 \times 10^{-5}$ ) (Figure 4B), however, there was no difference in the proportion of up- or down-regulated antisense RNAs for genes associated with R-loops (Table S8).

To determine if the sense genes that accumulate in *dbp2* cells and form R-loops in wild type cells fall into common biological categories, we conducted functional annotation clustering analysis using DAVID (Huang et al., 2009a, b). This revealed over representation of four significant clusters including energy homeostasis, carbohydrate metabolism, oxidative phosphorylation and cellular responses to stress (Figure 4C, top; Table S9). The same analysis with antisense transcripts revealed carbohydrate transport as the sole cluster with significant enrichment (Figure 4C, bottom; Table S9). This suggests that *Dbp2* may regulate formation of R-loops at specific genes involved in nutrient utilization and environmental responses.

### Loss of DBP2 Does Not Result in Significant Hyper-recombination but May Mimic *hpr1* at Some Loci

*Hpr1* is a member of the THO complex required for efficient elongation whose loss causes widespread R-loop formation and hyper-recombination (Chan et al., 2014; Chavez and Aguilera, 1997; Huertas and Aguilera, 2003). To determine if loss of *DBP2* results in hyper-recombination, we measured recombination frequencies in isogenic wild type, *dbp2* or *hpr1* cells (Figure S4A). *HPR1*-deficient cells displayed the expected hyper-recombination phenotype (Figure S4B). Loss of *DBP2* also resulted in a length-dependent increase in recombination, but at levels significantly below the definition for hyper-recombination (Prado et al., 1997) (Figure S4B). This suggests that loss of *DBP2* either does not cause widespread R loop accumulation or that the *LEU2* gene within the hyper-recombination reporter is not a *Dbp2* target.

We then used bioinformatics to ask if *Dbp2*-dependent transcripts correlate with genes that exhibit increased R-loops in other mutant strains (Chan et al., 2014). This revealed that genes with significantly enriched R-loop formation in *hpr1* tend to coincide with up-regulated genes in *dbp2* (two-sided Fisher's test,  $p = 7.3 \times 10^{-3}$ ), but not the RNase H-deficient *rnh1 rnh201* strain or in cells harboring a *sen1-1* termination mutation (Chan et al., 2014; Table S10). This suggests that *Dbp2* may have a role in repressing transcription-dependent R-loop formation at some loci similar to *Hpr1*.

### The GAL lncRNAs Promote Gene Looping at the GAL Cluster

Gene loops are higher order chromatin structures that promote interaction between distant gene elements (Hampsey et al., 2011). *GAL10* has been shown to form gene loops upon transcriptional activation of the gene in *S. cerevisiae* (Laine et al., 2009). To determine if the GAL lncRNAs promote gene looping, we utilized chromatin confirmation capture or 3C (Ansari and Hampsey, 2005; Laine et al., 2009; Medler et al., 2011). Consistent with prior characterization of *GAL10* (Laine et al., 2009), we observed a time-dependent increase in promoter-terminator looping following transcriptional activation in wild type cells (Figures 5B and 5C). Analysis of *dbp2* cells revealed a slight but detectable increase in *GAL10* looping at early time points post-activation, representing a ~50% and ~25% increase as compared to wild type cells at 90 and 180 min (Figures 5B and 5C). In contrast, neither the lncRNA nor the *dbp2* lncRNA strain exhibited any detectable increase in the P1/T1 PCR product above the 0 min time point, indicating that the GAL lncRNAs, and/or the Reb1-binding sites, are necessary for *GAL10* looping and are epistatic to *DBP2*.

To determine if the GAL lncRNAs alter the association of *trans*-acting factors with the GAL cluster, we conducted chromatin immunoprecipitation (ChIP) of the Cyc8 co-repressor in wild type, *lncRNA*, *dbp2* and *dbp2 lncRNA* cells grown in transcriptionally repressive conditions. Consistent with our prior studies, we observed reduced association of Cyc8-FLAG with the *GAL1/10* and *GAL7* promoter in *dbp2* cells as compared to wild type ((Cloutier et al., 2013); Figures 5D and 5E). Interestingly, removal of the GAL lncRNAs in *dbp2* cells restored Cyc8 binding to wild type levels, an effect that is not due to altered abundance of Cyc8 (Figure 5F). This suggests that the GAL lncRNAs reduce repressor binding at GAL promoters, a necessary step prior to activation and gene looping.

### The GAL lncRNAs Promote Faster Adaptation to A Nutritional Shift

The GAL cluster genes are part of a network whose induction is necessary to metabolize galactose as an alternative carbon source from glucose (Lohr et al., 1995). To determine if the GAL lncRNAs provide a fitness advantage to yeast cells when challenged with adaptation to galactose, we measured the growth of wild type and *lncRNA* strains grown in synthetic complete media over the course of a glucose to galactose or a mock carbon source shift (Figure 6). Interestingly, we observed an ~30 min faster doubling time for wild type cells as compared to the *lncRNA* strain (Figure 6A). Faster growth was nutrient-specific as a mock shift from glucose to glucose resulted in superimposable growth curves for the two strains (Figure 6B). We then conducted a growth assay in wild type and *lncRNA* cells expressing human RNase H1 or an empty vector to determine if loss of R-loops abolished the growth advantage of wild type cells in a carbon shift. Strikingly, expression of RNase H1 in wild type cells caused a profound increase in lag time of ~6 hours when cells were shifted to galactose media as compared to vector alone (Figure 6C). This lag time was similar to *lncRNA* cells harboring empty vector or pRNH1. However, both wild type and *lncRNA* cells expressing RNase H1 showed reduced doubling times overall, suggesting that overexpression of RNase H1 has other effects on cell growth (Figure 6C).

To determine if cells expressing the GAL lncRNAs can better compete for food sources in a diverse population, we conducted a competition assay using a mixed culture of wild type and *lncRNA* cells. This revealed that wild type cells markedly outcompete *lncRNA* cells in a mixed genetic population when challenged with a carbon source switch (Figure 6D). In fact, wild type cells represented ~60% and 95% of the culture within 12 and 24 hours after the shift to galactose, respectively. In contrast, the two strains remained largely equal through the duration of a mock shift (Figure 6E). The GAL lncRNAs encoded in trans (*trans-lncRNA*) also conferred a competitive advantage over *lncRNA* cells during the nutritional switch (Figure 6F), whereas we saw no difference in fitness between the *cis* versus *trans*-encoded GAL lncRNA strains (Figure 6G). Thus, the GAL lncRNAs promote a fitness advantage to yeast cells in response to changing nutritional availability irrespective of genomic location (*cis* versus *trans*). Taken together, our work suggests that Dbp2 regulates formation of lncRNA-DNA hybrids in response to nutrients, thereby facilitating rapid transcriptional induction for environmental adaptation (Figure 7).

## Discussion

R-loops have been shown to function as transcriptional regulators at the level of initiation, elongation and termination, as well as etiological agents of DNA damage (Costantino and Koshland, 2015). As transcriptional regulators, R-loops protect CpG-containing promoters from transcriptional silencing by blocking recruitment of the DNA methyltransferase DNMT3B1 (Ginno et al., 2013; Ginno et al., 2012), promote efficient termination by pausing RNA polymerase (Skourti-Stathaki et al., 2011), and repress transcription by promoter occlusion (Sun et al., 2013). Our results now show that R-loops formed with the GAL lncRNAs promote gene induction, thus positively regulating transcription.

Consistently, lncRNA-dependent R-loops in mammalian cells have very recently been shown to promote activation of the vimentin (VIM) gene (Boque-Sastre et al., 2015). This suggests a conserved mechanism for R-loops in lncRNA-dependent gene activation.

There are two proposed mechanisms for formation of R loops *in vivo* (Costantino and Koshland, 2015). The first envisions that nascent RNA emerging from RNA polymerase folds back onto the DNA, forming an R-loop in *cis* from defects in elongation, termination and mRNA-protein packaging (Costantino and Koshland, 2015; Dominguez-Sanchez et al., 2011; Gomez-Gonzalez et al., 2011; Stirling et al., 2012). The second mechanism follows the observation that R-loops can form in *trans* through the action of homologous recombination machinery (Wahba et al., 2013). Interestingly, the fact that the GAL lncRNAs function both in *cis* and in *trans* upon loss of *DBP2* suggests that RNA-protein assembly may be a determinant of R-loop formation in both cases. This finding also suggests that many *cis*-acting lncRNAs may be much more similar mechanistically to *trans*-acting molecules than previously thought. If this is the case, the prevalence of *cis*-encoded lncRNAs near targeted gene loci (Necsulea et al., 2014) may reflect evolutionary constraints to maintain base pairing between the target DNA and lncRNA, rather than a reflection of molecular function.

Export of the RNA helicase Dbp2 promotes lncRNA-dependent R-loop spreading, addressing a long-standing question regarding how functional R-loops may be regulated. Interestingly, this regulation appears to be specific for carbon source metabolic genes (Beck et al., 2014). Because Dbp2 is required for efficient 3' end formation and assembly of RNA-binding proteins (Ma et al., 2013), factors that also associate with lncRNAs (Tuck and Tollervey, 2013), this suggests that specificity may arise from differential requirements for Dbp2 in RNA processing and packaging. Structured elements in the nascent RNA that require Dbp2 for resolution are the most likely determinants, as Dbp2 is an efficient RNA helicase *in vitro* and structured RNA may be refractory to mRNP/lncRNP assembly (Ma et al., 2013). Failure to properly assemble nascent mRNPs/lncRNPs would be consistent with co-occurrence of derepressed genes in *dbp2* cells with ones that form R-loops in *hpr1* cells, as Hpr1 is a member of the THO/TREX complex required for mRNP packaging, transcriptional elongation, and mRNA export (Gomez-Gonzalez et al., 2011; Strasser et al., 2002; Zenklusen et al., 2002). Alternatively, loss of Dbp2 may stabilize lncRNAs in the nucleus. The latter is consistent with evidence that the exosome component Rrp6 prevents R-loop formation at enhancers in mouse B cells and ESCs (Pefanis et al., 2015) and the fact that simultaneous loss of *DBP2* and *RRP6* is lethal in *S. cerevisiae* (Cloutier et al., 2012).



Consistent with promoting activation, the GAL lncRNAs are also essential for gene looping at *GAL10*. Alternatively, gene looping may be mediated by the Reb1-binding sites in the DNA. Regardless, this is reminiscent of mammalian enhancers and associated non-coding RNAs, which have recently been shown to form R-loops (Lai et al., 2013; Orom et al., 2010; Pefanis et al., 2015). However, whereas *GAL1* and *GAL10* form gene loops (Laine et al., 2009; Tan-Wong et al., 2009), we have not detected these structures at the *GAL7* gene (data not shown). This may be a technical limitation or reflective of an alternative mechanism for enhanced induction at this locus. Indeed, recruitment of the *GAL* genes to the nuclear periphery facilitates deSUMOylation of Cyc8 and subsequent derepression (Texari et al., 2013), suggesting that the GAL lncRNAs and/or R-loops may also promote rearrangements within the 3-dimensional nuclear space.

Our observations appear to be in conflict with previous studies noting a role for the GAL lncRNAs in transcriptional repression and it is important to integrate our results with the field (Geisler et al., 2012; Houseley et al., 2008; Lenstra et al., 2015; Pinskaya et al., 2009). Our data and model suggest that lncRNA-dependent induction only occurs in the context of a repressive (glucose) to activated (galactose) switch, consistent with our prior studies (Cloutier et al., 2013) and a recent report published during submission of this study showing no role for the GAL lncRNAs from a non-induced (raffinose) state (Lenstra et al., 2015). However, the latter does not establish that the GAL lncRNAs have no positive role as concluded (Lenstra et al., 2015), as raffinose is not a transcriptionally repressed state (Sellick et al., 2008). Another point of confusion are conclusions from studies of the RNA decapping factor *DCP2* that the GAL lncRNAs are strongly repressive from the same non-inducing conditions (Geisler et al., 2012). In this case, the strong transcriptional attenuation appears to be specific for *dcp2* cells (Cloutier et al., 2013; Geisler et al., 2012), a finding that may be explained by a role for Dcp2 in transcription itself (Haimovich et al., 2013), as well as the use of a strain harboring a genomic deletion of *GAL10* to analyze GAL lncRNA-dependent repression of *GAL1* (Geisler et al., 2012).

Regardless, several lines of evidence suggest that transcriptional repression does occur to prevent low level, leaky expression of galactose metabolic genes in the context of mixed sugars (i.e., glucose and galactose) (Houseley et al., 2008; Lenstra et al., 2015). These findings are fully compatible with our results and suggest a dual role for the GAL lncRNAs: one in transcriptional repression in *cis* by transcriptional interference and one in derepression/enhanced induction in *trans* by the lncRNA molecule itself (see Graphical Abstract). Importantly, our work shows that the GAL lncRNAs confer a strong competitive advantage over otherwise genetically identical yeast cells during a glucose to galactose switch, establishing a biologically beneficial role for these molecules that outweighs the subtle transcriptional benefit in wild type cells (Cloutier et al., 2013). This has widespread implications as investigators pursue functions of individual lncRNAs by underscoring the value of simple model organisms and utilization of mutant strains to tease apart complex molecular processes.

## Experimental Procedures

For detailed protocols, see supplemental experimental procedures.

### **Plasmids, Cloning and Yeast Strain Construction**

Plasmids are listed in Table S1. Yeast strains were constructed using classical yeast genetics (Table S2). Oligos for homologous recombination are listed in Table S3.

### **Northern Blotting**

Northern blotting was performed as described previously (Cloutier et al., 2013). Probes were generated from PCR products using plasmids and primers in Tables S1 and S4.

### **Strand-specific Reverse Transcriptase-quantitative PCR (ssRT-qPCR)**

Strand-specific cDNA preparation and qPCR were performed as described (Beck et al., 2014) using oligos listed in Table S5.

### **DNA-RNA Immunoprecipitation (DRIP)**

DRIP was conducted similar to prior studies (El Hage et al., 2010) using the S9.6 antibody (Boguslawski et al., 1986; Hu et al., 2006). Primer/probes for qPCR are in Table S6.

### **Determination of Recombination Frequencies**

Strains were transformed with direct repeat constructs pRS314-L or pSch204 to assay the recombination frequencies as described previously (Chavez and Aguilera, 1997; Prado et al., 1997).

### **RNA-seq Bioinformatics**

RNA-Seq data was previously deposited in the Gene Expression Omnibus (GSE58097, (Beck et al., 2014)). Bioinformatics was conducted as detailed in Supplemental Experimental Procedures.

### **Chromosome Conformation Capture (3C)**

3C was performed as described previously (El Kaderi et al., 2012). Primers are listed in Table S7.

### **Chromatin Immunoprecipitation (ChIP)**

ChIP and qPCR were performed as described (Cloutier et al., 2013) using primer/probes listed in Table S6.

### **Growth Curves and Competition Assay**

Cell density was assessed by absorbance (600nm) over 30 hours of growth in synthetic complete media and indicated carbon source. For competition assays, equal amounts of wild type and lncRNA TRP1, wild type and trans-lncRNA, or lncRNA and trans-lncRNA cells were mixed and resuspended in liquid culture and subjected to a carbon shift followed by plating on selective media.

### **Supplementary Material**

Refer to Web version on PubMed Central for supplementary material.

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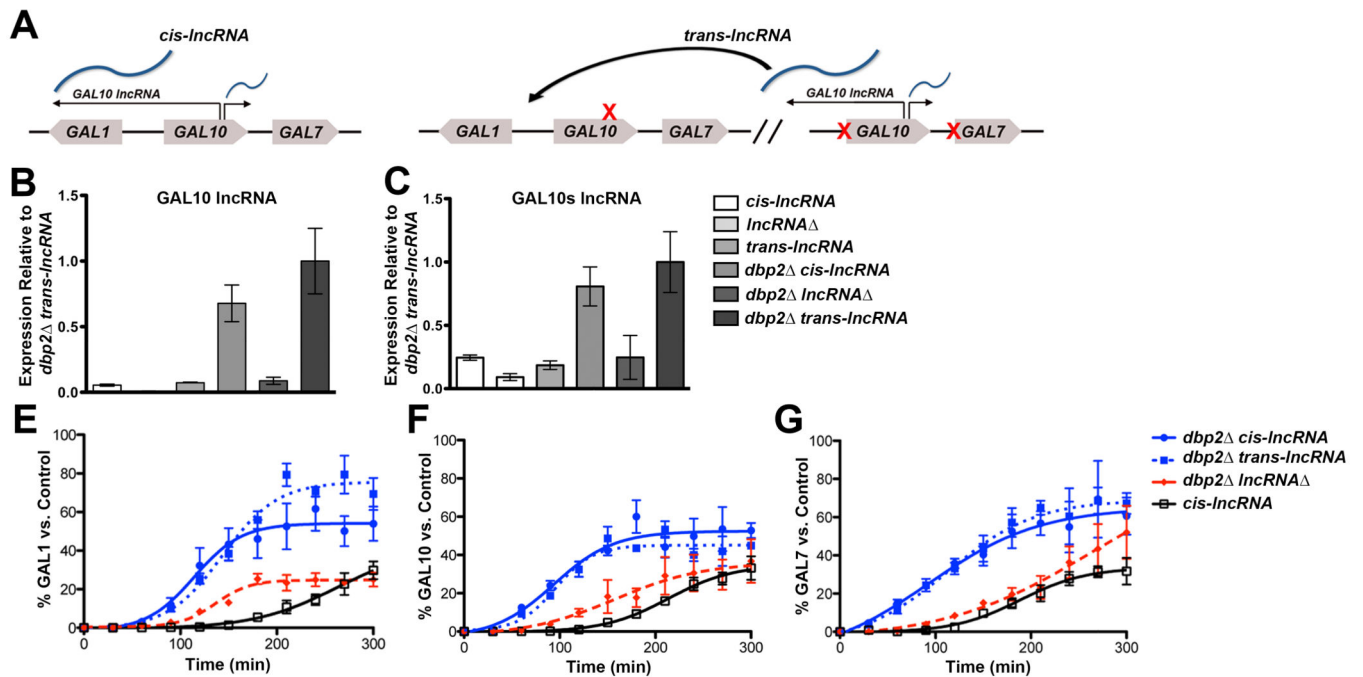
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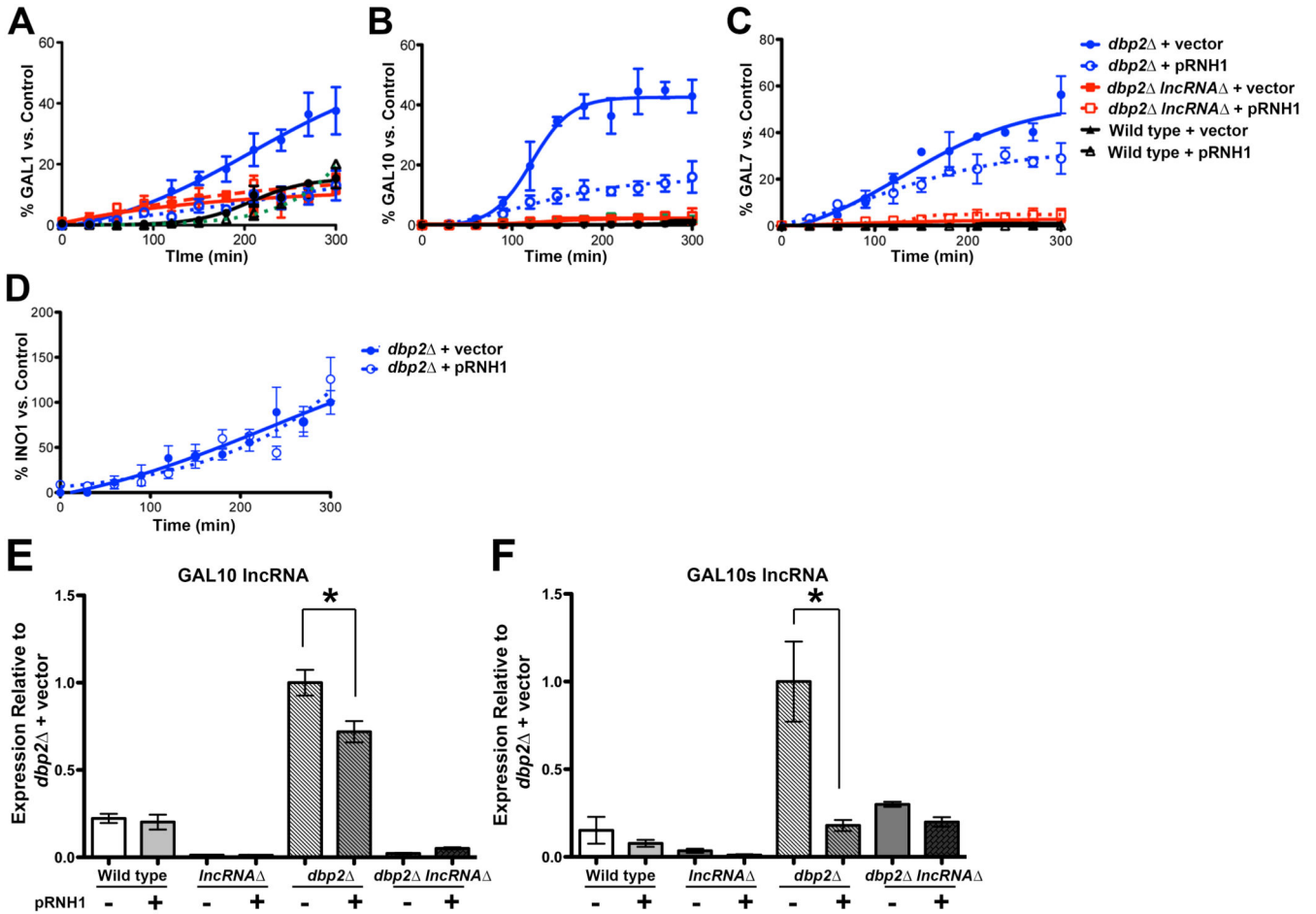
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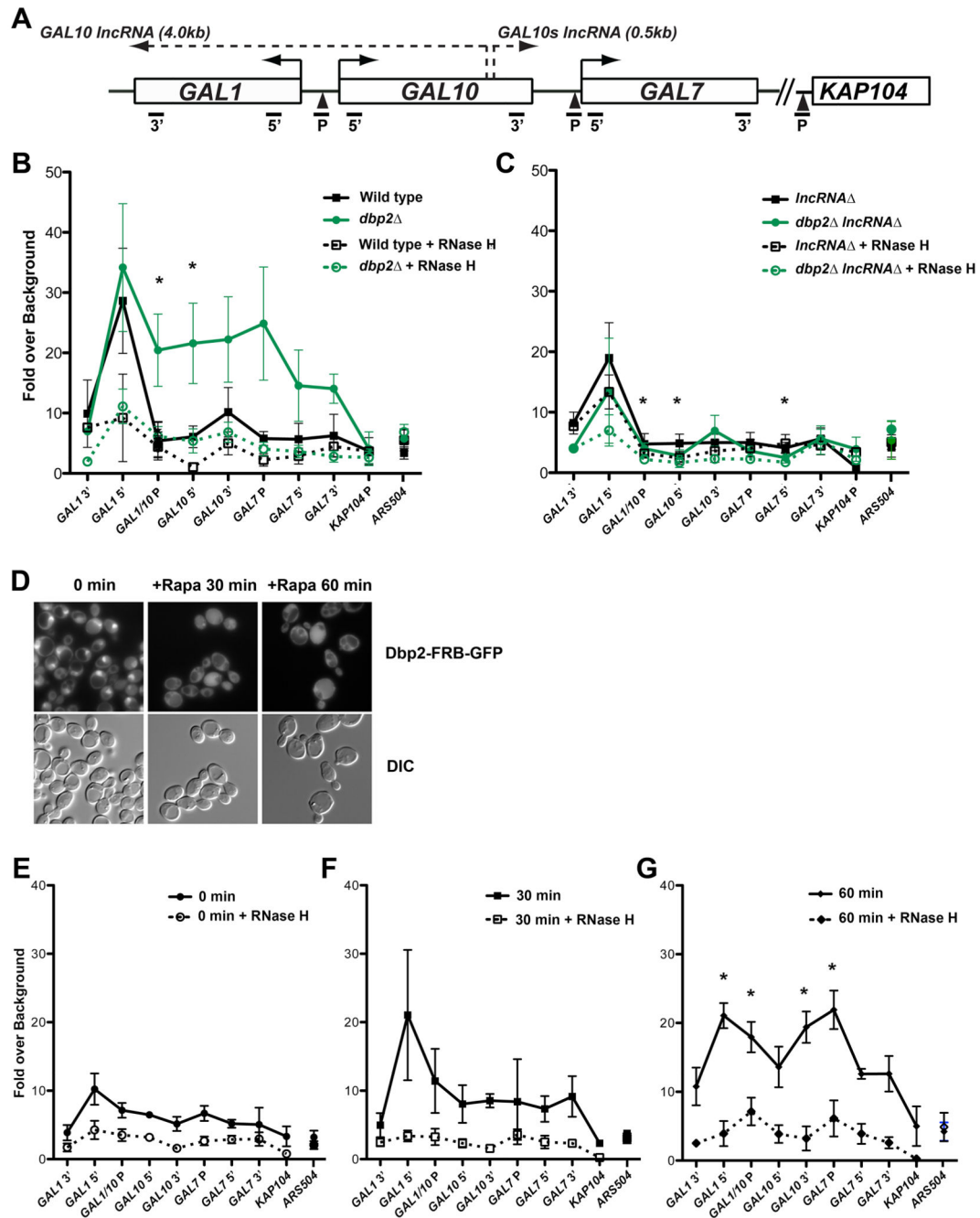
**Figure 1. GAL lncRNAs function in *trans* to promote induction of the GAL cluster genes** (A) A schematic of “*cis-lncRNAs*” and “*trans-lncRNAs*” at the GAL cluster. (B-C) The *trans-lncRNA* strain expresses the GAL lncRNAs at similar levels to *cis*-encoded strains. Levels of the antisense GAL10 lncRNA (B) and GAL10s lncRNA (C) were assayed in wild type, *lncRNAΔ*, *dbp2*, *dbp2 lncRNAΔ*, *trans-lncRNA* and *dbp2 trans-lncRNA* cells by strand-specific RT-qPCR (ssRT-qPCR) as described (Beck et al., 2014) using cells grown in repressive (+glucose) conditions and primers listed in Table S5. Results are presented as the mean of 3 biological replicates relative to ACT1 with S.E.M. (D-F) Graphical representation of GAL gene induction of isogenic wild type, *dbp2*, and *dbp2 lncRNAΔ* and *dbp2 trans-lncRNA* strains. Transcriptional induction of GAL1 (D), GAL10 (E) and GAL7 (F) transcripts during a shift from *repressive* (+glucose) to activated conditions (+galactose) was assayed by Northern blotting, quantified with respect to sCR1, and plotted as a percentage of a fully induced wild type “control” as previously described (Cloutier et al., 2013). Results are presented as the mean with SEM of three biological replicates. Also see Figure S1.



**Figure 2. Ectopic expression of RNase H1 suppresses rapid, GAL IncRNA-dependent induction in *dbp2* cells**

Graphical representation of GAL1 (A), GAL10 (B) and GAL7 (C) gene induction assays of wild type, *dbp2* and *dbp2 IncRNA* cells harboring a plasmid encoding the human RNase H1 (pRNH1) gene or empty vector. Strains were grown as in Figure 1 but in –LEU selective media for plasmid maintenance with transcript induction and graphical representation as above across three biological replicates. (D) Graphical representation of INO1 induction assays in *dbp2* cells expressing pRNH1 or empty vector. Induction assays were performed by growing *dbp2* cells with vector or pRNH1 to mid-log in synthetic complete media (SC) + inositol, before shifting to SC – inositol to induce *INO1* expression. (E-F) RNase H1 expression reduces the levels of the GAL10 (E) and GAL10s (F) IncRNAs in *dbp2* cells. Wild type, *IncRNAΔ*, *dbp2* and *dbp2 IncRNAΔ* cells harboring either empty vector or pRNH1 were grown in SC-LEU + glucose and transcript abundance was assayed by ssRT-qPCR as in Figure 1. \* =  $p < 0.05$ . See also Figures S2 and S3.





**Figure 3. Loss of Dbp2 promotes accumulation of GAL lncRNA R-loops across the GAL cluster** (A) Schematic of the GAL gene cluster showing coding regions, GAL10 and GAL10s lncRNA loci, and qPCR primer-probe positions. (B-C) DNA-RNA-immunoprecipitation (DRIP) of wild type and *dbp2* $\Delta$  (B) or *lncRNA* $\Delta$  and *dbp2* *lncRNA* $\Delta$  (C) cells grown under transcriptionally repressive (+glucose) conditions. DRIP was conducted using the S9.6 antibody (Boguslawski et al., 1986; El Hage et al., 2010; Hu et al., 2006) or beads alone (no antibody control) followed by qPCR using the indicated primer-probe sets (A) or the non-protein coding region ARS504, which lacks both RNase H-dependent R-loops (Chan et al.,

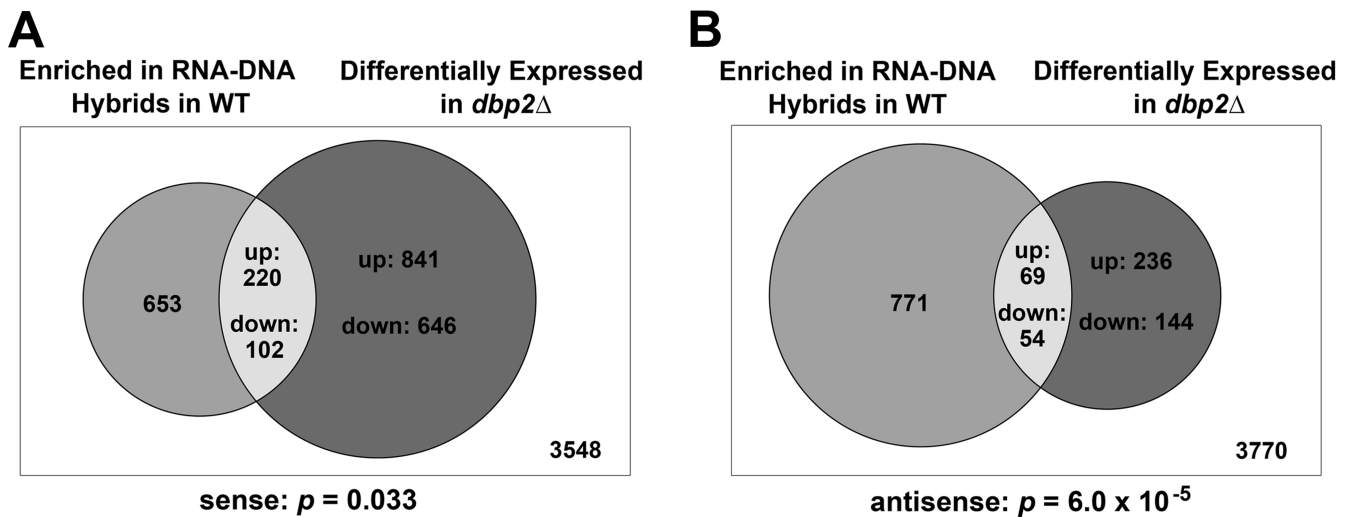
2014) and Dbp2-dependent transcripts (Beck et al., 2014). R-loop levels were determined across 5 biological replicates as the increase in signal of above the no antibody control presented as the mean and S.E.M. \* =  $p < 0.05$ . (D) Rapamycin-dependent cytoplasmic localization of Dbp2-GFP via anchor away. Dbp2-FRB-GFP was visualized by fluorescent microscopy 0 min (-Rapa), 30 min or 60 min after treatment with 10 $\mu$ g/mL rapamycin. (E-G) Cytoplasmic relocalization of Dbp2 promotes time-dependent accumulation and spreading of R-loops across the GAL cluster. DRIP was conducted as in B with cells following 0, 30 or 60 min rapamycin treatment. \* =  $p < 0.05$  with respect to 0 min.

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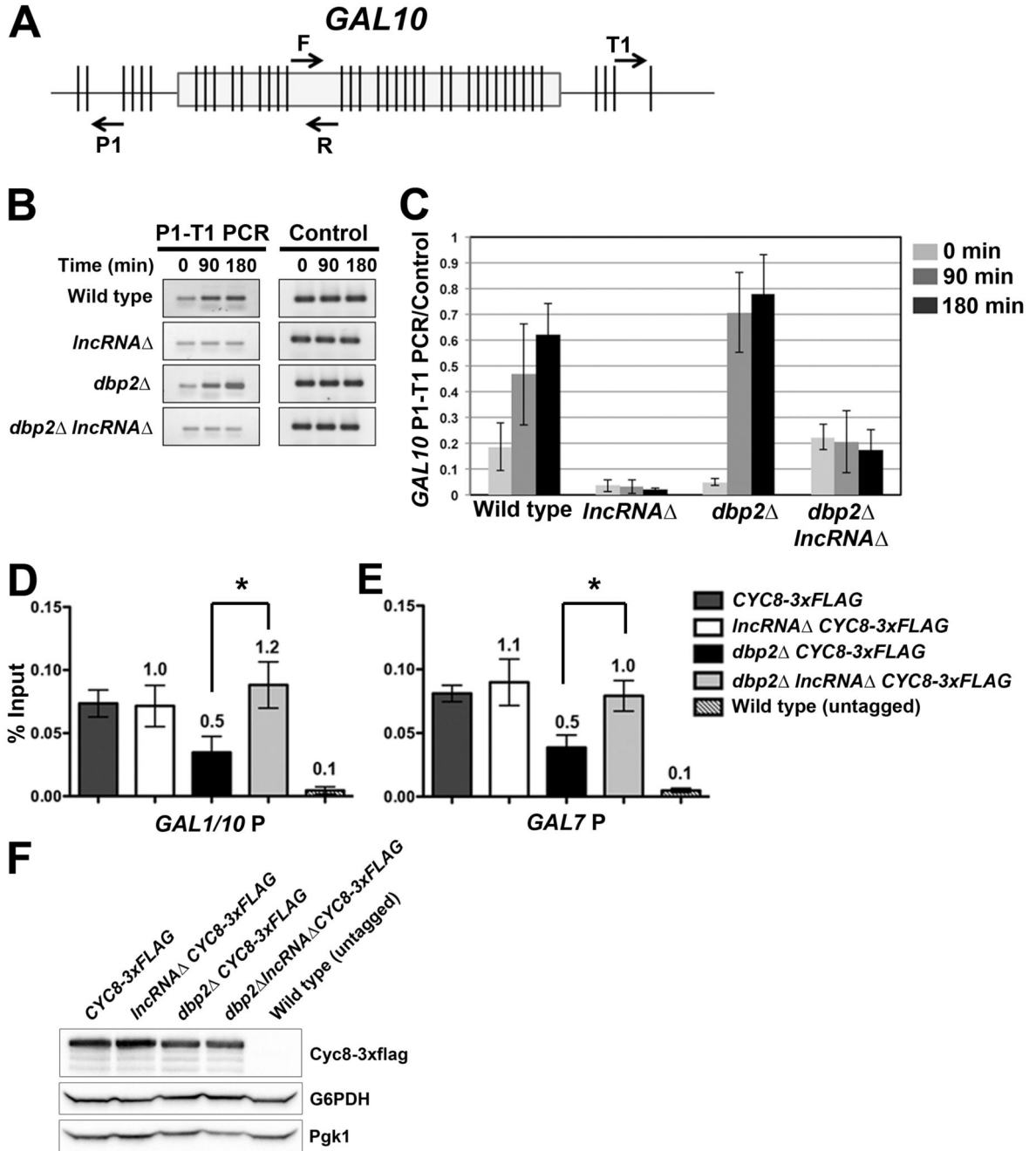
Annotation cluster	Enrichment score <sup>a</sup>	Description of GO terms <sup>b</sup>	Gene count <sup>c</sup>
1	2.604	Carbohydrate metabolic process	21
2	2.316	Oxidative phosphorylation/ cell death	52
3	1.624	Cellular response to stress	39
4	1.391	ATP metabolic process/ cation transport	24

Antisense

Annotation cluster	Enrichment score <sup>a</sup>	Description of GO terms <sup>b</sup>	Gene count <sup>c</sup>
1	1.927	Carbohydrate transport/ transmembrane transport	11

**Figure 4. Functional analysis of genes that are both differentially expressed in *dbp2* and form R loops in wild type cells**

(A-B) Venn diagrams showing the numbers of R loop-enriched ORFs in wild type (Chan et al., 2014) with differentially expressed sense (A) or antisense (B) transcripts in *dbp2* (this study and (Beck et al., 2014)). Numbers (inset) correspond to the total up- or down-regulated transcripts in *dbp2* or ORFs with R-loops in wild type cells. The number of ORFs that are neither category are shown in the box. (C) Functional annotation clustering for sense and antisense transcripts that are both DBP2-dependent and whose ORFs form R loops in wild type cells. <sup>a</sup>ORF groups with significant enrichment score of at least 1.3 ( $p =$  or  $< 0.05$ ). <sup>b</sup>GO term descriptions. <sup>c</sup>The number of unique ORFs in each cluster. See Tables S8-S9. See Figure S4 and Table S10 for comparison to *hpr1*.



**Figure 5. *GAL10* forms lncRNA-dependent gene loops**

(A) Schematic of *GAL10* showing restriction sites (vertical lines) and primers for Chromosome Conformation Capture (3C) (arrows). P1 and T1 correspond to the *GAL1/10* promoter and *GAL10* terminator, respectively, whereas F and R are internal control primers. (B-C) 3C of *GAL10* in wild type, *lncRNA*Δ, *dbp2*Δ and *dbp2*Δ *lncRNA*Δ cells during a glucose to galactose shift. Samples were quantified across three biological replicates relative to the control PCR with error bars representing SEM. (B) Ethidium bromide-stained agarose gel showing representative PCR products. (C) Quantification of looping at 0, 90 and 180

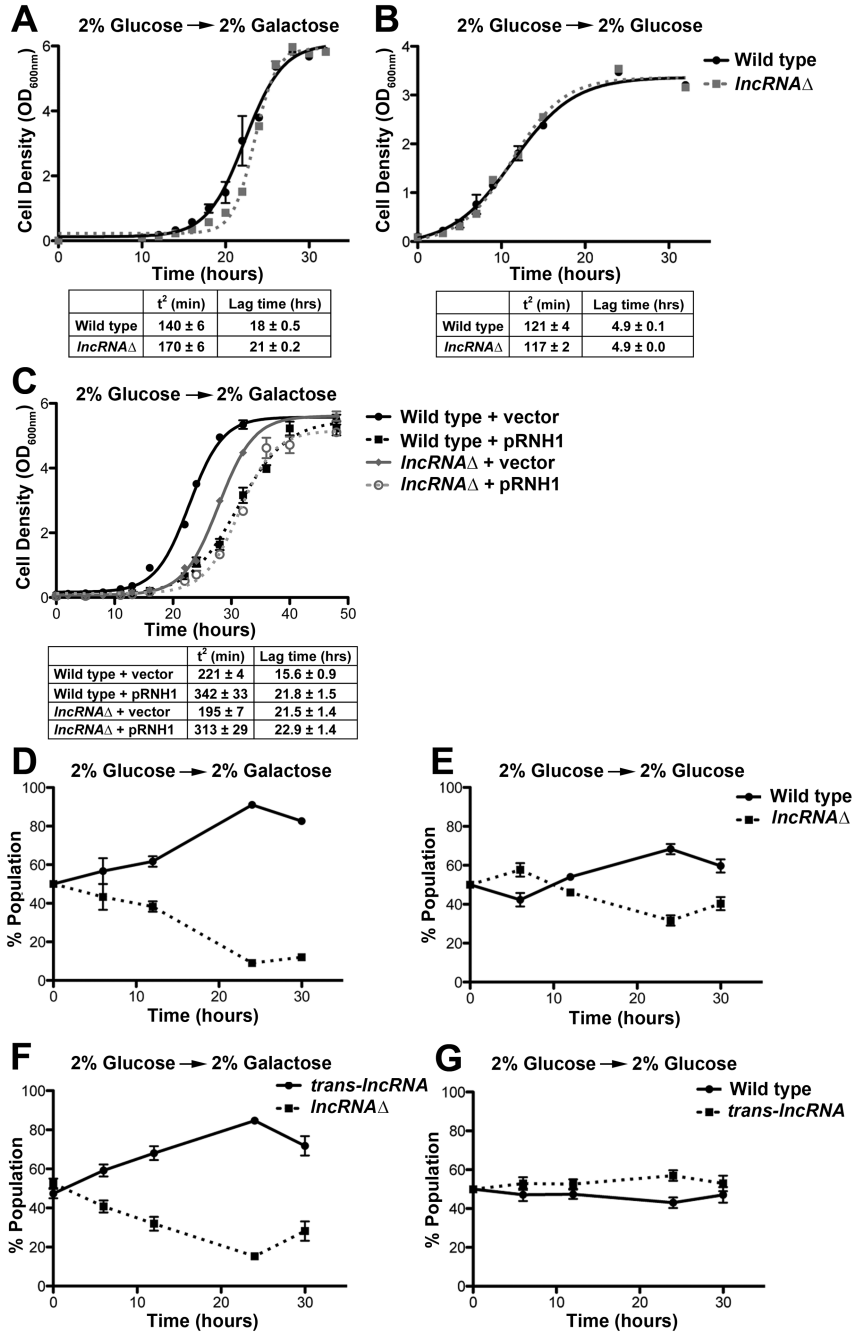
min post-induction. (D-E) Chromatin immunoprecipitation (ChIP) of 3XFLAG-tagged Cyc8 in wild type, *lncRNA*, *dbp2* and *dbp2 lncRNA* strains grown under transcriptionally repressive (+glucose) conditions. ChIP-qPCR was performed as described (Cloutier et al., 2013). Results are presented as the mean percent of input with SEM. \* =  $p < 0.05$  with respect to the *lncRNA* strain. (F) *Western blot of Cyc8-3XFLAG*. Whole cell lysate was extracted and resolved by SDS-PAGE. Cyc8, G6PDH and Pgk1 were detected using antibodies specific to FLAG, G6PDH and Pgk1, respectively, from the induced strains.

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**Figure 6. GAL IncRNAs promote faster growth and adaptation to a changing environment** (A) Growth curves of wild type and *IncRNA* $\Delta$  cells during a carbon source switch. Cell density was measured by absorbance ( $A_{600nm}$ ) across a glucose to galactose switch in synthetic complete (SC) media. Results are presented as the mean of 6 biological replicates with the SEM. (B) Growth curves of wild type and *IncRNA* $\Delta$  cells during a mock (glucose to glucose) shift. Doubling times ( $t^2$ ) were determined by fitting to an exponential growth curve in GraphPad Prism whereas lag times were calculated using DMFit (Baranyi and Roberts, 1994) (<http://www.combase.cc/tools/>). (C) RNase H1 represses growth of wild type

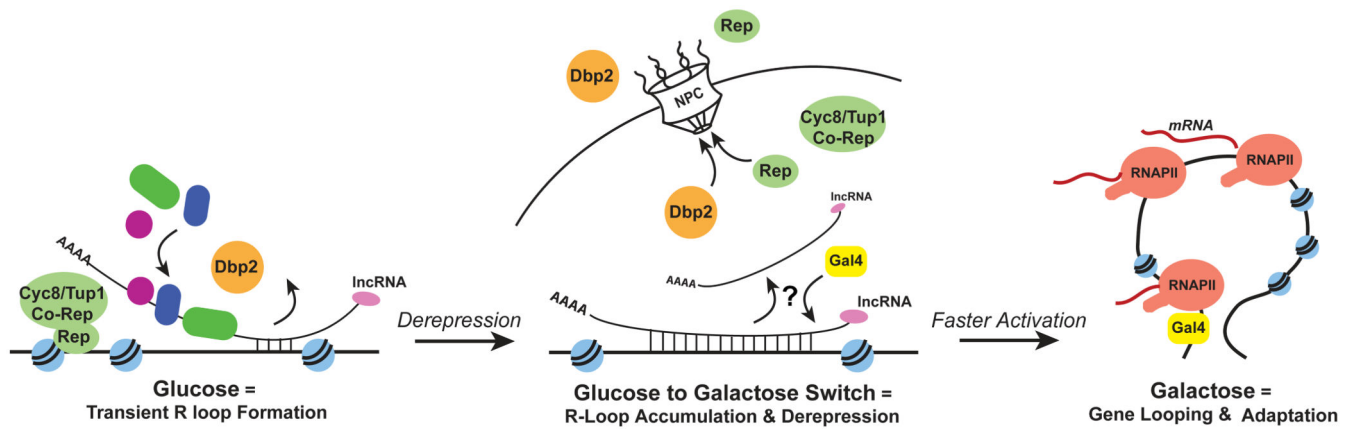
cells during a glucose to galactose shift. Doubling and lag times of cells were determined as above. (D-E) Competition assay of wild type and *lncRNA* cells during a carbon source switch. A *TRP1* allele was integrated into the *trp1-63* locus of the *lncRNA* strain to differentiate from wild type. Colony forming units (c.f.u.'s) were determined on both rich and selective media at each time point with the proportion of wild type or *lncRNA* cells in the culture corresponding to the number of *trp-* or *trp+* cells over the total. Results represent the mean of 3 biological replicates with SEM. (F-G) Competition assays of trans-*lncRNA* versus *lncRNA* (F) and cis-versus trans-*lncRNA* strains (G) reveals equal benefit of trans versus cis-encoded GAL *lncRNAs* during a carbon source shift. Competition assays were performed as above.

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**Figure 7. Regulation of Dbp2 stimulates transcriptional induction by promoting formation of transient GAL lncRNA-DNA hybrids**

Loss of Dbp2 from the nucleus upon removal of glucose promotes formation of GAL lncRNA-DNA hybrids, or R-loops, likely by preventing Dbp2-dependent lncRNA-protein complex assembly (Ma et al., 2013). These R-loops promote derepression and subsequent assembly of transcriptional activators, acting in concert with nutrient-responsive transcription factors and signaling pathways (Sellick et al., 2008). GAL lncRNA R-loops are presumably cleared by the Gal4 activator and subsequent transcriptional interference from active *GAL* gene expression and/or re-import of Dbp2 in the nucleus upon prolonged exposure to galactose (Beck et al., 2014). This rapid activation promotes gene looping and faster metabolic adaptation to a new sugar source.