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Decapping of Long Noncoding RNAs Regulates Inducible Genes

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SUMMARY

Decapping represents a critical control point in regulating expression of protein coding genes. Here, we demonstrate that decapping also modulates expression of long noncoding RNAs (lncRNAs). Specifically, levels of >100 lncRNAs in yeast are controlled by decapping and are degraded by a pathway that occurs independent of decapping regulators. We find many lncRNAs degraded by DCP2 are expressed proximal to inducible genes. Of these, we show several genes required for galactose utilization are associated with lncRNAs that have expression patterns inversely correlated with their mRNA counterpart. Moreover, decapping of these lncRNAs is critical for rapid and robust induction of *GAL* gene expression. Failure to destabilize a lncRNA known to exert repressive histone modifications results in perpetuation of a repressive chromatin state that contributes to reduced plasticity of gene activation. We propose that decapping and lncRNA degradation serve a vital role in transcriptional regulation specifically at inducible genes.

INTRODUCTION

Gene transcription in the nucleus and degradation in the cytoplasm together dictate the level of messenger RNA (mRNA) that is available as a template for protein synthesis. mRNA turnover, therefore, represents a critical control point in regulating gene expression (Franks and Lykke-Andersen, 2008). In eukaryotes, mRNA decay is initiated by removal of the 3′ poly(A) tail (deadenylation) and is typically followed by cleavage of the 5′ end 7-methyl-guanosine (m^7G) cap and rapid 5′→3′ exonucleolytic degradation of the transcript body. Cleavage of the mRNA 5′ cap is catalyzed by a holoenzyme composed of the decapping proteins, DCP1 and DCP2, with DCP2 harboring a conserved NUDIX domain required for catalysis (Dunckley and Parker, 1999). mRNA decapping is regulated by a suite of activators, including DHH1, PAT1, and the LSM1-7 complex (Franks and Lykke-Andersen, 2008). While the role of decapping in controlling mRNA levels is well documented, the contribution of decapping in modulating the levels and function of other RNAs has been largely unexplored.

Eukaryotic genomes express a complex repertoire of RNA molecules that are not protein coding—thousands of which are classified as small noncoding RNAs (i.e., microRNA, small interfering RNA, Piwi-interacting RNA) or large noncoding RNAs (lncRNAs; i.e., intergenic, antisense, and intronic) (Wilusz et al., 2009; Djuranovic et al., 2011). While some lncRNA transcripts may represent transcriptional "noise," several lncRNAs have now been shown to have biological function as bona fide regulators of gene expression both transcriptionally and post-transcriptionally (Wilusz et al., 2009; Nagano and Fraser, 2011). Notwithstanding, our understanding of the mechanisms and biological importance of lncRNAs is comparatively scant to that of small noncoding RNAs, which have been the recent focus of intense research (Djuranovic et al., 2011).

lncRNAs have been implicated in regulating a large array of processes in eukaryotic cells, including gene imprinting, dosage compensation, cell-cycle regulation, innate immunity, pluripotency, retrotransposon silencing, meiotic entry, and telomere length (Wilusz et al., 2009; Nagano and Fraser, 2011). Moreover, altered expression of lncRNAs has been linked to disease states such as cancer and neurological disorders (Qureshi et al., 2010; Tsai et al., 2011). Regulation of gene expression by lncRNAs can be mediated at the level of transcription by interference with mRNA expression, competition at genomic loci for transcription factors, or chromatin remodeling (Berretta and Morillon, 2009; Wilusz et al., 2009). Posttranscriptionally, lncRNAs influence pre-mRNA splicing, nuclear trafficking, and mRNA degradation (Wilusz et al., 2009; Nagano and Fraser, 2011; Gong and Maquat, 2011). Based on the emerging emphasis of lncRNAs on regulating gene expression, the metabolism of the lncRNA itself will likely be a vital aspect of its function.

Similar to most mRNAs transcribed by RNA polymerase II, lncRNAs are both capped and polyadenylated (Berretta and Morillon, 2009; Khalil et al., 2009; Guttman et al., 2009). We therefore set out to evaluate whether the decapping enzyme, DCP2, and its associated factors play a role in lncRNA metabolism and whether lncRNA turnover impinges on the ability of lncRNAs to regulate gene expression. Using RNA sequencing to profile transcriptome-wide expression patterns, we determined that over 100 lncRNAs are elevated in cells lacking RNA decapping activity. Importantly, decapping of lncRNA occurs independently of all known regulators of the decapping holoenzyme and thus represents a unique pathway for RNA turnover. Our study reveals that lncRNAs are often found proximal to inducible genes, and degradation of a lncRNA is required for proper induction of genes involved in galactose metabolism. We propose that lncRNAs are used as a means to tightly

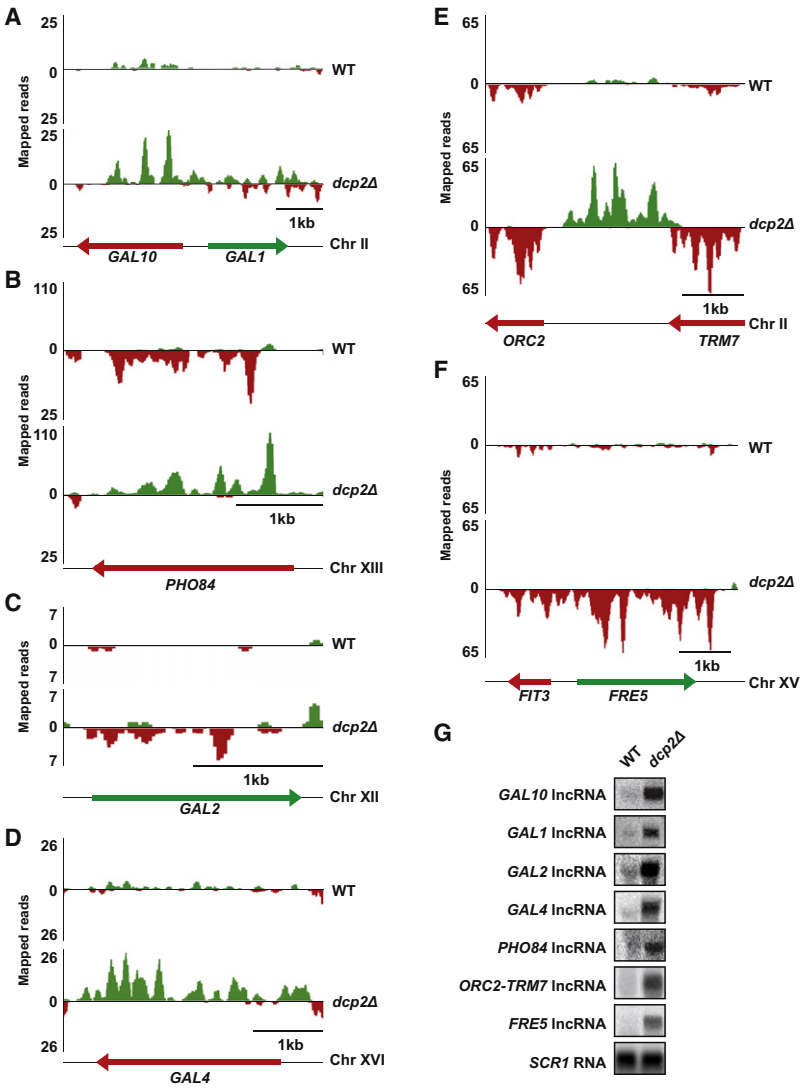

Figure 1. lncRNA Transcripts Accumulate When Decapping-Dependent Decay Is Blocked

(A–F) Total RNA from WT and *dcp2Δ* cells was prepared for strand-specific RNA-seq. Mapped reads are displayed with diagrams of each locus. Color corresponds to the strand of origin (green, Watson; red, Crick) and arrows indicate direction of orientation 5′ to 3′. Shown are *GAL10-GAL1* (A), *PHO84* (B), *GAL2* (C), *GAL4* (D), *ORC2-TRM7* (E), and *FRE5* (F).

(G) lncRNAs at the *GAL10*, *GAL1*, *GAL2*, *GAL4*, *PHO84*, *ORC2-TRM7*, and *FRE5* loci were confirmed by northern analysis. *SCR1* RNA, a RNA polymerase III transcript, is the loading control.

See also Tables S1 and S2.

maintain repression at inducible genes and that efficient clearance of the lncRNA by DCP2-dependent decapping is vital for robust gene activation.

RESULTS

lncRNAs Accumulate When DCP2-Dependent Decapping Is Blocked

RNA polymerase II transcribes a large number of lncRNAs that are predicted to receive a 5′ m^7G cap structure cotranscriptionally (Berretta and Morillon, 2009; Bentley, 2005). We anticipated that decapping might, therefore, play an important role in modulating abundance and perhaps biological activity of lncRNAs. We monitored the contribution of decapping to global lncRNA levels in budding yeast by high-throughput RNA sequencing (RNA-seq). Total RNA was isolated from wild-type (WT) cells and a strain lacking the catalytic subunit of the decapping enzyme (i.e., *dcp2Δ*), and complementary DNA (cDNA) libraries were prepared for analysis by the Illumina sequencing platform (see the Experimental Procedures). Notably, RNA was not subjected to poly(A)$^+$ selection since, without decapping, mRNA (and perhaps lncRNA) accumulate as a deadenylated [i.e., poly(A)$^-$] species (Dunckley and Parker, 1999). In addition, cDNA libraries were constructed from RNA with strand-specific information retained (see the Experimental Procedures). RNA-seq analysis resulted in 84.4 million and 61.2 million mappable reads from WT and *dcp2Δ* libraries, respectively. Of these, 5.2 million WT and 5.5 million *dcp2Δ* reads mapped to nonribosomal loci.

Consistent with our prediction that lncRNAs would be substrates for decapping, we observed a dramatic elevation in the level of several previously characterized lncRNAs in decapping-deficient cells compared to the WT (Figures 1A and 1B and Table S1 available online). Moreover, our analysis identified approximately 100 putative and previously unannotated lncRNAs based on their accumulation in *dcp2Δ* cells (Figures 1C–1F and Table S2). Notably, several of the putative lncRNAs we identified were predicted in previous studies interrogating the yeast transcriptome, but remain uncharacterized (Nagalakshmi et al., 2008; Xu et al., 2009; Yassour et al., 2010; van Dijk et al., 2011).

In general, lncRNAs that accumulated in *dcp2Δ* cells mapped to three types of genomic loci: (1) intergenic regions between previously annotated protein-coding genes, (2) locations proximal to telomeres, and (3) antisense to either the 5′ end or the entire length of known protein-coding genes (Table S2). Unexpectedly, the majority of DCP2-sensitive lncRNAs map proximal to genes that could be grouped into specific biological pathways. These pathways include, but are not limited to, iron sensing (i.e., *FRE1*, *FRE5*, and *FRE7*), glucose usage (i.e., *HXT5*, *HXT8*, *HXT10*, and *RGS2*), maltose metabolism (i.e., *MAL11*, *MAL12*, *MAL13*, and *MAL32*), flocculation (i.e., *FLO5*, *FLO9*, *FLO10*, and *FLO11*), inorganic phosphate uptake and utilization (i.e., *PHO5* and *PHO84*), and galactose utilization (i.e., *GAL1*, *GAL10*, *GAL2*, and *GAL4*). Importantly, most genes

within this subset were repressed, and therefore not transcriptionally active, under the conditions assayed in our RNA-seq analysis (Table S3). Their expression is, however, induced by specific environmental cues, which suggests that a large proportion of these lncRNAs map to highly regulated genes.

We analyzed RNA by Northern blot to confirm that the steady-state levels of seven of our identified lncRNAs were indeed elevated in *dcp2Δ* cells compared to the WT. Specifically, lncRNAs that map antisense to *GAL10*, *GAL1*, *GAL2*, *GAL4*, *PHO84*, and *FRE5* genes were dramatically increased in decapping-deficient cells (Figure 1G, WT versus *dcp2Δ*). Similarly, a lncRNA mapping intergenic to the *OCR2* and *TRM7* genes was poorly expressed in WT cells but accumulated in *dcp2Δ* mutants (Figure 1G).

Our analyses in *dcp2Δ* cells confirm decapping modulates levels of lncRNAs. Recently, lncRNAs termed XUTs were identified based on their sensitivity to XRN1, a cytoplasmic 5′→3′ exonuclease implicated in degrading decapped mRNA (van Dijk et al., 2011). Consistent with the requirement for removal of the 5′ m^7Gpp cap before RNA degradation by XRN1 (Stevens and Poole, 1995), 70% of the lncRNAs upregulated in *dcp2Δ* cells were also classified as XUTs (van Dijk et al., 2011). Interestingly, 30% of lncRNAs we identified were not identified as XUTs (Table S2). It is unclear whether this discrepancy represents differences in annotation of RNA-seq data or whether some lncRNAs are degraded by an alternative pathway. Indeed, in eukaryotes there are two 5′→3′ exonucleases (i.e., XRN1 and RAT1), both of which act downstream of RNA decapping to degrade RNA.

lncRNA Levels Are Regulated by DCP2

Our observation that lncRNAs are sensitive to decapping prompted us to evaluate whether they are also modulated by additional proteins implicated in mRNA turnover. Degradation of cytoplasmic mRNA is initiated by removal of the 3′ poly(A) tail by the CCR4-NOT deadenylase and is followed by decapping catalyzed by the DCP1/DCP2 holoenzyme and 5′→3′ exonucleolytic degradation by XRN1. Additional factors, including DHH1, PAT1, and LSM1-7, play an important role in mRNA stability as activators of decapping (Franks and Lykke-Andersen, 2008) (Figure S1). We performed northern blot analysis on RNA isolated from WT or cells lacking an activity important for mRNA turnover. As shown in Figures 2A and 2B, RNA levels for seven lncRNAs elevated in *DCP2*-deficient cells (Figure 1G) were also increased in cells lacking *XRN1* (5- to 47-fold). Consistent with our northern analysis, qRT-PCR of the *GAL10* lncRNA confirmed its accumulation in *dcp2Δ* and *xrn1Δ* cells compared to the WT (4.8- and 6-fold, respectively; Figure 2E, top). Surprisingly, lncRNA levels were unchanged in cells lacking either deadenylase activity (i.e., *ccr4Δ*) or regulators of mRNA decapping (i.e., *dhh1Δ* and *lsm1Δ*; Figures 2A and 2B). Additionally, inactivation of either nuclear or cytoplasmic 3′→5′ exosome activity (i.e., *rrp6Δ*, *trf4Δ*, and *ski2Δ*) failed to result in elevated levels of these lncRNAs (Figures 2A and 2B). The abundance, therefore, of lncRNAs we identified as decapping substrates, unlike mRNAs, is unaffected by deadenylation, 3′→5′ degradation, and, most unexpectedly, proteins required for activating mRNA decapping.

Eukaryotic cells possess two enzymes catalyzing 5′→3′ exonucleolytic degradation, XRN1 and RAT1, which are predominantly present in the cytoplasm and nucleus, respectively (Johnson, 1997). To determine whether RAT1 plays a role in modulating lncRNA levels, we used a temperature-sensitive allele of the essential *RAT1* gene, *rat1-1*, that abrogates RAT1 function at restrictive temperature of 37°C (Amberg et al., 1992). WT and *rat1-1* cells were grown at permissive temperature and shifted to 37°C for 2 hr before harvesting and isolation of RNA. Northern analysis determined several but not all lncRNAs elevated in *dcp2Δ* cells also accumulated when RAT1 was inactivated (Figures 2C and 2D). qRT-PCR analysis of the *GAL10* lncRNA confirmed these findings (Figure 2E, bottom). These observations suggest that nuclear 5′→3′ exonucleolytic digestion by RAT1 contributes to lncRNA decay.

lncRNA Degradation Is Regulated by a Distinct Decapping Pathway

Steady-state accumulation of lncRNAs in the absence of DCP2, XRN1, or RAT1 activity strongly implies a role in their degradation. To demonstrate direct involvement of these proteins in mediating lncRNA turnover, we performed kinetic analysis of RNA degradation. We specifically focused on the *GAL10* lncRNA because its transcription is regulated by the sugar in the growth media (Houseley et al., 2008). Cells grown in raffinose, where the *GAL10* lncRNA is transcriptionally active, were shifted to galactose-containing media, which represses *GAL10* lncRNA transcription. RNA isolated from cells at various times after galactose addition was analyzed by northern blot to evaluate changes in *GAL10* lncRNA levels over time and determine lncRNA half-life. As seen in Figure 2F, the half-life of *GAL10* lncRNA in WT cells was 17 min. In contrast, *GAL10* lncRNA stability increased to 49 min in *dcp2Δ* cells, demonstrating that DCP2 is directly involved in the turnover of this lncRNA. Stabilization of *GAL10* lncRNA is dependent on DCP2's pyrophosphatase activity (Figures S2B and S2C). In cells lacking the noncatalytic subunit of the decapping enzyme, *DCP1* (*dcp1Δ*), *GAL10* lncRNA was also stabilized with a half-life of 39 min. This result is consistent with observations that DCP1 is required for decapping activity both in vivo and in vitro and that the two proteins constitute a holoenzyme (Steiger et al., 2003). Moreover, as expected from steady-state analysis, *GAL10* lncRNA was stabilized in cells lacking *XRN1* (*xrn1Δ*; half-life of 36 min), but unaffected by inactivation of mRNA decapping activators (*lsm1Δ*, *lsm6Δ*, *dhh1Δ*), the deadenylase complex (*ccr4Δ*), nonsense-mediated mRNA decay (*upf1Δ*), the nuclear exosome (*rrp6Δ*), and the TRAMP complex (*trf4Δ*) (Figure 2F).

We also evaluated the role of the nuclear 5′→3′ exonuclease RAT1 in *GAL10* lncRNA stability. Transcriptional shut-off analysis of *GAL10* lncRNA in *rat1-1* cells at the permissive temperature, where RAT1 function is impaired but not completely abrogated, demonstrated that RAT1 plays an important role in its stability (half-life of 42 min; Figure 2F). In contrast, RAI1, a known cofactor of RAT1 that itself has pyrophosphatase activity (Jiao et al., 2010), did not impact either the steady-state level or stability of *GAL10* lncRNA (Figures 2A and 2F). Taken together, our data demonstrate that *GAL10* lncRNA turnover is mediated by DCP2, DCP1, XRN1, and RAT1, but not other

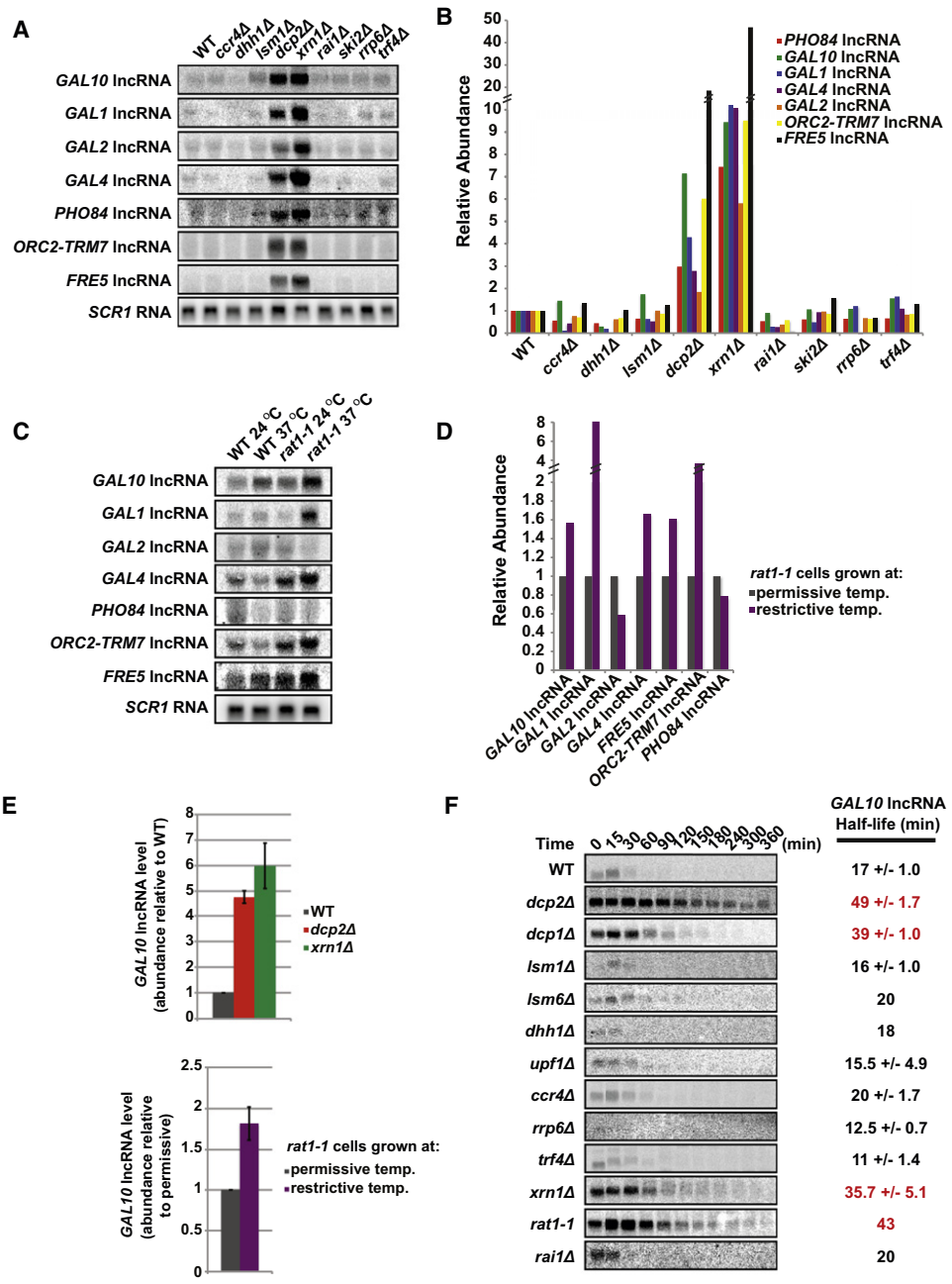

Figure 2. lncRNA Stability Is DCP2 Dependent

(A–E) Cells were grown in the presence of glucose and total RNA from WT and RNA decay mutant cells were analyzed by northern and qRT-PCR. *SCR1* RNA (northerns) and *U1* RNA (qRT-PCR) are the loading control. Northern analysis probing for *GAL10*, *GAL1*, *GAL2*, *GAL4*, *PHO84*, *ORC2-TRM7*, and *FRE5* are shown in (A)–(D).

(B) Quantification of (A) displaying abundance relative to WT with values normalized to *SCR1* RNA.

(C) WT and *rat1-1* cells were grown at permissive temperature (24°C) then shifted to restrictive temperature (37°C) for 2 hr.

(D) Quantification of (C) displaying the lncRNA increase in *rat1-1* cells with values normalized to *SCR1* RNA.

(E) qRT-PCR analysis of relative *GAL10* lncRNA abundance (top) WT, *dcp2Δ* and *xrn1Δ* cells (bottom) *rat1-1* cells comparing restrictive and permissive temperature. Error bars represent standard deviation between three experiments.

(F) Northern analysis of *GAL10* lncRNA half-life analysis in RNA decay defective cells. Time points were taken after transcriptional shut off (shift from raffinose to galactose growth), and half-lives were determined after normalization to *SCR1* RNA.

See also Figures S1 and S2.

proteins implicated in decay of mRNA, and therefore its metabolism involves a distinct decay pathway. Given the steady-state data for a number of lncRNAs (Figure 2A), we predict that this decay pathway is used to degrade many, if not most, lncRNAs in the cell. Moreover, these observations support the existence of two separate, yet partially redundant pathways for decay of lncRNAs—a cytoplasmic, XRN1-dependent pathway and a nuclear, RAT1-dependent pathway. Considering that decapping is required to generate a substrate for both 5′→3′ exonuclease enzymes (i.e., XRN1 or RAT1), DCP2 constitutes a critical regulator in lncRNA metabolism that is likely to function in both the cytoplasm and the nucleus based on observations that DCP2 can shuttle (Grousl et al., 2009).

GAL lncRNA Expression Is Regulated by Environmental Conditions

GAL-inducible gene regulation represents a classic genetic switch regulating sugar metabolism in eukaryotic cells (Lohr et al., 1995). The *GAL* system has been extensively characterized in yeast and consists of four structural genes—*GAL1*, *GAL10*, *GAL7*, and *GAL2*—that are coordinately regulated at the level of transcription by GAL4, GAL80, and GAL3. In repressed or noninduced conditions (glucose and raffinose sugar sources, respectively), GAL80 inhibits the ability of GAL4 to recruit the transcription machinery to drive expression of the structural genes. In the presence of galactose, GAL3 sequesters GAL80 in the cytoplasm, thus allowing robust transcriptional activation of *GAL1*, *GAL10*, *GAL7*, and *GAL2* by GAL4.

We were interested in determining whether *GAL* lncRNAs, like their mRNA counterparts, exhibit expression patterns in response to sugar availability. To this end, we grew WT and *dcp2Δ* cells under conditions where *GAL* gene transcription is either repressed (glucose), noninduced (raffinose), or induced (galactose) with respect to the mRNA at these loci (Figure 3A) and analyzed lncRNA levels by northern blot. In WT cells grown in glucose, *GAL2*, *GAL1*, and *GAL10* lncRNAs are present at very low or undetectable levels (Figure 3B, lane 1), consistent with our RNA-seq data and previous reports characterizing *GAL10* lncRNA expression (Figures 1A, 1C, 2A, and 2B) (Houseley et al., 2008). Similarly, low levels of these lncRNA were observed for WT cells grown in raffinose or galactose (Figure 3B, lanes 3 and 5). *GAL* lncRNAs from cells lacking *DCP2* were significantly elevated in glucose-grown cells (Figure 3B, lane 2), in agreement with their detection by RNA-seq. These lncRNA levels were also elevated in raffinose-grown cells (Figure 3B, lane 4), but not in cells grown in galactose (Figure 3B, lane 6). Critically, *GAL* lncRNAs fail to accumulate in cells where *GAL* gene expression is induced, despite the absence of destabilizing DCP2 activity, indicating a reciprocal pattern of expression and suggesting a role for these lncRNAs in regulating their cognate protein-coding genes (see below).

GAL4 lncRNA Influences Expression of *GAL4* mRNA

Our RNA-Seq data indicated a lncRNA expressed antisense to the *GAL4* locus (Figure 1D), and we were interested in determining whether the *GAL4* lncRNA also displayed reciprocal expression patterns with regards to its cognate mRNA. Importantly, *GAL4*, the transcriptional activator of *GAL*-inducible genes, while subject to glucose repression, is itself not induced by galactose (Lohr et al., 1995). We determined that, similar to *GAL2*, *GAL1*, and *GAL10* lncRNAs, *GAL4* lncRNA levels were low in WT cells grown under all conditions tested (Figure 3C, lanes 1, 3, and 5). Abrogation of decapping activity resulted in robust accumulation of *GAL4* lncRNA in glucose-grown cells as expected (Figure 3C, lane 2) but also led to elevated levels in raffinose and galactose-grown cells, where *GAL4* mRNA is also expressed (Figure 3C, lanes 4 and 6). *GAL4* mRNA and lncRNA do not, therefore, show an inverse expression pattern. *GAL4* mRNA levels were, however, reduced in decapping-deficient cells in which *GAL4* lncRNA accumulated (*dcp2Δ;* Figure 3C, lanes 4 and 6), suggesting that *GAL4* lncRNA may impair *GAL4* mRNA expression.

To evaluate whether *GAL4* lncRNA impinges upon *GAL4* mRNA levels, we attenuated *GAL4* lncRNA expression by introducing mutations within the region of its promoter (see the Experimental Procedures). WT and *dcp2Δ* cells in which *GAL4* lncRNA was either present or absent (*gal4 lncRNAmut*) were grown under conditions in which *GAL4* mRNA is expressed (i.e., raffinose or galactose-containing media) to determine the influence of *GAL4* lncRNA on *GAL4* mRNA expression. Importantly, *GAL4* lncRNA levels were reduced greater than 90% in *dcp2Δ* cells harboring the *gal4 lncRNAmut* mutation (Figures 3D and 3E). Critically, in the absence of *GAL4* lncRNA, *GAL4* mRNA levels increased 2- to 3-fold in *dcp2Δ* cells grown in either condition (Figures 3D and 3F, lanes 2 versus 4; lanes 6 versus 8). Our data indicate that *GAL4* lncRNA levels regulate expression of *GAL4* mRNA.

Decapping of a lncRNA at the *GAL10-GAL1* Locus Is Required for Efficient Activation upon Galactose Addition

We observed that in *dcp2Δ* cells, several *GAL* lncRNAs accumulate in cells grown in glucose or raffinose but fail to accumulate under conditions where *GAL* mRNAs are expressed (i.e., in the presence of galactose; Figures 3A and 3B). We hypothesized that *GAL* lncRNAs are absent in cells grown in galactose because their presence would impinge upon the transcriptional induction of *GAL* structural genes. *GAL* lncRNAs would, therefore, need to be rapidly removed from the cell upon an environmental shift from transcriptionally inactive to active conditions. We evaluated whether the *GAL10* lncRNA, which spans both *GAL10* and *GAL1* gene loci, influenced transcriptional activation of *GAL1* mRNA upon induction by galactose. Prior to induction, cells were grown in raffinose, noninducing conditions for *GAL1* mRNA, and samples were removed over time after galactose addition. In WT cells, *GAL10* lncRNA was present at low levels and decayed quickly upon addition of galactose (half-life = 17 min; Figure 4B). Consistent with reciprocal expression patterns, *GAL1* mRNA levels accumulated quickly in these cells and attained maximum levels after 90 min (Figures 4B and 4C). In cells lacking decapping activity, *GAL10* lncRNA persisted for hours after addition of galactose (half-life = 51 min), and induction of *GAL1* mRNA was significantly delayed (Figures 4B and 4C). Importantly, however, decay of *GAL10* lncRNA

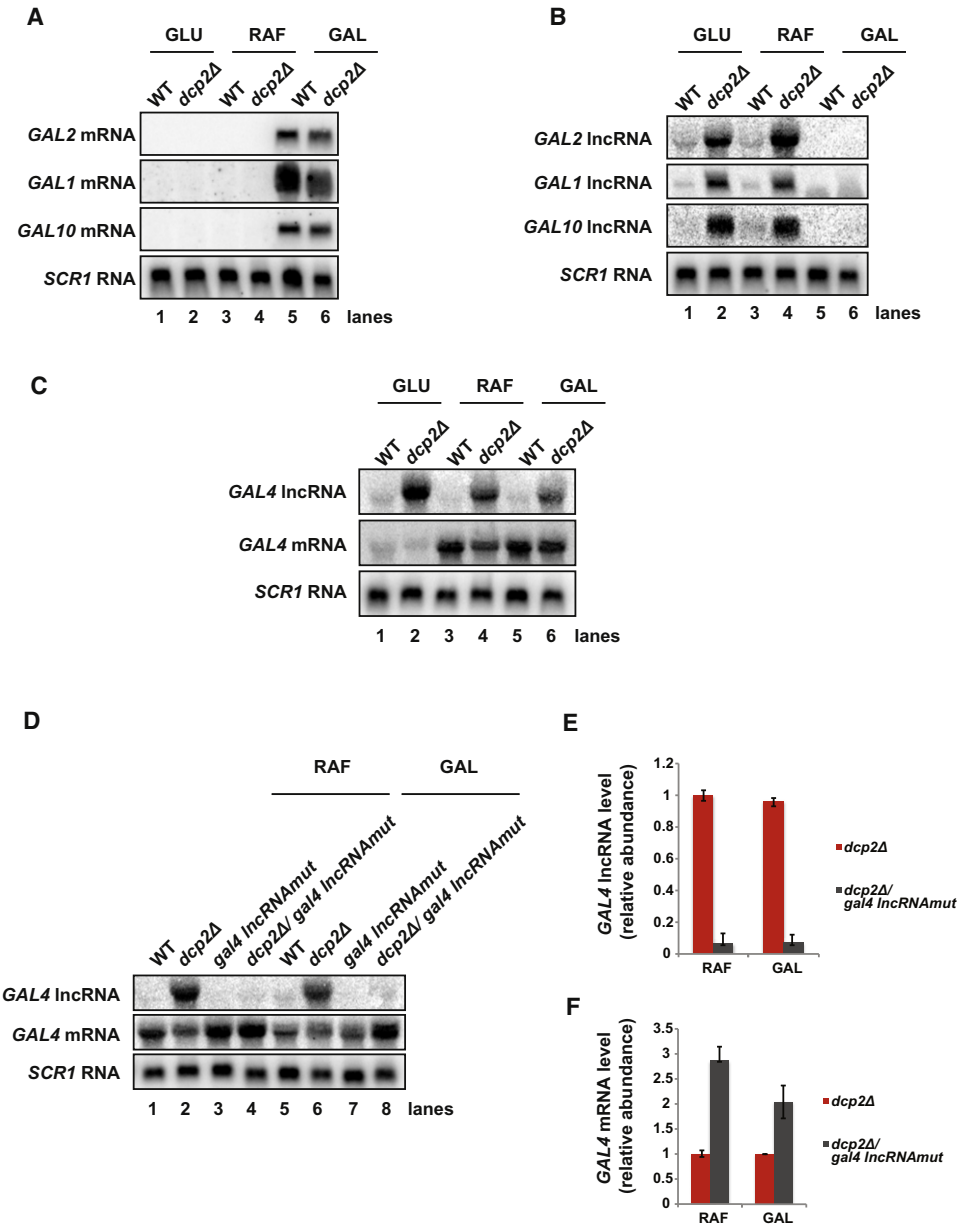

Figure 3. *GAL* lncRNA Expression Is Regulated by Environmental Conditions, and lncRNA Levels Influence mRNA Expression

(A–D) Cells were grown in glucose (GLU), raffinose (RAF), or galactose (GAL), and total RNA was analyzed by northern analysis probing for mRNAs at *GAL*-inducible loci (*GAL2, GAL1*, and *GAL10*) (A), lncRNAs at *GAL*-inducible loci (*GAL2, GAL1*, and *GAL10*) (B), and lncRNA and mRNA at the *GAL4* locus (C). *GAL4* lncRNA expression was attenuated, and levels of *GAL4* lncRNA and mRNA were determined by Northern analysis in *dcp2Δ* cells in raffinose (RAF) and galactose (GAL) (D).

(E and F) Relative fold changes between *dcp2Δ* and *dcp2Δ/gal4 lncRNAmut* cells for *GAL4* lncRNA (E) and *GAL4* mRNA (F) are displayed with values normalized to *SCR1* RNA. Error bars represent standard deviation between three experiments.

strongly correlated with *GAL1* mRNA induction in both WT and d*cp2Δ* cells (Figures 4B and 4C). This demonstrates that degradation of *GAL10* lncRNA through decapping plays an important role in the cells ability to respond to and utilize galactose as a sugar source. Consistent with this, d*cp2Δ* cells display a pronounced growth defect on galactose containing media (Figure S3).

Degradation of *GAL10* lncRNA Requires RAT1 for Efficient Induction of *GAL1* mRNA

We have demonstrated that *GAL10* lncRNA is stabilized in the absence of DCP2-dependent decapping and downstream XRN1 or RAT1-dependent 5′→3′ exonucleolytic degradation (Figure 2F). Since RAT1 and XRN1 are localized in distinct cellular compartments, and function in nuclear and cytoplasmic

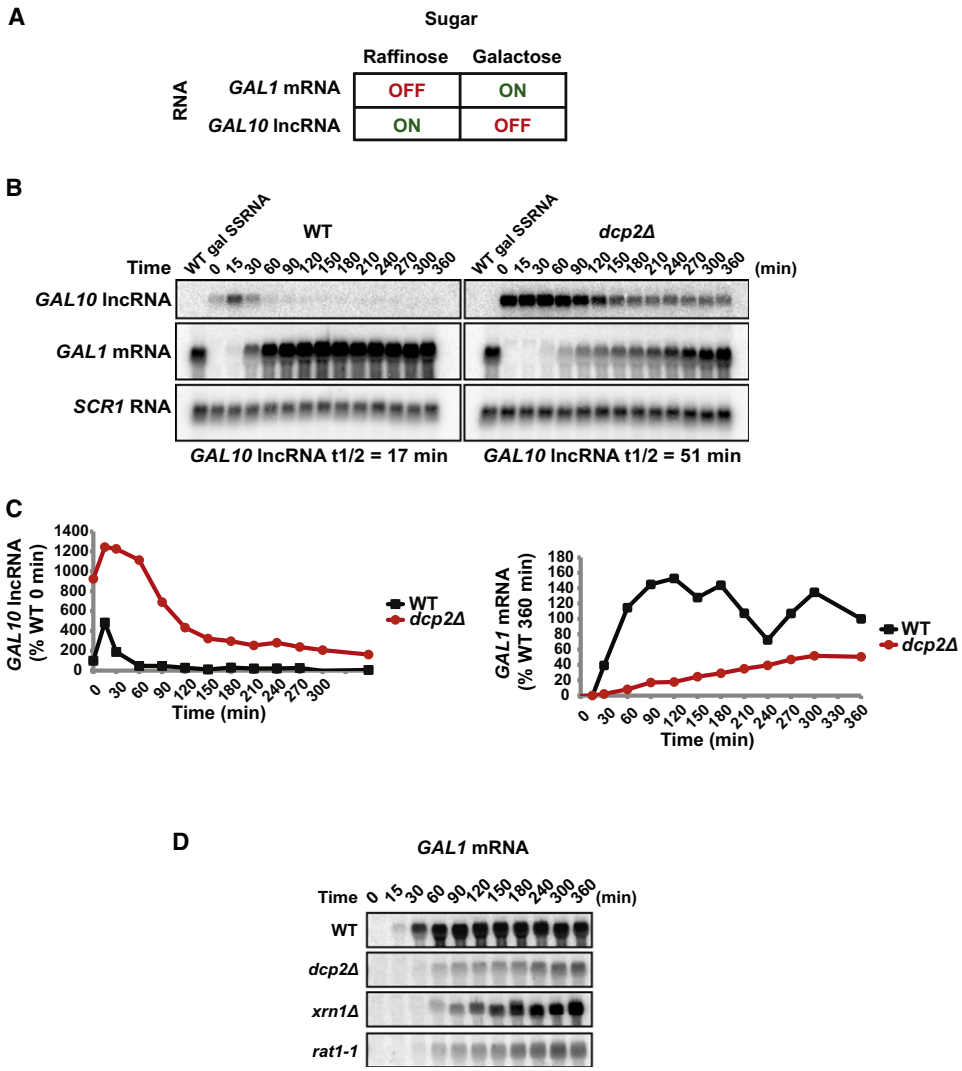

Figure 4. Decapping of a lncRNA at the *GAL10-GAL1* Locus Is Required for Efficient Activation upon Galactose Addition

(A) Schematic of *GAL10* lncRNA and *GAL1* mRNA expression in raffinose and galactose.

(B) Northern analysis of WT and *dcp2Δ* cells grown in raffinose then shifted to galactose. *GAL10* lncRNA decay and *GAL1* mRNA accumulation were measured over time with *SCR1* RNA as the loading control.

(C) Quantification of band intensities of (B) normalized to *SCR1* RNA. *GAL10* lncRNA levels are represented as a percentage of WT 0 min and *GAL1* mRNA levels are represented as a percentage of WT 360 min time points.

(D) Northern analysis of WT, *dcp2Δ*, *xrn1Δ* and *rat1-1* cells grown as in (B) after *GAL1* mRNA accumulation.

See also Figure S3.

RNA degradation, respectively, we evaluated whether either exonuclease was required for proper induction of *GAL1* mRNA expression. *GAL1* mRNA induction upon addition of galactose to the media was impaired in cells lacking *XRN1*, but not to the extent observed in d*cp2Δ* cells (Figures 4D and 4E). In contrast, cells only partially active for RAT1 (*rat1-1* cells grown at permissive temperature) demonstrated a significant delay in induction of *GAL1* mRNA similar to cells lacking decapping activity. These observations suggest a major role for nuclear 5′→3′ exonucleolytic degradation in regulating the function of *GAL10* lncRNA.

Decapping Influences Chromatin at the *GAL10-GAL1* Locus

We find that failure to destabilize *GAL10* lncRNA by decapping (or nuclear 5′→3′ decay) negatively influences induction of *GAL1* mRNA in response to galactose. Expression of *GAL10* lncRNA has been shown to exert transcriptional repression through methylation of histone H3 at lysine 4 and 36 (H3K4 and H3K36) and, ultimately, deacetylation of the *GAL10-GAL1* locus (Houseley et al., 2008; Pinskaya et al., 2009). The influence of *GAL10* lncRNA turnover on its function in chromatin remodeling, however, has not been addressed. In cells lacking

decapping activity, *GAL10* lncRNA is stabilized and we predicted that chromatin surrounding the locus should be hypoacetylated. Moreover, we hypothesized that failure to rapidly degrade *GAL10* lncRNA in decapping-deficient cells would correlate with delayed resolution of this hypoacetylated state upon a shift in conditions that transcriptionally activate *GAL1* mRNA. We performed chromatin immunoprecipitation (ChIP) followed by quantitative PCR (qPCR) to evaluate the acetylation status at the *GAL10-GAL1* locus in WT and *dcp2Δ* cells. Importantly, acetylation was surveyed over time after cells were shifted from noninducing to inducing growth conditions (i.e., raffinose to galactose). Acetylation levels were determined based on the percentage of immunoprecipitated DNA normalized against a telomeric region and represented relative to the WT.

Acetylation of histone H3 lysine 18 (H3K18) at the *GAL10-GAL1* locus (Figure 5A) was reduced in *dcp2Δ* cells compared to WT cells before galactose addition (Figure 5B; 0 min), indicative of hypoacetylation within this region when *GAL10* lncRNA levels are high, as anticipated. We predicted that elimination of *GAL10* lncRNA would be required to resolve the repressive chromatin state, and we measured H3K18 acetylation at various times after addition of galactose. After 30 min postinduction, when *GAL10* lncRNA is absent in WT cells but still at high levels in cells lacking *DCP2* (Figure 5C), chromatin at the *GAL10-GAL1* locus remains hypoacetylated in *dcp2Δ* cells compared to WT cells (Figure 5B; 30 min). At 180 min when a only paucity of *GAL10* lncRNA is present in either WT or *dcp2Δ* cells (Figure 5C), the locus is no longer hypoacetylated with H3K18 levels similar in both cell types (Figure 5B). Consistent with an attenuation in lncRNA repression, *GAL1* mRNA levels in *dcp2Δ* cells have begun to accumulate (Figure 5C; 180 min). By 360 min, H3K18 acetylation between WT and *dcp2Δ* cells are indistinguishable and *GAL1* mRNA levels in *dcp2Δ* cells are nearing WT (Figures 5B and 5C). The observed changes in chromatin state at the *GAL10-GAL1* locus over time correlate with changing *GAL10* lncRNA and *GAL1* mRNA levels in the two cell types. As shown in Figure 5C, *GAL10* lncRNA levels must fall below a critical threshold (~50%) before productive *GAL1* mRNA accumulation occurs. Importantly, in WT this threshold is met approximately 30 min after induction, while in *dcp2Δ* cells this threshold is not achieved until after 2 hr.

To directly test whether the repressive chromatin state induced by *GAL10* lncRNA in *dcp2Δ* cells causes the reduced kinetics of *GAL1* mRNA induction, we analyzed *GAL1* mRNA induction in cultures containing trichostatin A (TSA), a selective inhibitor of class I and II histone deacetylases (Codd et al., 2009). Importantly, RPD3, the histone deacetylase implicated in *GAL10* lncRNA repression is sensitive to TSA (Houseley et al., 2008; Pinskaya et al., 2009; Bernstein et al., 2000). Northern blot analysis indicated that TSA did not significantly influence the decay rate of *GAL10* lncRNA in *dcp2Δ* cells (versus untreated cells; Figure 5D versus Figures 4B and 4C). Induction of *GAL1* mRNA, however, was substantially improved compared to untreated cells (Figure 5D versus Figures 4B and 4C) and followed kinetics closer to those observed in WT cells (Figure 5D). These results indicate that destabilization of *GAL10* lncRNA is important for alleviating its function in histone deacetylation that impinge upon expression of the proximal *GAL1* gene.

DCP2 Mediates *GAL1* mRNA Expression through Destabilization of lncRNAs

Collectively our data suggest that the decapping enzyme, DCP2, mediates decapping of *GAL10* lncRNA and that proper metabolism of the lncRNA has a direct impact on transcriptional activation of *GAL1* mRNA. If *GAL10* lncRNA stabilization in decapping-deficient cells causes impaired rates of *GAL1* mRNA induction, then we would expect that removal of this lncRNA in *dcp2Δ* cells would result in increased rates of *GAL1* mRNA accumulation. To test this hypothesis directly, we introduced mutations in the *GAL10* lncRNA promoter that block *GAL10* lncRNA expression (Houseley et al., 2008) and determined the impact on induction of *GAL1* mRNA. As shown in Figure 6, these mutations completely abrogate expression of *GAL10* lncRNA, even in cells where *DCP2* is absent. Importantly, loss of *GAL10* lncRNA accumulation in *dcp2Δ* cells resulted in robust and rapid induction of *GAL1* mRNA upon addition of galactose to the growth media (Figure 6).

Interestingly, while *GAL1* mRNA induction was much higher in *dcp2Δ/GAL10 lncRNAΔ* cells compared *dcp2Δ* alone, restoration of *GAL1* mRNA induction in *dcp2Δ/GAL10 lncRNAΔ* cells was still delayed compared to that observed in WT cells. We attribute this observation to indicate that repressive factors in addition to *GAL10* lncRNA exist that regulate *GAL1* mRNA induction. In agreement with this, we annotated a lncRNA expressed antisense to *GAL1* based on our RNA-seq data (*GAL1* lncRNA; Figures 1A and 1G). Moreover, we have shown that expression of *GAL4,* the transcriptional activator of *GAL1*, is, in part, controlled by a lncRNA that is itself stabilized in *dcp2Δ* cells (Figures 3C and 3D), suggesting that reduced *GAL4* expression may also contribute to less robust *GAL1* mRNA induction. Together, we suggest that galactose utilization is regulated by a network of partially redundant lncRNAs, and that degradation by DCP2, RAT1, and to a lesser extent XRN1, is required to rapidly and robustly activate expression of *GAL* mRNAs.

DISCUSSION

Decapping Modulates lncRNA Levels to Regulate Inducible Genes

In recent years, the complexity of the eukaryotic transcriptome has become a subject of intense curiosity as well as debate (Wilusz et al., 2009; Ponting and Belgard, 2010). It is now well established, however, that RNA polymerase II transcribes hundreds of lncRNAs, and that some of these function in such processes as transcriptional regulation, imprinting, and chromosome inactivation (Nagano and Fraser, 2011). The metabolism of these lncRNAs and the impact of turnover on their function has not, until recently, been extensively addressed.

We find that many of the lncRNAs we identified by RNA-seq are expressed antisense to highly regulated genes whose transcription is responsive to a variety of environmental cues. Specifically, our lncRNAs map to genes involved in regulatory networks for glucose metabolism, maltose utilization, flocculation, iron sensing, meiosis, mating, and sporulation (Table S3). Interestingly, target genes of these pathways are transcriptionally repressed in the absence of stimulation but are rapidly

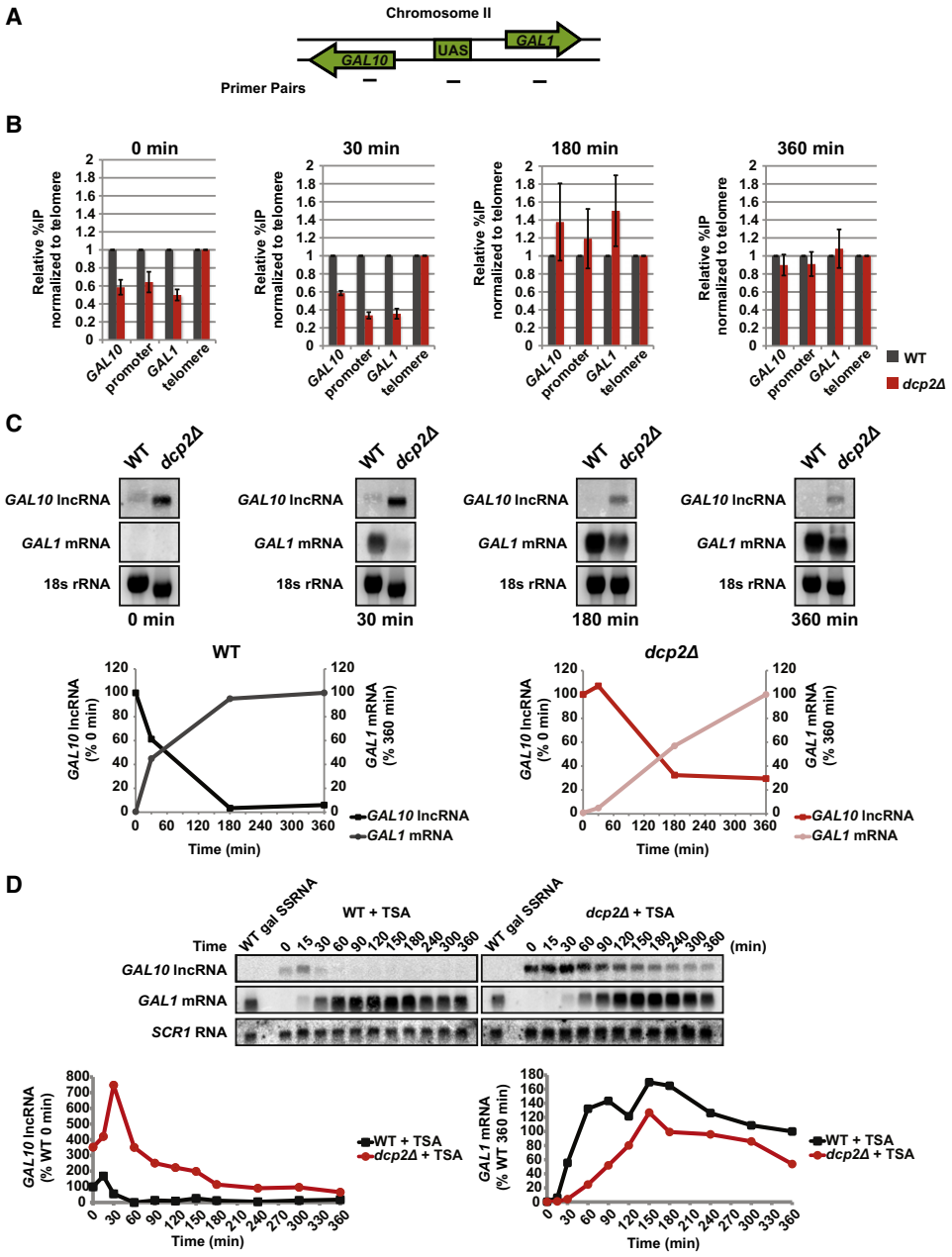

Figure 5. Decapping Influences Chromatin at the *GAL10-GAL1* Locus

(A) Diagram of ChIP primer set positions at the *GAL10-GAL1* locus.

(B) Cells were grown as in Figure 4B, and aliquots were crosslinked at 0, 30, 180, and 360 min of galactose exposure. Coimmunoprecipitated DNA from ChIP against acetylated H3K18 was amplified by qPCR. Acetylation relative to the WT is displayed as a percentage of input normalized to a telomeric location. Error bars represent standard deviation between three experiments.

(C) Cells were grown as in Figure 4B and RNA was analyzed by northern probing for *GAL10* lncRNA and *GAL1* mRNA with 18S RNA ethidium stain as the loading control. RNA levels for WT and *dcp2Δ* were plotted with *GAL10* lncRNA levels represented as a percentage of the 0 min and *GAL1* mRNA levels as a percentage of the 360 min time points.

(D) *GAL1* mRNA expression was induced by galactose in cells grown in raffinose and 10 μM trichostatin A (TSA). *GAL10* lncRNA decay and *GAL1* mRNA accumulation was measured by northern with *SCR1* RNA as the loading control. *GAL10* lncRNA levels are represented as a percentage of WT 0 min and *GAL1* mRNA levels as a percentage of WT 360 min time points.

derepressed upon introduction of the effector (Campbell et al., 2008; Chow et al., 1989; Teunissen and Steensma, 1995; Shakoury-Elizeh et al., 2004).

Galactose utilization in yeast presents a classical paradigm for understanding gene regulation in eukaryotes (Lohr et al., 1995). Our analysis confirms a previously characterized lncRNA

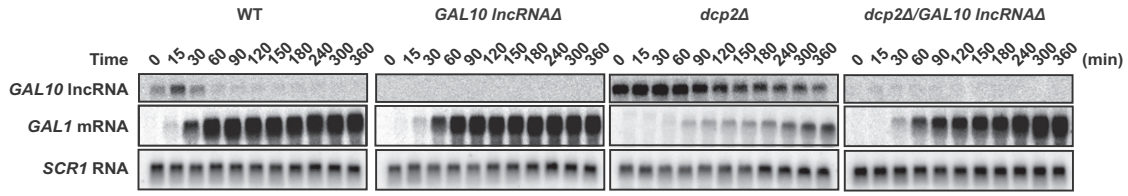

Figure 6. DCP2 Mediates *GAL1* mRNA Expression through Stabilization of lncRNAs

GAL10 lncRNA was deleted by genetically removing the *GAL10* gene and complementing *GAL10* mRNA expression with a plasmid born copy with silent mutations that disrupt expression of *GAL10* lncRNA. Cells were grown in raffinose and *GAL1* mRNA expression was induced by shifting to galactose. *GAL10* lncRNA decay and *GAL1* mRNA accumulation were measured by northern analysis. *SCR1* RNA was the loading control.

antisense to *GAL10* (*GAL10* lncRNA) (Houseley et al., 2008) and reveals lncRNAs mapping antisense to the structural genes *GAL2* and *GAL1* and to the master transcriptional regulator *GAL4*. We have, therefore, taken advantage of the wealth of information and robust regulatory circuit underlying the *GAL* regulon to determine a role for decay in lncRNA function. We demonstrate that decapping of *GAL4* lncRNA is required for *GAL4* mRNA to reach levels observed in the WT (Figures 3C and 3D). Moreover, transcriptional activation of *GAL1* mRNA upon induction by galactose absolutely requires *GAL10* lncRNA decapping for rapid and robust expression (Figure 4B).

Our data provide the first evidence that *GAL* lncRNAs are degraded by a DCP2-dependent process and that decapping of lncRNAs is required for robust expression of their cognate protein-coding genes. We propose that, in yeast, regulatory pathways have evolved to include lncRNAs and that these RNA species play a critical role in maintaining and/or reinforcing gene repression in the absence of activating signals (Figure 7). Moreover, upon stimulation, proper clearance of the lncRNA by decapping-dependent decay is required for rapid and robust gene activation. Our discovery of a network of regulatory *GAL* antisense lncRNAs adds an additional layer of complexity to this classic genetic switch and suggests that other inducible genes may also require lncRNA degradation for proper transcriptional regulation (Figure 7).

Mechanism of lncRNA-Dependent Transcriptional Control and Derepression by DCP2

An important and unresolved issue is the precise mechanism by which lncRNA degradation leads to derepression of lncRNA-regulated genes. In yeast and metazoans, lncRNAs have been documented to function both in *cis* and *trans* to alter methylation and acetylation states of histones through association with chromatin-modifying complexes (Berretta and Morillon, 2009; Nagano and Fraser, 2011). Modulation of lncRNA levels either transcriptionally or by degradation would, therefore, be expected to significantly affect chromatin state and, consequently, expression of lncRNA-regulated genes. In the case of *GAL10* lncRNA, transcription of the lncRNA has been suggested to recruit histone-modifying enzymes to the C-terminal domain of RNA polymerase II cotranscriptionally that alter chromatin structure and create a chromatin environment that represses spurious transcription (Houseley et al., 2008). In this study, we demonstrate that destabilization of *GAL10* lncRNA by DCP2 is essential in order to maintain a chromatin structure that is repressed yet poised for rapid activation.

GAL10 lncRNA regulates the expression of proximal mRNA genes by altering the acetylation status of histones associated with its own locus (Houseley et al., 2008) (Figure 5B). We find that in cells deficient for decapping, the *GAL10-GAL1* locus is hypoacetylated compared to the wild-type, and that pharmacological inhibition of histone deacetylation bypasses *GAL10* lncRNA-mediated repression and restores robust *GAL1* mRNA induction to near WT levels (Figure 5D). These observations indicate that stabilization of *GAL10* lncRNA represses induction of *GAL1* mRNA expression through persistent deacetylation of histones.

It remains to be established whether impaired induction of *GAL1* mRNA expression by stabilized *GAL10* lncRNA results from inhibition in *cis* or in *trans*. The initial characterization of the *GAL10* lncRNA suggested that this lncRNA exerts its effects in *cis* because transcription of *GAL10* lncRNA from one allele in heterozygous diploid cells, failed to downregulate *GAL1* mRNA expression from the other allele (Houseley et al., 2008). Although in the absence of changes in transcription, it would be anticipated that elevated lncRNA levels would likely manifest their function in *trans*, we find no apparent reason to suggest that decapping of *GAL10* lncRNA could not modulate its function in *cis*. Indeed, several lines of evidence place DCP2 as well as *GAL10* lncRNA decapping in the right compartment of the cell (i.e., the nucleus) to modulate *GAL10* lncRNA function in *cis*. First, although predominately cytoplasmic, DCP2 has been documented to shuttle and reside in both nuclear and cytoplasmic compartments of the cell (Grousl et al., 2009). Second, a decapping activity involved in turnover of nuclear retained pre-mRNA and mature mRNA has been characterized (Kufel et al., 2004). Third, the elevated level of *GAL10* lncRNA in the absence of *XRN1* has only a modest effect on *GAL1* mRNA induction, while conditional loss of the nuclear exonuclease, RAT1, strongly impairs induction of *GAL1* mRNA transcription (Figure 4D). Together, these observations support a model in which DCP2, in concert with RAT1, regulates the function of *GAL10* lncRNA by promoting its decapping/destruction in the nucleus and perhaps at its site of transcription.

We envision two possible mechanistic models for how stabilization of *GAL10* lncRNA by DCP2 (and RAT1) impairs galactose-induced *GAL1* mRNA transcriptional activation. First, stabilization of the lncRNA promotes formation of an R loop around the transcriptional bubble inhibiting transcriptional

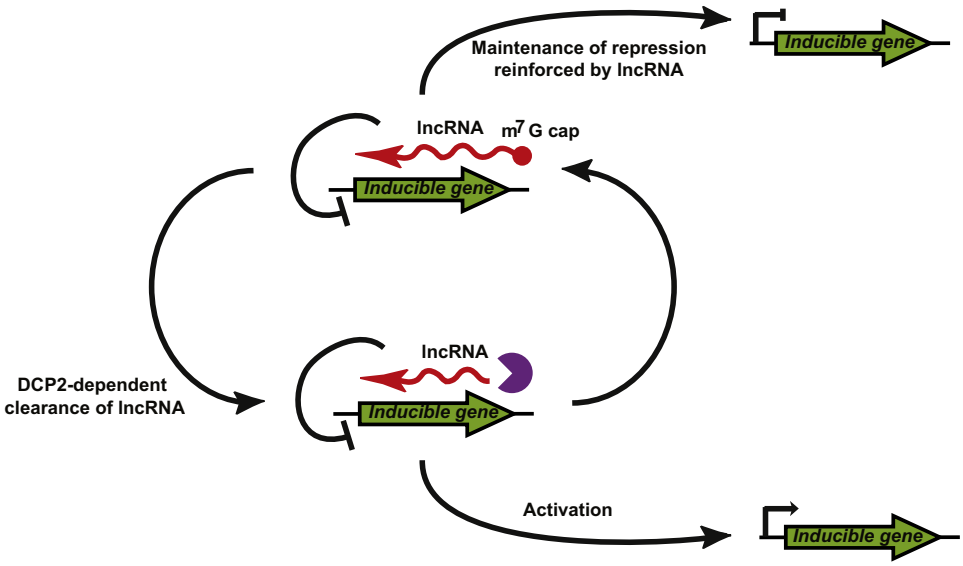

Figure 7. DCP2-Dependent Clearance of lncRNAs Is Required for Rapid Responses to Environmental Cues
Inducible genes have evolved lncRNA regulators that are important for maintenance of inactive states. Upon stimulation DCP2 is required to clear these lncRNAs from the cell, allowing for the activation of inducible genes in response to stimulus. See also Table S3.

elongation, as recently described by Belotserkovskii and Hanawalt (2011). Considering that stabilization of the lncRNA exacerbates *GAL10* lncRNA repression, the physical presence of lncRNA might be an important and currently unaccounted for aspect of its mechanism. An R loop-based model reconciles the observed *cis*-acting nature of *GAL10* lncRNA repression (Houseley et al., 2008) with our observations that stabilization and perhaps the physical presence of the lncRNA influences function. Consistent with R loop structures playing a role in lncRNA physiology, RNA/DNA hybrids have been implicated in the mechanism by which *TERRA* lncRNA stabilization causes telomere length defects (Luke et al., 2008). It is unclear, however, exactly how an RNA/DNA hybrid model would account for the observed *GAL10* lncRNA-mediated histone deacetylation. In the second model, transcription of lncRNA is sufficient to impose a repressive chromatin environment but DCP2 and RAT1 dampen this effect by degrading lncRNA cotranscriptionally. Indeed, RAT1 is vital for termination of RNA polymerase II transcription by the "torpedo" model in which mRNA cleavage and polyadenylation generates a downstream monophosphorylated product that is a substrate of RAT1 (Kawauchi et al., 2008). Since lncRNAs have 5′ m^7G caps, cotranscriptional decapping by DCP2 would provide RAT1 access to nascent RNA and an opportunity to promote transcriptional termination. Limiting lncRNA transcription by this mechanism would circumvent the repressive alterations in chromatin architecture that result from *GAL10* lncRNA transcription. An important next step will, therefore, be to determine precisely how the stability of lncRNAs alter transcriptional events.

Other Roles of Decapping in lncRNA Function

Our observation that RNA decapping and 5′ exonucleolytic decay play a major role in modulating the levels of lncRNA is consistent with a previous observation that *SRG1* lncRNA (controlling expression of the serine biosynthetic gene, *SER3*) is also a substrate for DCP2 and XRN1 (Thompson and Parker, 2007). Importantly, *SRG1* lncRNA turnover was also mediated by the auxiliary decay factors, DHH1 and UPF1. In contrast, none of the lncRNAs analyzed in this study were affected by loss of other factors involved in mRNA metabolism, including deadenylation, activation of decapping, or nuclear and cytoplasmic 3′ exonucleolytic decay (Figures 2A and 2B). This observation raises an important question regarding how lncRNAs are distinguished from mRNAs and targeted for a distinct decapping pathway. One obvious distinction between mRNA and lncRNAs is their association with the translation apparatus and, considering mRNA decay occurs cotranslationally (Hu et al., 2009), a potential determinant in eliciting mRNA decapping rather than lncRNA decapping could be the act of translation itself.

Many of the lncRNAs identified by RNA-seq that are sensitive to decapping are also substrates of XRN1 (van Dijk et al., 2011). Importantly, however, approximately 30% of lncRNAs that are elevated in cells lacking *DCP2* (Table S2) do not appear to be substrates of XRN1 (i.e., XUTs). Although this observation may reflect differences in annotating RNA-seq data, it also suggests that an alternative pathway exists for the degradation of these RNAs. Indeed, we show evidence that several lncRNAs are sensitive to degradation by RAT1. Differences in lncRNA metabolism likely represent differences in their subcellular distribution. Accordingly, we predict that XUTs and *SRG1* lncRNA are predominantly present in the cytoplasm where they are subject to XRN1-mediated decay. In contrast, *GAL10* lncRNA is both nuclear and cytoplasmic, since the decapped product is a substrate for both XRN1 and RAT1. Critically, stabilization of only the nuclear pool of *GAL10* lncRNA led to an alteration in

GAL1 mRNA induction (Figures 4D and 4E) indicating that degradation of the lncRNA in the correct cellular compartment is critical for its proper function. The presence of lncRNAs in the nucleus is consistent with their functional importance in chromatin organization and suggest that decapping can influence gene expression patterns much more broadly than in its characterized role in mRNA stability.

EXPERIMENTAL PROCEDURES

All strains used are the BY4741 genetic background unless indicated; genotypes are listed in Table S4. Cells were grown at 24°C into mid-log phase (3.0×10^7 cells ml^{-1}) in standard synthetic medium (pH 6.5) with the appropriate amino acids supplemented and 2% glucose, 2% raffinose, or 2% galactose. Cultures used for RNA-seq were grown in glucose media. For RNA-seq library construction, libraries were prepared according to Illumina's Directional mRNA-Seq Sample Prep Guide (Part # 15018460 Rev. A). Total RNA was isolated from yeast cells via standard methods. Chromatin immunoprecipitation was carried out using standard methods. All plasmids and oligos used are listed in Tables S5 and S6. See the Supplemental Experimental Procedures and methods for a detailed description of experimental procedures.

ACCESSION NUMBERS

RNA-seq data have been deposited in the NCBI Sequence Read Archive (accession number SRA048128.1).

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, three figures, and six tables and can be found with this article online at doi:10.1016/j.molcel.2011.11.025.

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