Control of Cdc28 CDK1 by a Stress-Induced IncRNA

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SUMMARY

Genomic analysis has revealed the existence of a large number of long noncoding RNAs (lncRNAs) with different functions in a variety of organisms, including yeast. Cells display dramatic changes of gene expression upon environmental changes. Upon osmostress, hundreds of stress-responsive genes are induced by the stress-activated protein kinase (SAPK) p38/Hog1. Using whole-genome tiling arrays, we found that Hog1 induces a set of IncRNAs upon stress. One of the genes expressing a Hog1-dependent IncRNA in antisense orientation is CDC28, the cyclin-dependent kinase 1 (CDK1) that controls the cell cycle in yeast. Cdc28 IncRNA mediates the establishment of gene looping and the relocalization of Hog1 and RSC from the 3′ UTR to the +1 nucleosome to induce CDC28 expression. The increase in the levels of Cdc28 results in cells able to reenter the cell cycle more efficiently after stress. This may represent a general mechanism to prime expression of genes needed after stresses are alleviated.

INTRODUCTION

The existence of long noncoding RNAs (lncRNAs) is widespread in eukaryotes from yeast to mammals (Guttman and Rinn, 2012; Jacquier, 2009). Long noncoding transcripts in yeast influence gene expression, revealing a new layer of transcriptional regulation (Wei et al., 2011; Wu et al., 2012). LncRNAs might regulate transcription at multiple levels. Sense-oriented lncRNAs of IMD2 and URA2 alter expression by transcriptional interference and transcription start site (TSS) selection (Kuehner and Brow, 2008; Thiebaut et al., 2008). Expression of lncRNAs can also trigger changes in chromatin epigenetic state or nucleosome occupancy (Houseley et al., 2008; Kim et al., 2012; Margaritis et al., 2012; Pinskaya et al., 2009; van Werven et al., 2012; Hainer et al., 2011; Uhler et al., 2007). Although in a few cases expression of specific lncRNAs alters normal mRNA biogenesis, the general biological relevance and functionality of lncRNAs remains elusive. Remarkably, changes in nutrient availability result in changes in lncRNA expression (Xu et al., 2009, 2011), indicating that environmental insults and signal transduction pathways might affect lncRNA transcription.

Exposure of cells to stress requires immediate and specific cellular responses for proper adaptation (Hohmann et al., 2007). Thus, environmental insults require adaptive responses for maximal cell survival (de Nadal et al., 2011). Stress-activated protein kinases (SAPKs) serve to respond and adapt to extracellular changes. Exposure of yeast to high osmolarity results in activation of the p38-related Hog1 SAPK (Saito and Posas, 2012), which is essential to control cell cycle (Clotet and Posas, 2007; Duch et al., 2013) and gene expression (de Nadal and Posas, 2010).

The Hog1 SAPK acts in multiple stages of the cell cycle by targeting several core components of the cell cycle machinery. For instance, Hog1 controls G1/S transition by the downregulation of cyclin expression and the stabilization of the Sic1 cyclin-dependent kinase inhibitor (CDKl) (Adrover et al., 2011; Escoté et al., 2004). Hog1 also modulates other phases of the cell cycle, such as S phase (Duch et al., 2013). Cells unable to delay cell cycle progression upon osmostress display reduced viability upon those conditions, suggesting the need to delay cell cycle for proper adaptation.

The Hog1 SAPK is a key element for reprogramming gene expression in response to osmostress by acting on hundreds of stress-responsive genes. Hog1 is recruited to chromatin to recruit RNA polymerase II (Alepuz et al., 2003; Nadal-Ribelles et al., 2010) and associated factors (De Nadal et al., 2004; Solé et al., 2011; Zapater et al., 2007). Hog1 is present also at the open reading frames (ORFs) of stress-responsive genes (Cook and O’Shea, 2012; Nadal-Ribelles et al., 2012; Pokholok et al., 2006; Proud et al., 2008), where it stimulates strong chromatin remodeling by the interplay of the INO80 and the RSC complexes (Klopf et al., 2009; Mas et al., 2009). Chromatin dynamics set a threshold for gene induction upon Hog1 activation (Pelet et al., 2011). In addition to gene induction, Hog1 controls mRNA stability (Miller et al., 2011; Molin et al., 2009; Romero-Santacreu et al., 2009), export (Regot et al., 2013), and translation (Warringer et al., 2010). Thus, Hog1 plays a key role in the regulation of mRNA biogenesis (de Nadal et al., 2011; de Nadal and Posas, 2010; Martínez-Montañés et al., 2010; Weake and Workman, 2010).

Here, we show that the Hog1 SAPK also associates with and controls the induction of a set of IncRNAs in response to osmostress. One of the genes expressing a stress-induced IncRNA in antisense orientation is CDC28, the cyclin-dependent kinase 1 (CDK1) that controls the cell cycle in yeast. Induction of the CDC28 IncRNA permits the increase in the levels of Cdc28, allowing cells more efficient reentry into the cell cycle after stress.

Therefore, Hog1 directly coordinates the regulation of
transcription and cell cycle progression by controlling expression of a stress-induced lncRNA in CDC28.

RESULTS

Hog1 Mediates the Expression of a Set of Stress-Inducible lncRNAs

Most transcriptome studies performed to define stress genes have analyzed coding genes. To cover the expression of the whole genome upon stress, we monitored transcription using strand-specific tiling arrays (David et al., 2006). The number of coding genes induced upon stress was 343 at 0.4 M NaCl (15 min) and 294 at 1.2 M NaCl (100 min) using a stringent threshold (see Experimental Procedures). Expression of 56% and 84% of the stress-induced genes depended on Hog1 at 0.4 M and 1.2 M NaCl, respectively. Overall, the number of stress-responsive genes was similar to that in previous reports (Capaldi et al., 2008; Gasch et al., 2000; Nadal-Ribelles et al., 2012; Posas et al., 2000).

Remarkably, in addition to coding genes, up to 173 lncRNAs were strongly induced upon treatment with 0.4 M NaCl and up to 216 with 1.2 M NaCl (Figure 1). Almost a hundred of them were shared between the two stress conditions (Figure S1 available online). The average length of these stress-induced lncRNAs is about 843 nt (Figure S1). Expression of 50% and 91% of the lncRNAs induced by treatment with 0.4 M and 1.2 M NaCl, respectively, depended on the presence of Hog1 (http://steinmetzlab.embl.de/francescData/arrayProfile/index.html) (Figure 1B). Some overlapped with previously annotated cryptic unstable transcripts (CUTs) or stable unannotated transcripts (SUTs) (Wu et al., 2012; Xu et al., 2009). However, most of them were not expressed in the absence of RRP6, TRF4, or XRN1 and were present only upon stress (Figures 1C and S1). Thus, Hog1 mediates the expression of a set of stress-inducible lncRNAs.

Hog1 Associates with the Promoters of Stress-Induced lncRNAs and Stimulates RNA Pol II Recruitment and Gene Expression

Hog1 associates with chromatin of stress-responsive genes upon stress (Alepuz et al., 2001, 2003; Cook and O’Shea, 2012; Pohlokol et al., 2006; Proft et al., 2006). Actually, Hog1 is present in at least 80% of the Hog1-induced genes upon stress (Nadal-Ribelles et al., 2012). We also found that Hog1 is present in ~63% of Hog1-dependent lncRNA promoters, whereas it is recruited to <30% of Hog1-independent lncRNAs (Figure 2A). Genome-wide association of RNA Pol II showed that it strongly associates with stress-responsive loci upon stress (Cook and O’Shea, 2012; Nadal-Ribelles et al., 2012).

RNA Pol II was also recruited at the stress-induced lncRNAs (2.3-fold increase) upon stress. In contrast, RNA Pol II was not recruited at the Hog1-dependent lncRNA promoters in a hog1 strain (Figures 2B and S2A). Therefore, Hog1 associates to and stimulates the recruitment of RNA Pol II at the promoters of stress-induced lncRNAs.

Once recruited to stress-responsive genes, Hog1 mediates chromatin remodeling (Mas et al., 2009; Pelet et al., 2011). Genome-wide micrococcal nuclease (MNase) digestion of chromatin and deep sequencing (MNase-seq) showed that upon osmostress, Hog1 mediates dramatic change of nucleosome occupancy (Nadal-Ribelles et al., 2012). We analyzed the chromatin organization at genes that expressed Hog1-induced lncRNAs in antisense orientation and found that regions beyond the transcription termination site (TTS) also suffered strong Hog1-dependent chromatin remodeling (Figure 2C). Although levels of nucleosome occupancy decreased slightly upon stress in hog1Δ cells, this decrease was clearly more prominent in the wild-type strain. In contrast, no changes in chromatin structure were observed in promoters of lncRNAs that do not respond to stress (Figure S2B).

Expression from stress-responsive promoters can be quantitatively measured by the fusion to quadruple Venus (qV) fluorescent protein (Pelet et al., 2011; Regot et al., 2013). To characterize one of these lncRNA promoters further, we fused the 3’ untranslated region (3’ UTR) of CDC28 in both the sense and the antisense orientation to a qV-yellow fluorescent protein (YFP) and assessed gene expression by flow cytometry in wild-type and hog1 strains. Expression of qV-YFP was induced upon stress, depending on the presence of Hog1 and only when placed in the antisense orientation (Figure 2D). Therefore, the 5’ regions of stress-induced lncRNAs behave as bona fide stress-responsive promoters.

Induction of CDC28 lncRNA Expression Promotes the Induction of CDC28 Gene Expression upon Stress

To functionally characterize the role of stress-induced lncRNAs, we asked whether there is a correlation between expression of the sense and antisense-induced transcription. We found a correlation for only a few relevant cases (8 out of 91; Hog1-dependent lncRNAs at 0.4 M NaCl) in which an increase of the antisense was associated with an increase in the sense transcript (Figure 3A). Tiling arrays are not very sensitive for slight increases on transcription; thus, we analyzed the expression of the 91 genes with stress-induced lncRNAs from previous run-on assays and found that 41 out of 91 genes displayed a positive correlation (Romero-Santacreu et al., 2009).

One of the genes with a clear correlation of the sense and lncRNA expression was CDC28 (Figure 3B). CDC28 encodes the main CDK that drives progression of the cell cycle in yeast. We found that upon osmostress, there is an increase in CDC28 expression that was not observed in a hog1 strain (Figure 3B). Systematic insertion analysis at the 3’ UTR of CDC28 showed that insertion of a KanR marker at 180 nt downstream of the TTS (lncRNA) abolished expression of the lncRNA. In this strain, the induction of CDC28 upon osmotic stress was impaired (Figures 3B and S3A). Notably, the rrp6 mutation did not alter the induction of the CDC28 sense transcript (Figure S3B). Thus, the presence of the stress-inducible CDC28 lncRNA correlates with induction of the CDC28 gene expression.

We then assessed whether it was the expression of the lncRNA from the 3’ UTR or solely the presence of the lncRNA that induced CDC28 expression. We created a strain containing a CDC28::GFP that recapitulated CDC28 gene expression (CDC28::GFP) and then abolished lncRNA expression...
We then expressed CDC28 and its terminator region from a plasmid. This permitted us to distinguish transcription from the plasmid or the endogenous locus within the same cell. Remarkably, in response to osmostress, we could detect an increase in expression of CDC28 sense from the plasmid, but we did not observe an increase from the endogenous locus.

Figure S3 C. We then expressed CDC28::GFP lncRNA (Figure S3C).
Figure 2. Hog1 Binds and Recruits RNA Pol II at Genes with lncRNA

(A) Hog1 associates with lncRNA promoters. The percentage of Hog1 binding at the Hog1-dependent or -independent lncRNAs determined by ChIP-seq (0.4 M NaCl, p < 0.05).

(B) Hog1 stimulates Pol II recruitment to lncRNA promoters. Distribution of RNA Pol II binding (normalized reads, TRPKs) was determined by ChIP-seq (0.4 M NaCl for 10 min) for Hog1-dependent lncRNAs in wild-type (WT) and hog1Δ. ***p < 0.001 (t test).

(C) Hog1 mediates changes in chromatin architecture at lncRNA promoters. Distribution of nucleosome hits (RPKM; reads per kilobase per million) expanding 1 kb up and downstream from the TTS of wild-type and hog1Δ mutant strains under basal conditions (dark blue and red) and 0.4 M NaCl (light blue and yellow). Plot represents coverage of reads of approximately 90 genes: Hog1-dependent (upper graph), Hog1-independent (lower graph), and non-stress responsive (see Figure S2). Dotted black line marks TTS.

(D) Antisense-oriented 3’ UTR of CDC28 is an osmoreponsive promoter. The 3’ UTR of CDC28 fused to the qV-YFP in the antisense orientation (promoter lncRNA) or in its natural orientation (terminator sense). Fluorescence intensity was measured by flow cytometry.
The RSC Chromatin Remodeling Complex Mediates Chromatin Remodeling at the CDC28 +1 Nucleosome Region upon Stress

Hog1 stimulates chromatin remodeling at specific stress-responsive loci by recruiting the RSC complex (Mas et al., 2009). Induction of the CDC28 IncRNA in cells deficient in the RSC complex (rsc9Δ) under nonpermissive temperature was similar to that of wild-type but reduced in a SAGA mutant (Figures 4C and S4A). Thus, RSC is not necessary for IncRNA expression. In clear contrast, rsc9Δ mutant cells did not induce CDC28 expression upon stress. This suggests a key role of RSC for the increase of CDC28 sense upon stress.

Then, we assessed the recruitment of RSC and Hog1 to various regions of CDC28 before and after the addition of NaCl. We found that RSC associates with 3′ UTR and +1 nucleosome regions of CDC28 in response to stress only in the presence of Hog1 (Figure 4D). Induction of IncRNA and CDC28 required active Hog1, since a catalytically inactive Hog1 was unable to associate to chromatin and promote RSC association (Figures S4B–S4D). By contrast, association of Hog1 was not altered in the rsc9Δ mutant under nonpermissive temperature (Figure S4E), suggesting that Hog1 mediates the recruitment of RSC at CDC28 to remodel chromatin upon stress. Correspondingly, chromatin remodeling at the +1 nucleosome, assessed by MNase digestion, was impaired in the rsc9Δ mutant under nonpermissive temperature (Figure 4E). Thus, recruitment of RSC by Hog1 is essential to mediate chromatin reorganization at the +1 nucleosome region and CDC28 gene induction.

The Expression of Both the CDC28 IncRNA and Hog1 Is Required for CDC28 Induction upon Stress

In the absence of CDC28 IncRNA expression, the presence of Hog1 at the 3′ UTR of CDC28 is not sufficient to increase CDC28 expression. Then, we asked whether the expression of the CDC28 IncRNA alone was sufficient for CDC28 induction. Thus, we inserted an inducible GAL1 promoter in the presence of β-estradiol (Louvion et al., 1993). Although the presence of estradiol strongly induced expression of the CDC28 IncRNA, this was not sufficient to stimulate sense transcription (Figure 5A). We then monitored Hog1 recruitment and found that the presence of estradiol did not mediate Hog1 recruitment in CDC28 (Figure 5B). Correspondingly, chromatin remodeling at the +1 nucleosome did not occur by the sole induction of the CDC28 IncRNA from the GAL1 promoter in the presence of the Gal4-ER-VP16 activator (Figure 5C). Thus, the induction of the CDC28 IncRNA alone is not sufficient to mediate chromatin remodeling and CDC28 gene induction.

Then, we assessed whether the recruitment of Hog1 together with the CDC28 IncRNA expression from the GAL1 promoter could induce CDC28 gene expression. Expression from CDC28::pGAL1 was then driven by Gal4DBD-Msn2 activator. Msn2 mediates the recruitment of Hog1 to Msn2-dependent genes (Alepuz et al., 2001). Tethering Msn2 to the Gal4-binding domain stimulated stress-inducible transcription of the IncRNA in the CDC28::pGAL1 strain upon stress and restored CDC28
induction upon stress (Figure 5D). We then inserted a terminator downstream of the GAL1 promoter (IT) (Kopcewicz et al., 2007; Loya et al., 2012) that permitted transcription initiation but prevented the generation of the lncRNA. Here, the Gal4DBD-Msn2 activator did not promote CDC28 sense induction (Figure 5E). Correspondingly, Hog1 was recruited at the GAL1 promoter in the 3′ UTR region of CDC28 as well as at the +1 region of CDC28 (Figure 5F), whereas it was not recruited at the +1 region in the IT construct (Figure 5G). We then monitored chromatin remodeling at the +1 region. In contrast to Gal4-ER-VP16 activator, expression of the CDC28 lncRNA from the GAL1 promoter by the Gal4DBD-Msn2 activator caused remodeling of the +1 nucleosome upon stress (Figure 5H). Thus, induction of the CDC28 lncRNA and the recruitment of Hog1 at the +1 region are required for chromatin remodeling at the 5′ region of CDC28 and CDC28 gene expression.

The Establishment of Gene Looping Permits the Recruitment of Hog1 at the +1 Nucleosome Region and Induction of CDC28

The absence of Hog1 recruitment and remodeling at the 5′ region of CDC28 in cells deficient in lncRNA induction prompted us to assess whether the presence of Hog1 at this region was mediated by gene looping formation (O’Sullivan et al., 2004; Tan-Wong et al., 2012). Gene loop formation depends on the essential protein Ssu72 (Ansari and Hampsey, 2005). Expression of SSU72 under GAL1 is repressed in the presence of glucose (YPD). Cells were grown in galactose, shifted to glucose, and subjected to osmolarity. Depletion of Ssu72 did not alter induction of the CDC28 lncRNA but prevented induction of the CDC28 (Figure 6A). Similar results were obtained in a sua7-1 mutant (Singh and Hampsey, 2007) with impaired gene looping (Figure 5A). Then, we assessed the recruitment of Ssu72 and found that there was a clear Hog1-dependent increase in Ssu72 binding upon stress at both the 3′ UTR and +1 nucleosome regions (Figure 5B). Thus, the enhanced recruitment of Ssu72 in response to stress does not alter CDC28
Figure 5. Antisense Transcription and Hog1 Recruitment Are Required to Induce CDC28 Expression

(A) IncRNA alone is not sufficient for CDC28 expression. Graphical representation of the CDC28 locus with inducible IncRNA expression achieved by insertion of the pGAL1 promoter (CDC28::pGAL1). Gal4-ER-VP16 activator induces IncRNA in the presence of β-estradiol (black bars) or β-estradiol and NaCl (gray bars). CDC28 sense and IncRNA transcripts were assessed by northern blot.

(B and C) We measured Hog1 association (5 min) by ChIP (B) and +1 nucleosome eviction (10 min) by MNase (C) in the indicated strains upon induction with β-estradiol.

(D) Hog1 and IncRNA induction are necessary for CDC28 induction. Graphical representation of the CDC28::pGAL1 locus induced by the Gal4-Msn2DBD. Transcript levels were followed as in (A).

(E) Graphical representation of the CDC28::pGAL1 strain containing an internal terminator (IT).

(F) Hog1 and Rsc1 recruitment (5 min) in cells expressing and empty or Gal4-Msn2DBD.

(G) Presence of the IncRNA is required for Hog1 recruitment at the +1 nucleosome. Hog1 recruitment (5 min) in cells in the IT strain.

(H) +1 nucleosome eviction (10 min) was assessed in CDC28::pGAL1 cells harboring empty vector (Ø) or Gal4-Msn2DBD upon stress. Normalized quantification of CDC28 is shown. Error bars represent SD.
lncRNA expression, but it is essential for the increase of CDC28 expression. The lack of CDC28 induction in the absence of Ssu72 and in the sua7-1 mutant suggested that gene looping might mediate the transfer of activities from the 3' UTR to +1 nucleosome. The absence of Ssu72 and in the sua7-1 mutant suggested that gene looping might mediate the transfer of activities from the 3' UTR to +1 nucleosome.

Figure 6. Gene Looping Allows Recruitment of Hog1 at the +1 Nucleosome Region as well as Induction of CDC28

(A) CDC28 induction depends on Ssu72. Wild-type and GAL1::SSU72 strains grown as stated were subjected to osmotic stress. CDC28 sense and lncRNA transcripts were detected.

(B) Hog1 binding at the +1 nucleosome of CDC28 depends on Ssu72. The GAL1::SSU72 strain was grown as stated, and Hog1 recruitment was analyzed by ChIP after 5 min of NaCl at the indicated regions (as in Figure 4A).

(C) Binding of RSC at the +1 nucleosome requires gene looping. Recruitment of Rsc1 was assessed by ChIP at the indicated regions.

(D) Chromatin remodeling at the +1 region depends on Ssu72. The GAL1::SSU72 strain was grown as stated, and remodeling at the +1 nucleosome was assayed by MNase digestion after 10 min of NaCl. Bars represent the average of the stressed (black bars) compared to the unstressed (white bars) cells ± SD.

(E) Recruitment of RNA Pol II requires gene looping. Binding of RNA Pol II at the +1 nucleosome was assessed by ChIP. Levels in the wild-type upon stress were used as a reference for the other indicated strains.

(F) Hog1 induces physical interaction between the +1 nucleosome region and 3' UTR of CDC28. Association of Hog1 at the 3' UTR region of CDC28 upon stress was not altered by the absence of Ssu72. In clear contrast, the absence of Ssu72 (YPD) completely abrogated the association of Hog1 at the +1 nucleosome region of CDC28 (Figure 6B). Correspondingly, recruitment of Rsc1 was also abolished at the +1 region in the absence of Ssu72 (Figure 6C), which prevented chromatin remodeling at the +1 region upon stress (Figure 6D). Moreover, the increase in RNA Pol II association upon stress at the CDC28 5' region observed in the wild-type strain was abolished in cells deficient in hog1, CDC28 lncRNA, and sua7-1 (Figure 6E). Thus, gene looping mediates the recruitment of Hog1 at the 5' region of CDC28 to induce chromatin remodeling and RNA Pol II recruitment upon stress.
Molecular Cell

CDC28 IncRNA Regulates Cell Cycle upon Stress

To further confirm the establishment of gene looping between the 3’ UTR and promoter regions, we applied the 3C assay (see Experimental Procedures). We found that there was a clear increase in gene looping formation upon stress between the 3’ UTR and the +1 nucleosome regions, as detected by the presence of O1-T PCR products. The O1-T PCR product was ligation dependent (D), and it was not detected when an alternative region (O2) was assessed (Figures 6F and S5C). Notably, the increase in gene looping formation upon stress was dependent on Hog1 and abolished in the absence of SSU72 (YPD) and sua7-1 mutant cells (Figures 6F and S5C), but not in a rsc9Δ mutant (Figure S5D). Therefore, gene looping is critical for the recruitment of Hog1 from 3’ UTR to the +1 nucleosome region of CDC28 to promote chromatin remodeling and induce CDC28 gene expression.

An IncRNA in MMF1 Induces Hog1 Recruitment and Chromatin Remodeling at the +1 Nucleosome Region

To assess whether genes other than CDC28 displayed a similar regulatory mechanism, we chose MMF1 because it expresses a strong, stress-induced IncRNA (Figure S6A) and, albeit not seen in the tiling arrays due to insufficient sensitivity, it was reported to be induced upon stress by Hog1 in run-on and dynamic transcriptome analysis (DTA) experiments (Miller et al., 2011; Romero-Santacreu et al., 2009). We created a mutant in antisense MMF1 IncRNA expression (Figure S6A). Hog1 was recruited at the 3’ UTR and the 5’ region, but not in the region of the MMF1 gene, depending on the presence of the IncRNA (Figure S6B). Nucleosome eviction occurred in response to stress in a Hog1- and IncRNA-dependent manner (Figure S6C). Then, we performed 3C experiments in wild-type, hog1Δ, and SSU72 shut-off system (pGAL::SSU72). Notably, in the absence of stress, we could already detect gene looping between the P and T regions. But most remarkably, the wild-type strain showed an increase of P-T association in response to stress, which was fully dependent on the presence of Hog1 and gene looping (Figure S6D). Thus, albeit some particularities, MMF1 seems to stimulate chromatin remodeling via Hog1 and IncRNA expression as in CDC28.

We then asked whether the 3’ UTR region of CDC28 could confer osmoinduction in a nonosmoresponsive gene. We replaced the 3’ UTR region of a non-stress responsive gene, MBA1, with the 3’ UTR region of CDC28 (300 bp downstream of STOP codon). We chose MBA1 because its expression under normal conditions and the length of the gene are similar to those of CDC28. Replacement of the MBA1 terminator by CDC28 led to the transcription of an IncRNA from the 3’ UTR in MBA1, and most important, it conferred Hog1-dependent osmoinduction (Figure S6E), suggesting that the role of CDC28 3’ UTR is to confer stress-inducible regulation of gene expression from the terminator region of the gene.

Stress-Induced CDC28 IncRNA Results in an Increase of Cdc28 that Permits Cells to Reenter the Cell Cycle More Efficiently in Response to Stress

We then asked whether an increase of CDC28 mRNA results in an increase of Cdc28 protein production upon stress. Endogenous [35S]methionine Cdc28 protein increased ~2-fold in response to stress in wild-type cells, whereas no increase was observed in a CDC28 IncRNA-deficient strain (Figure 7A). Thus, stress-induced Cdc28 IncRNA expression leads to increased levels of Cdc28 kinase.

Hog1 promotes an immediate, but transient, cell cycle delay that permits stress adaptation (Clotet and Posas, 2007; Duch et al., 2012). The increase in Cdc28 levels occurred when cells were already recovering from the initial arrest caused by stress. Thus, we hypothesized that this increase of Cdc28 can serve to accelerate cell cycle reentry after stress. To test this hypothesis, we assessed the exit from the arrest caused by osmostress in a phase of cell cycle with high Cdc28 by synchronizing cells using a temperature-sensitive allele of cdc15 (cdc15Δ) (see Experimental Procedures). Cells deficient in CDC28 IncRNA were able to arrest and exit the cell cycle from cdc15Δ synchronization as efficiently as wild-type in the absence of stress. In contrast, compared to the wild-type, cells deficient in CDC28 IncRNA production delayed cell cycle reentry by approximately 20 min upon stress (Figure 7B). Notably, overexpression of CDC28 from a plasmid can suppress the delay on cell cycle progression observed upon osmostress in CDC28 IncRNAΔ cells (Figure S7). Thus, stress-induced CDC28 IncRNA results in an increase of Cdc28 that permits cells to reenter the cell cycle more efficiently in response to stress.

DISCUSSION

A Specific Set of Hog1-Dependent IncRNAs Is Induced in Response to Osmostress

Stress-activated protein kinases regulate gene expression to maximize cellular adaptation to environmental stresses (de Nadal et al., 2011; Weake and Workman, 2010). In yeast, activation of Hog1 leads to major changes in gene expression. Here, we provide evidence that in addition to controlling expression of coding genes, Hog1 also induces a dedicated set of stress-responsive IncRNAs. Upon osmostress, about 200 IncRNAs are rapidly induced. The induction of these stress-induced IncRNAs depends mostly on the presence of Hog1. Correspondingly, similar to osmoresponsive genes, Hog1 associates to the promoters of IncRNAs upon stress and stimulates RNA Pol II recruitment and chromatin remodeling. In fact, fusing the promoter of one of these IncRNAs (CDC28) to a GFP reporter showed that expression occurred only upon stress, in antisense orientation, and depending on Hog1. This observation is remarkable, since most of the described antisense transcripts have been proposed to arise from bidirectional promoters (Tang et al., 2012; Xu et al., 2009). The fact that this terminator can function as a heterologous promoter suggests that a different transcriptional unit recruited to this region that is independent of the neighboring genes must exist. Accordingly, a recent study by chromatin immunoprecipitation-exonuclease (ChiP-exo) precisely positioned distinct transcriptional machineries at bidirectional promoters, supporting the idea of unique transcription units (Rhee and Pugh, 2012).

Most of the stress-induced IncRNAs are transcribed in response to osmostress. Except for SUTs, which are stable transcripts, the rest of IncRNAs are only detectable in strains with deleted components of the nuclear or cytosolic exosome
(CUTs and XUTs) (Xu et al., 2009), when gene looping is impaired (Ssu72-restricted transcripts), or by deletion of SET3 (Kim et al., 2012; Tan-Wong et al., 2012). Expression analysis of some representative Hog1-dependent lncRNAs showed that they were not expressed under basal conditions in the absence of RRPI, XRN1, or TRF4. Transcription was only induced upon stress, but stability was altered in these mutants. Thus, Hog1 regulates the transcription of a distinctive class of stress-induced lncRNAs whose induction might have relevant implications for proper cellular adaptation.

The CDC28 IncRNA and Hog1 Induce Chromatin Remodeling and CDC28 Expression via Gene Looping

To unravel the biological function of the stress-induced lncRNAs, we investigated whether there was correlation between the expression of sense and lncRNAs. Overall, this was not evident except for in the cases of some genes. Remarkably, there was a positive correlation between the induction of CDC28 and an lncRNA in CDC28 expressed in antisense orientation. CDC28 expression was dependent on Hog1, since there was induction of neither the lncRNA nor the CDC28 sense in hog1 cells. This posed the question of how the SAPK and the induction of an lncRNA lead to increased gene expression. In clear contrast to typical osmoreponsive genes in which the SAPK associates all along the gene (Proft et al., 2006), Hog1 associated at the 3’ region of CDC28, which corresponds to the promoter region of the CDC28 lncRNA, and at a region surrounding the +1 nucleosome of CDC28. Transcription of CDC28 is not controlled by any of the transcription factors targeted by Hog1, thus opening the possibility that Hog1 uses the 3’ UTR region to mediate its association to the +1 nucleosome region to promote gene expression. This interesting Hog1-binding pattern resembles some of the features of osmoreponsive genes in which Hog1 recruitment at the ORFs depends on the 3’ UTR region (Proft et al., 2006).

Cells deficient in lncRNA still recruit Hog1 at the 3’ UTR, but not at the +1 nucleosome region in CDC28. Remarkably, these cells induce neither chromatin remodeling nor CDC28 gene induction. On the other hand, when CDC28 lncRNA was induced by a heterologous activator that does not promote recruitment of Hog1 at the 3’ UTR, the SAPK did not bind at the +1 nucleosome region and cells could induce neither chromatin remodeling nor gene expression. Therefore, the combination of the induction of CDC28 lncRNA transcription and the recruitment of Hog1 is necessary for gene induction. Correspondingly, the combination of artificial tethering of Hog1 to a strain containing the GAL1 at the CDC28 3’ UTR, together with the expression of the lncRNA, allows chromatin remodeling and induction of CDC28. RSC mediates chromatin remodeling at specific stress-responsive genes (Mas et al., 2009). Here, we found that expression of the CDC28 lncRNA was affected not by conditions. Cell cycle progression was analyzed by fluorescence-activated cell sorting (FACS), and percentage of cells in G2/M is shown. See also Figure S7.
depletion of RSC, but by depletion of SAGA. In contrast, RSC was absolutely necessary for chromatin remodeling at the +1 nucleosome region and CDC28 gene induction. Thus, the targeting of RSC by Hog1 at the +1 nucleosome region is required for gene induction.

Unlike osmosresponsive genes in which Hog1 travels with elongating polymerase (Proft et al., 2006), the Hog1-binding pattern at the CDC28 locus suggested that Hog1 could reach the 5′ end of the gene without traveling through the coding region. In yeast, gene looping has been shown to juxtapose promoter-terminator regions during active transcription (O’Sullivan et al., 2004). Indeed, osmotic stress stimulates Hog1-mediated gene looping in CDC28. Looping can be prevented by impairing expression of SSU72 or in cells containing the sua7-1 mutation (Ansari and Hampsey, 2005; Singh and Hampsey, 2007). Depletion of Ssu72 or sua7-1 mutation did not alter induction of CDC28 lncRNA but completely abolished CDC28 gene induction, most likely because Hog1 and RSC cannot be transferred from the 3′ UTR to the +1 nucleosome position.

Altogether, these data suggest the following tentative model for the induction of CDC28 by Hog1 (Figure 7C). In response to osmotic stress, Hog1 associates at the 3′ UTR region of CDC28 and induces lncRNA transcription. Once antisense transcription is induced, gene looping is established and Hog1 is transferred to the +1 nucleosome region in CDC28. The recruitment of Hog1 serves to target the RSC chromatin remodeler, which remodels the +1 region, thus permitting an increase of the transcription of the CDC28 gene. It is worth noting that another example has recently demonstrated that DNA looping facilitates targeting of chromatin remodeling complexes (Yadon et al., 2013). Taken together, the regulation of CDC28 transcription by the induction of a stress-responsive lncRNA provides a paradigm by which an lncRNA mediates gene induction through changes in chromatin architecture.

**Induction of the CDC28 lncRNA Controls Cell Cycle Reentry upon Stress**

The CDC28 gene encodes the main CDK kinase (CDK1) that drives progression of the cell cycle in yeast. Cdc28 is regulated by several mechanisms, including cyclin association and CDK inhibitors (Bloom and Cross, 2007). However, the increase in transcription of CDC28 observed upon stress was unexpected since transcription of CDC28 was assumed to be constant (Spellman et al., 1998). The increase in CDC28 transcription resulted in an increase of de novo synthesis of Cdc28. In response to osmotic stress, Hog1 mediates a rapid, but transient, arrest of cell cycle progression to allow adaptation (Clotet and Posas, 2012). One of the mechanisms under the control of Hog1 consists in the downregulation of Cdc28 activity, which seems to be contradictory with an increase of Cdc28 protein. Nevertheless, the increase in Cdc28 protein levels occurred when cells started to recover from stress; thus, we postulated that this increase in Cdc28 protein levels should have an effect during the recovery phase. Indeed, cells deficient in CDC28 lncRNA arrested similar to wild-type upon stress but reentered cell cycle less efficiently, suggesting that the increase in Cdc28 permits a faster recovery of the cell cycle delay caused by stress. Although cell cycle reentry delay in CDC28 lncRNAΔ cells might seem modest (20 min), this lapse of time has proven to be important to maximize cell survival upon stress (Escoté et al., 2004; Duch et al., 2013). Therefore, Hog1 is able to induce a cell cycle delay and promote the recovery by controlling transcriptional CDC28 modulation, thus achieving a different temporal outcome.

In summary, we present here a mechanism of Cdc28 regulation through a stress-inducible lncRNA production that is able to alter cell cycle progression in response to environmental challenges. Cdc28 regulation provides a mechanism by which an lncRNA together with a SAPK can mediate gene induction through changes of chromatin architecture. Moreover, this study provides insights into how lncRNAs might affect the regulation of gene expression through chromatin changes in eukaryotic cells.

**EXPERIMENTAL PROCEDURES**

**Yeast Strains and Plasmids**

Full list and description of strains and plasmids used in this study is included in the Supplemental Experimental Procedures.

**Tiling Array**

Wild-type (BY4741) and hog1Δ cells were grown to mid-log phase and subjected (or not) to mild osmotic stress (0.4 M NaCl for 15 min) or hyper osmotic stress (1.2 M NaCl for 100 min). Hybridization of tiling array was performed as described (David et al., 2006).

**Definition of Stress-Induced lncRNAs**

Stress-induced IncRNAs were defined as upregulated, with a minimum change of 2-fold in response to stress (at the indicated osmolarity) in the wild-type (BY4741) strain. Hog1 dependence was determined by the percentage of expression in a hog1Δ mutant with respect to the wild-type strain. Table S1 provides the entire list of the osmosresponsive lncRNA, their relationship with the sense transcript, and Hog1 dependence.

**ChIP-Seq and MNase-Seq**

Wild-type and hog1Δ mutant S. cerevisiae strains were grown to mid-log phase and exposed to 5 min of osmotic stress (0.4 M NaCl) for Hog1 immunoprecipitation and 10 min for RNA Pol II immunoprecipitation and nucleosome positioning. ChIP and MNase protocols were performed, and purified DNA was sequenced as described. Enrichment of Hog1 and RNA Pol II was done by running the Pyicos enrichment protocol comparing untreated to treated samples (Nadal-Ribelles et al., 2012).

**MNase Nucleosome Mapping**

Spheroplasts and digestion with MNase were done with the indicated strains subjected (or not) to osmotic stress (0.4 M NaCl for 10 min) or treated with β-estradiol (100 nM, 10 min). For the analysis of CDC28, DNA was used in a real-time PCR.

**ChIP Assays**

Chromatin immunoprecipitation was done as described previously (Zapater et al., 2007). Briefly, indicated yeast cultures were grown to mid-log phase and exposed (or not) to osmotic stress (0.4 M NaCl, 5 min) or treated with β-estradiol (100 nM, 5 min). Real-time PCR of the indicated regions was performed.

**3C Analysis**

Cells were grown to mid-log phase before being subjected (or not) to osmostress (0.4 M NaCl, 10 min). 3C analysis was performed as described previously, with minor modifications indicated in the Supplemental Experimental Procedures.
Molecular Cell

CDC28 IncRNA Regulates Cell Cycle upon Stress

Metabolic Labeling
Briefly, indicated PHO85-TAP strains were grown in YPD and shifted to MET media for 2 hr before being stressed. A mixture of 35S[methionine] and 0.4 M NaCl was added simultaneously.

Flow Cytometry
Flow cytometry experiments were performed as described (Pelet et al., 2011). Cells were stressed for 45 min with 0.4 M NaCl. To study cell cycle progression, cdc15G (cdc15-2) cells were synchronized at 37°C (incubated for 2 hr) and released at 25°C to allow cell cycle progression.

ACCESSION NUMBERS
The link http://steinmetzlab.embl.de/francescData/arrayProfile/index.html directs to an interface to visualize array expression data. Raw array data are available from ArrayExpress under accession number E-MTAB-1886.

SUPPLEMENTAL INFORMATION
Supplemental Information includes Supplemental Experimental Procedures, seven figures, and one table and can be found with this article online at http://dx.doi.org/10.1016/j.molcel.2014.01.006.

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REFERENCES


