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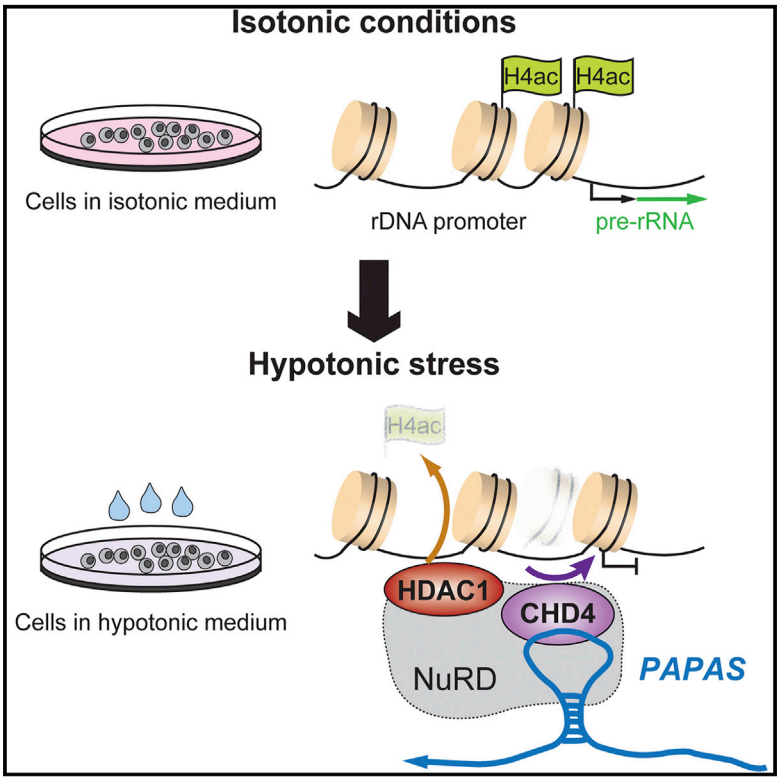
Report

lncRNA-Induced Nucleosome Repositioning Reinforces Transcriptional Repression of rRNA Genes upon Hypotonic Stress

Graphical Abstract



Highlights

- Synthesis of pre-rRNA is shut down under hypotonic conditions
- Hypotonic stress induces *PAPAS*, a transcript that is antisense to pre-rRNA
- *PAPAS* recruits the chromatin remodeling complex NuRD to rDNA
- NuRD repositions the promoter-bound nucleosome

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In Brief

The lncRNA *PAPAS* directs Suv4-20h2 to rDNA for repressive H4K20 trimethylation upon quiescence. Zhao et al. find that, upon hypotonic stress, *PAPAS* recruits the chromatin remodeling complex NuRD to rDNA. NuRD induces a nucleosomal "off" position, thereby reinforcing stress-dependent rDNA silencing.

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lncRNA-Induced Nucleosome Repositioning Reinforces Transcriptional Repression of rRNA Genes upon Hypotonic Stress

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SUMMARY

The activity of rRNA genes (rDNA) is regulated by pathways that target the transcription machinery or alter the epigenetic state of rDNA. Previous work has established that downregulation of rRNA synthesis in quiescent cells is accompanied by upregulation of *PAPAS*, a long noncoding RNA (lncRNA) that recruits the histone methyltransferase Suv4-20h2 to rDNA, thus triggering trimethylation of H4K20 (H4K20me3) and chromatin compaction. Here, we show that upregulation of *PAPAS* in response to hypoosmotic stress does not increase H4K20me3 because of Nedd4-dependent ubiquitinylation and proteasomal degradation of Suv4-20h2. Loss of Suv4-20h2 enables *PAPAS* to interact with CHD4, a subunit of the chromatin remodeling complex NuRD, which shifts the promoter-bound nucleosome into the transcriptional "off" position. Thus, *PAPAS* exerts a "stress-tailored" dual function in rDNA silencing, facilitating either Suv4-20h2-dependent chromatin compaction or NuRD-dependent changes in nucleosome positioning.

INTRODUCTION

The nucleolus has emerged as a central hub for coordinating the stress response, regulating cell growth, and promoting survival and recovery from stress. Environmental cues, including virtually any type of stress, have been shown to feed into the tight regulation of rRNA synthesis as part of ribosome biogenesis surveillance and growth control (Boulon et al., 2010; Grummt, 2013). As ribosome biogenesis consumes a tremendous amount of cellular energy, rRNA synthesis is tightly regulated to be responsive to specific environmental challenges. Actually, almost all signaling pathways that affect cell growth and proliferation directly regulate rRNA synthesis, their downstream effectors converging at the RNA polymerase I (Pol I) transcription machinery and at the chromatin structure of rRNA genes (Kusnadi et al., 2015).

We have recently discovered an epigenetic pathway that attenuates pre-rRNA synthesis in growth-factor-deprived or density-arrested cells, which is orchestrated by a long noncoding RNA (lncRNA) that is transcribed by RNA polymerase II (Pol II) from a fraction of rRNA genes in antisense orientation (Bierhoff et al., 2010, 2014). This antisense RNA, dubbed *PAPAS* (promoter and pre-rRNA antisense), is upregulated in quiescent cells and guides the histone methyltransferase Suv4-20h2 to rDNA, leading to trimethylation of histone H4 at lysine 20 (H4K20me3) and chromatin compaction. Thus, *PAPAS* reinforces transcriptional repression by inducing a chromatin environment that is incompatible with binding of Pol I to the rDNA promoter.

In this study, we have investigated whether *PAPAS*-mediated changes in chromatin structure is a general mechanism that contributes to shutdown of rDNA transcription under different stress conditions. Similar to growth factor deprivation, we observed a marked decrease in pre-rRNA and a strong increase in *PAPAS* upon hypotonic stress. In contrast to serum deprivation, however, hypotonicity increased the interaction of Suv4-20h2 with the E3-ubiquitin ligase Nedd4, leading to enhanced ubiquitinylation and degradation of Suv4-20h2. Depletion of Suv4-20h2 facilitates the interaction of *PAPAS* with CHD4, a subunit of the nucleosome remodeling and deacetylation complex (NuRD) (Zhang et al., 1998; Tong et al., 1998; Xue et al., 1998), which shifts the promoter-bound nucleosome into a position that is refractory to transcription initiation (Xie et al., 2012). The results reveal that *PAPAS* triggers epigenetic silencing of rDNA in different ways, emphasizing the versatility of lncRNAs in adapting the chromatin landscape to environmental cues.

RESULTS

Hypoosmotic Stress Leads to Upregulation of *PAPAS*

To investigate whether *PAPAS*-mediated rDNA silencing is induced by stress conditions other than growth factor deprivation and density arrest, we monitored the levels of pre-rRNA and *PAPAS* in mouse NIH 3T3 fibroblasts, comparing standard

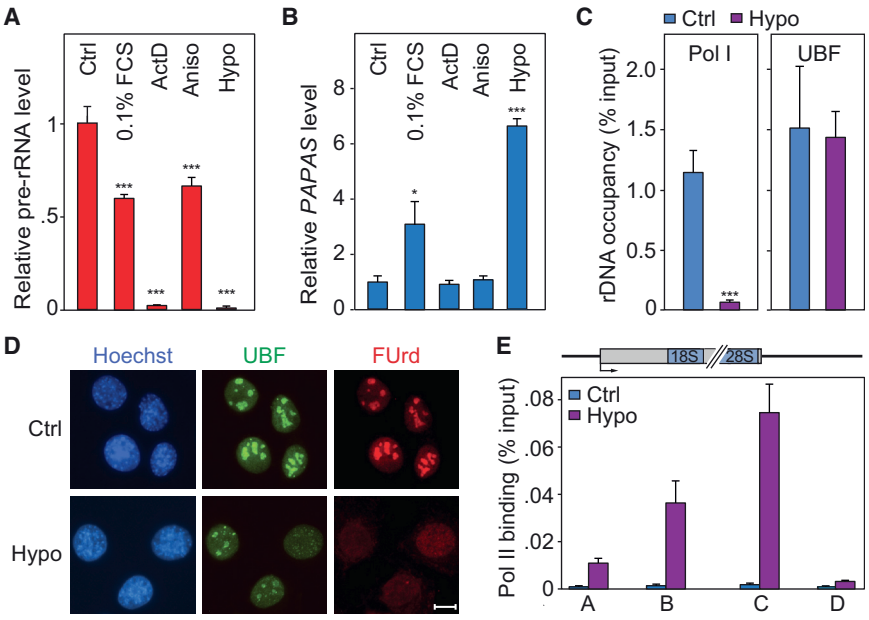

Figure 1. Hypoosmotic Stress Inhibits Pre-rRNA Synthesis and Upregulates *PAPAS*

(A) Impairment of pre-rRNA synthesis under different stress conditions. qRT-PCR showing levels of pre-rRNA normalized to mature 18S rRNA in NIH 3T3 cells cultured under normal conditions (Ctrl) or under stress, i.e., serum starvation (0.1% FCS, 48 hr) and treatments for 4 hr with ActD (0.1 μg/ml), anisomycin (Aniso, 10 μM), or hypoosmotic medium (Hypo, 70% water). For control, absolute quantification of 18S rRNA and levels of β-actin and GAPDH mRNAs normalized to 18S rRNA are shown in Figures S1A and S1B.

(B) *PAPAS* levels under different stress conditions. Cells were treated as in (A), and *PAPAS* levels relative to 18S rRNA were determined by qRT-PCR.

(C) ChIP assays monitoring occupancy of Pol I (RPA116 subunit) and UBF at the rDNA promoter (−160/−1) upon hypoosmotic stress. Precipitated rDNA was quantified by qPCR and normalized to input.

(D) Pol I transcription is inhibited in response to hypotonic stress. Immunofluorescence of NIH 3T3 cells cultured under isotonic (Ctrl) or hypotonic (Hypo) conditions. Nascent RNA was pulse-labeled with FUrd before it was immunostained together with the nucleolar marker protein UBF. DNA was stained with Hoechst 33342. Scale bar, 10 μm.

(E) Occupancy of Pol II at rDNA upon hypotonicity. ChIP with anti-Pol II antibodies followed by qPCR using the rDNA amplicons positioned as indicated in the scheme above. (A: promoter; B: 5′-18S rRNA region; C: 3′-28S rRNA region; D: IGS_{16} region) Data are represented relative to input.

Values are means ± SD from at least three independent replicates. *$p < 0.05$; ***$p < 0.001$. See also Figure S1.

growth conditions with serum deprivation, treatment with actinomycin D (ActD) or anisomycin, and exposure to hypoosmotic stress. Normalization of pre-rRNA and *PAPAS* to mature 18S rRNA, which was not affected in all tested conditions (Figure S1A), showed that hypoosmotic stress led to strong inhibition of rDNA transcription and upregulation of *PAPAS*, the increase of *PAPAS* being more pronounced upon hypotonicity than upon serum deprivation (Figures 1A and 1B; Figure S1B). In contrast, reduced pre-rRNA synthesis after treatment with ActD and anisomycin was not accompanied by increased *PAPAS* levels, indicating that upregulation of *PAPAS* in response to growth factor deprivation or hypoosmotic stress is not a mere consequence of impaired Pol I transcription.

Downregulation of Pol I transcription in hypotonically stressed cells was further demonstrated by chromatin immunoprecipitation (ChIP) experiments showing loss of Pol I from the rDNA promoter, while binding of the transcription factor UBF was not affected (Figure 1C). Moreover, co-immunostaining of UBF and nascent RNA labeled with 5-fluorouridine (FUrd) revealed an almost complete shutdown of pre-rRNA synthesis accompanied by partial loss of UBF from nucleoli, indicating that, under these conditions, nucleolar transcription and structure were impaired (Figure 1D). Downregulation of rRNA synthesis was reversible, with transcription being restored after transfer to isotonic medium (Figures S1C and S1D). In accord with *PAPAS* being initiated from a Pol II promoter at the 3′ end of the 28S rRNA coding region (Bierhoff et al., 2010), hypoosmotic stress led to increased Pol II occupancy at rDNA, which was highest at the end of the coding region and declined toward the 5′-terminal pre-rRNA promoter (Figure 1E). Pol II was not associated with the intergenic spacer (IGS_{16} region), which is transcribed by Pol I under certain stress conditions (Audas et al., 2012; Figure S1E). These results show that elevated *PAPAS* levels in hypotonic conditions are brought about by enhanced antisense transcription, which initiates at the 3′ end of the pre-rRNA coding region and covers the rDNA promoter.

Hypotonic Stress Causes Degradation of Suv4-20h2

The pronounced decrease of pre-rRNA and increase of *PAPAS* upon hypotonic shock suggested that similar epigenetic mechanisms, i.e., recruitment of Suv4-20h2 and H4K20me3-dependent chromatin compaction, reinforce downregulation of Pol I transcription in serum-starved and osmotically stressed cells. However, in contrast to serum deprivation, rDNA occupancy of Suv4-20h2 and H4K20me3 did not increase in response to hypoosmotic stress (Figure 2A). In RNA immunoprecipitation (RIP) experiments, less *PAPAS* was co-precipitated with Suv4-20h2 from stressed cells as compared to normal cells (Figure 2B), suggesting that the interaction between *PAPAS* and Suv4-20h2 was impaired upon hypotonic stress. In contrast to this prediction, in vitro pull-down assays revealed that Suv4-20h2 binds to *PAPAS* with similar efficiency, regardless of whether cells were grown under normal or hypotonic conditions (Figure 2C). The apparent discrepancy between the RIP and the pull-down experiments could be explained by stress-dependent degradation of Suv4-20h2. Indeed, upon hypoosmotic shock, the level of Suv4-20h2 was markedly reduced (Figure 2D). Depletion of Suv4-20h2 under hypotonicity occurred faster than the half-life of Suv4-20h2 in normal conditions and was accompanied by a global decrease in H4K20me3, indicating that Suv4-20h2 is targeted for degradation under hypoosmotic conditions (Figures S2A and S2B).

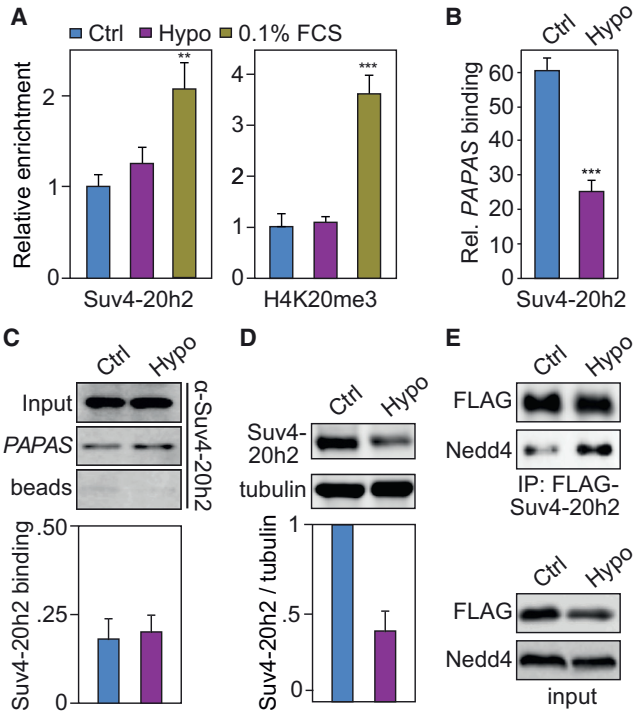

Figure 2. Degradation of Suv4-20h2 upon Hypotonic Stress Prevents *PAPAS*-Induced Upregulation of H4K20me3

(A) ChIP of Suv4-20h2 and H4K20me3 in NIH 3T3 cells under normal growth conditions (Ctrl), hypotonic stress (Hypo), or serum starvation (0.1% FCS). rDNA promoter occupancy of Suv4-20h2 was normalized to the occupancy at subtelomeres of chromosome 19. H4K20me3 levels were normalized to histone H3 levels at rDNA. Data represent the change in binding relative to control and are displayed as means ± SD from three independent experiments. $^{**}p < 0.01$; $^{***}p < 0.001$.

(B) RIP assay comparing the association of *PAPAS* with Suv4-20h2 in control and hypoosmotic conditions. GFP-tagged Suv4-20h2 was stably expressed in NIH 3T3 cells and was immunoprecipitated with anti-GFP antibodies. Co-precipitated *PAPAS* was assayed by qRT-PCR. Values are from five biological replicates and means ± SD are displayed relative to RIP experiments in cells without GFP-Suv4-20h2. Rel., relative. $^{***}p < 0.001$.

(C) Pull-down assay with bead-bound *PAPAS* or beads alone incubated with extracts from HEK293T cells overexpressing Suv4-20h2. Cells were left untreated or subjected to hypotonic stress before lysis. Suv4-20h2 bound to beads or in the input was monitored on western blots. The diagram shows quantification of *PAPAS*-bound Suv4-20h2 levels relative to input levels as means ± SD from three independent experiments.

(D) Degradation of Suv4-20h2 upon hypotonic stress. Immunoblot of Suv4-20h2 and β-tubulin from unstressed and stressed NIH 3T3 cells. The diagram below shows mean values ± SD of Suv4-20h2 levels normalized to β-tubulin from six independent experiments.

(E) The interaction between Suv4-20h2 and Nedd4 is increased under hypoosmotic stress. NIH 3T3 cells stably expressing FLAG-tagged Suv4-20h2 were cultured in isotonic or hypotonic conditions and were immunoprecipitated with anti-FLAG (M2) agarose. The immunoprecipitates and the inputs were analyzed on western blots using anti-FLAG and anti-Nedd4 antibodies.

See also Figure S2.

Suv4-20h2 interacts with the E3-ubiquitin ligase Nedd4/Rsp5p (Hahn et al., 2013), which plays a central role in stress-dependent proteolysis (Hoshikawa et al., 2003; Fang et al., 2014). Consistent with Suv4-20h2 being targeted by Nedd4, a stronger interaction of Nedd4 with Suv4-20h2 in response to hypoosmotic stress was detected by co-immunoprecipitation and mass spectrometry experiments (Figures 2E, S2C, and S2D; Table S2). Moreover, Suv4-20h2 was poly-ubiquitinylated in stressed cells, and treatment with the proteasome inhibitor MG132 further enhanced ubiquitinylation (Figure S2E). These results imply that Nedd4 targets Suv4-20h2 for proteasomal degradation and explain why upregulation of *PAPAS* in hypoosmotic conditions does not lead to increased H4K20me3 occupancy and chromatin compaction at rDNA.

PAPAS Recruits the Chromatin Remodeling Complex NuRD to rDNA

The observation that Suv4-20h2 is degraded in response to hypoosmotic stress suggested that *PAPAS* may reinforce transcriptional repression independent of Suv4-20h2. ChIPs monitoring the rDNA occupancy of several histone marks, i.e., H3K9me3, H3K27me3, H3K4me3, and acetylation of histone H4 (H4ac), in unstressed and stressed cells showed that H4ac was decreased after hypoosmotic shock, whereas methylation of histone H3 remained unaffected (Figure S3A). Upregulation of *PAPAS* and deacetylation of histone H4 occurred with similar kinetics, indicating that both processes are functionally linked (Figures 3A and 3B). Consistent with this view, binding of histone deacetylase 1 (HDAC1) to rDNA was increased in response to hypotonicity, suggesting that deacetylation of histone H4 is caused by *PAPAS*-dependent recruitment of HDAC1 (Figure 3C).

HDAC1 is part of several multiprotein complexes (Kelly and Cowley, 2013), including the chromatin remodeling complexes NoRC (nucleolar remodeling complex) and NuRD, which both modulate the epigenetic signature of rRNA genes (Strohner et al., 2001; Zhou et al., 2002; Xie et al., 2012). To examine whether the rDNA occupancy of either complex is enhanced in hypoosmotic conditions, we compared promoter binding of CHD4, the ATPase subunit of NuRD, and of TIP5, the large subunit of NoRC, in normal and stressed cells. As shown in Figure 3D, rDNA association of CHD4/NuRD, but not of TIP5/NoRC, was markedly increased upon hypotonicity. Consistent with *PAPAS* guiding NuRD to rDNA, the kinetics of NuRD binding to the rDNA promoter, monitored by ChIP of CHD4 and MTA2, correlated with the upregulation of *PAPAS* (Figure 3E). No binding of CHD4 was observed at the IGS_{16} region that is not covered by *PAPAS* (Figure S3B). Moreover, the levels of CHD4, MTA2, and HDAC1, as well as TIP5, remained largely unchanged, demonstrating that enhanced rDNA occupancy is not due to increased amounts of NuRD in hypotonic conditions (Figure S3C).

RIP experiments revealed that *PAPAS*, but not a control lncRNA (*HOTAIR*), was associated with CHD4, with the binding of *PAPAS* being enhanced upon hypotonic stress (Figure 3F). In contrast, the interaction of *PAPAS* with TIP5 was very low, being further decreased upon hypoosmotic stress (Figures S3D and S3E). The specificity of the interaction of *PAPAS* with CHD4/NuRD was further supported by in vitro pull-down experiments using either immobilized RNA or immobilized proteins (Figures 3G and S3F). These experiments showed that CHD4 binds preferentially to RNA comprising rDNA promoter

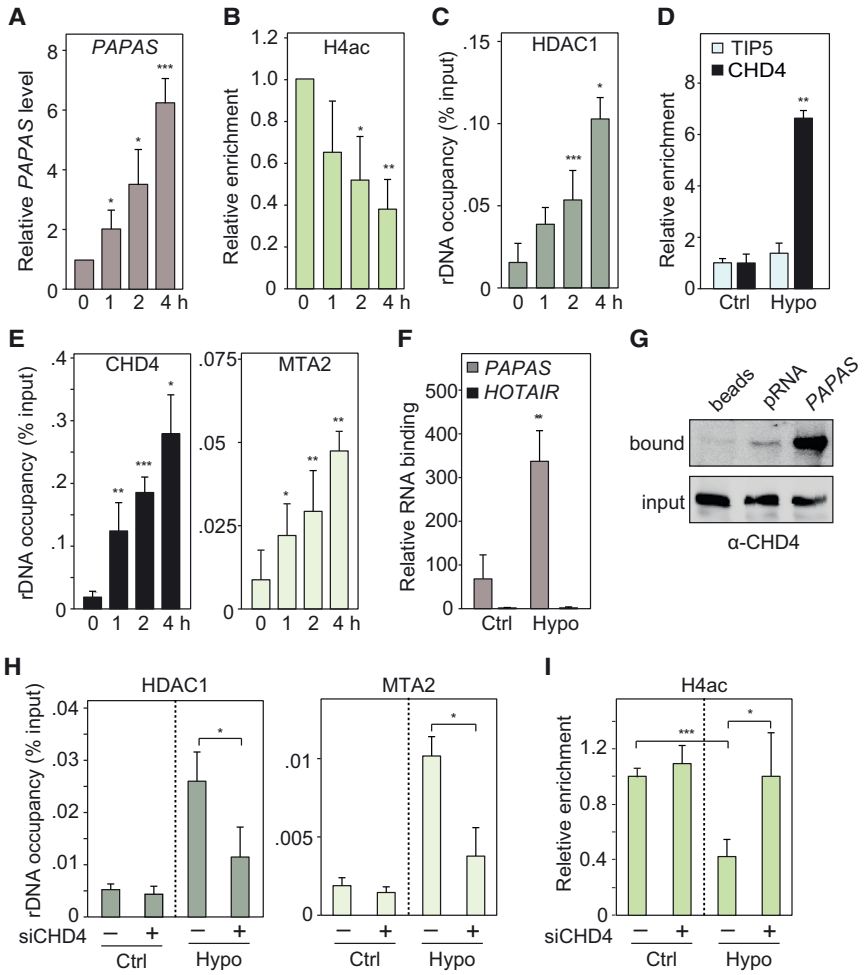

Figure 3. *PAPAS* Recruits NuRD to rDNA upon Hypotonicity

(A) Gradual upregulation of *PAPAS* under hypoosmotic stress. The level of *PAPAS* relative to 18S rRNA was measured by qRT-PCR in untreated NIH 3T3 cells (0 hr [h]) or cells subjected to hypotonicity for 1, 2, and 4 hr.

(B) ChIP at the rDNA promoter monitoring acetylation of histone H4 (H4ac) at the indicated times of hypotonic stress. H4ac levels were normalized to histone H3.

(C) HDAC1 recruitment parallels histone H4 deacetylation. HDAC1 binding to the rDNA promoter was monitored by ChIP at time points as in (A) and (B). ChIP enrichment is shown relative to input.

(D) Binding of NoRC and NuRD subunits to rDNA upon hypotonic stress. ChIP with antibodies against TIP5 or CHD4 in cells grown in hypotonic medium (Hypo) was normalized to ChIP under normal growth conditions. Ctrl, control.

(E) ChIP monitoring rDNA occupancy of CHD4 and MTA2 at the indicated times in hypotonic conditions. ChIP enrichment is shown relative to input.

(F) RIP of GFP-tagged CHD4 expressed in control and stressed HEK293T cells. *PAPAS* and *HOTAIR* co-precipitated with CHD4 were assayed by qRT-PCR. Values are displayed as fold change relative to control cells only expressing GFP.

(G) Pull-down assay monitoring binding of GFP-CHD4 to streptavidin beads without RNA or coated with biotinylated pRNA (−205/−1) or *PAPAS* (−1/−205). GFP-CHD4 captured from HEK293T cell extracts was monitored on western blots.

(H) Hypotonicity-induced recruitment of HDAC1 and MTA2 depends on CHD4. CHD4 was knocked down in NIH 3T3 cells by siRNA followed by hypotonic stress for 4 hr. rDNA occupancy of HDAC1 (left panel) and MTA2 (right panel) was monitored in ChIP and is displayed relative to input. Western blots showing the efficiency of the CHD4 knockdown are in Figure S3G.

(I) ChIP of H4ac in cells transfected with control siRNA or siRNA against CHD4 were cultured under isotonic or hypotonic conditions. H4ac levels were normalized to histone H3.

Values of qRT-PCR and ChIP experiments are means ± SD from at least three independent replicates. *p < 0.05; **p < 0.01; ***p < 0.001. See also Figure S3.

sequences (−1/−205) in an antisense orientation but barely to sense RNA (pRNA [NoRC-associated RNA]; −205/−1).

To demonstrate that the interaction of CHD4 with *PAPAS* targets NuRD to rDNA, we monitored the hypotonicity-induced occupancy of HDAC1 and MTA2, as well as deacetylation of H4 after the siRNA (small interfering RNA)-mediated knockdown of CHD4 (Figure S3G). Compared to cells treated with control siRNA, a marked decrease in rDNA occupancy of HDAC1 and MTA2 and an increased acetylation of histone H4 were observed under hypotonic conditions, supporting the finding that *PAPAS* tethers the NuRD complex to rDNA by binding to CHD4 (Figures 3H and 3I).

Stress-Induced Recruitment of NuRD Alters Nucleosome Positions at the rDNA Promoter

Previous work has established that the promoter of active and silent rRNA genes exhibits different nucleosome positions (Li et al., 2006). At active genes, the promoter-bound nucleosome, termed *NucU*, covers nucleotides from −157 to −2, which places the core promoter and the upstream control element into close proximity, thus nucleating transcription complex formation. At silent and poised genes, the nucleosome is positioned 24 nt further downstream (*NucD*), a position that is refractory to transcription initiation (Figure 4A). The *NucD* position is established at silent rDNA repeats by NoRC and at transcription-permissive, but poised, rRNA genes by NuRD (Li et al., 2006; Xie et al., 2012). To examine whether *PAPAS*-dependent recruitment of NuRD shifts the promoter-bound nucleosome into the *NucD* position, we used crosslinked, mononucleosomal DNA to assay nucleosome positions by ligation-mediated PCR (LM-PCR). Upon hypotonic stress, a shift of nucleosomes to the *NucD* position was observed in several cell lines, i.e., NIH 3T3, 3T3-L1, and L1210, which occurred with kinetics similar to those of the increase of *PAPAS* and the recruitment of NuRD (Figures 4B and 4C). In accord with transcription inhibition being reversible, the promoter-bound nucleosome was shifted

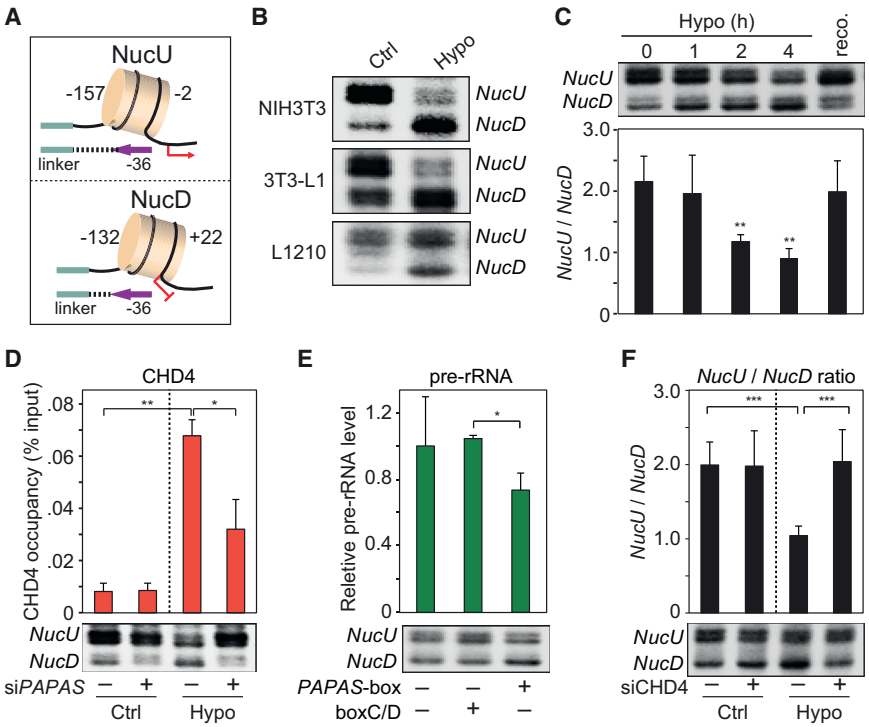

Figure 4. *PAPAS*-Mediated Targeting of NuRD Shifts the rDNA Promoter-Bound Nucleosome into a Poised Position

(A) Scheme depicting *NucU* and *NucD* positions of the nucleosome at the rDNA promoter. LM-PCR products amplified with linker and rDNA-specific primers are indicated.

(B) Nucleosome positioning assay in different mouse cell lines. Mononucleosomes were isolated from NIH 3T3 and 3T3-L1 fibroblasts and from L1210 lymphocytic leukemia cells cultured under isotonic (Ctrl) or hypotonic (Hypo) conditions. After radioactive LM-PCR, the two nucleosome positions, *NucU* and *NucD*, were visualized by electrophoresis and phosphorimaging.

(C) Changes of nucleosome positions upon hypotonic stress. Mononucleosomes were isolated from control cells (0 hr [h]); cells subjected to hypotonicity for 1, 2, and 4 hr; or cells that recovered in isotonic medium for 6 hr after stress (reco.). *NucU* and *NucD* were analyzed by LM-PCR (upper panel), and the *NucU*/*NucD* ratio was calculated from three independent experiments (lower panel).

(D) Knockdown of *PAPAS* prevents hypotonicity-induced nucleosome repositioning. Cells transfected with control siRNA (−) or *PAPAS* siRNA (si*PAPAS*) (+) were cultured in isotonic or hypotonic medium. rDNA occupancy of CHD4 was assayed by ChIP (upper panel) and *NucU* and *NucD* positions of the promoter nucleosome were monitored by LM-PCR (lower panel). qRT-PCR analysis of *PAPAS* levels and quantification of *NucU*/*NucD* ratios are shown in Figure S4C, a CHD4 western blot is in Figure S4D.

(E) Ectopic *PAPAS* induces nucleosome repositioning. NIH 3T3 cells were mock-transfected or transfected with *PAPAS* (−160/−1) fused to boxC/D sequences of U16 small nucleolar RNA (snoRNA) (*PAPAS*-box), or with boxC/D sequences alone. Levels of pre-rRNA normalized to 18S rRNA were monitored by qRT-PCR (upper panel), and nucleosome positions were monitored by LM-PCR (lower panel). Quantification of *NucU*/*NucD* ratios is displayed in Figure S4E.

(F) Depletion of CHD4 abolishes hypotonicity-dependent nucleosome movement. Mononucleosomes were isolated from cells transfected with control siRNA or siRNA against CHD4 and cultured under isotonic or hypotonic conditions. After radioactive LM-PCR, *NucU* and *NucD* positions were visualized by electrophoresis and phosphorimaging (lower panel), and the *NucU*/*NucD* ratios were calculated (upper panel).

Values in (C)–(F) are means ± SD from at least three independent replicates. *p < 0.05; **p < 0.01; ***p < 0.001. See also Figure S4.

back to the *NucU* position if cells were transferred to isotonic medium.

To examine whether *PAPAS* is required for NuRD-dependent changes in nucleosome positioning, we compared CHD4/NuRD recruitment and the *NucU*/*NucD* ratio in normal and stressed cells in which transcription of *PAPAS* was blocked by treatment with α-amanitin or, more specifically, with ActD, which preferentially intercalates into GC-rich rDNA (Perry and Kelley, 1970). Both drugs compromised stress-dependent recruitment of CHD4 to rDNA and movement of the nucleosome to the *NucD* position, indicating that upregulation of *PAPAS* is required for elevated levels of CHD4/NuRD and nucleosome remodeling (Figures S4A and S4B). Accordingly, knockdown of *PAPAS* by siRNA attenuated stress-dependent increase of CHD4 binding to rDNA and prevented changes in the *NucU*/*NucD* ratio (Figures 4D and S4C). Neither the drug treatments nor the knockdown of *PAPAS* affected CHD4 levels (Figure S4D), supporting the finding that *PAPAS* guides CHD4/NuRD to rDNA to move the promoter-bound nucleosome.

To corroborate the functional link between *PAPAS*, CHD4/NuRD and nucleosome positioning, we monitored the *NucU*/*NucD* ratio in cells transfected with the promoter-matching part of *PAPAS* (nucleotides −1 to −160), which was targeted to nucleoli by fusion with boxC/D sequences of U16 snoRNA (Bierhoff et al., 2014). Ectopic *PAPAS*-boxC/D triggered an increase in the *NucD* position and a modest reduction in pre-rRNA synthesis, whereas boxC/D sequences alone had no effect (Figures 4E and S4E). Moreover, upon siRNA-mediated knockdown of CHD4 no shift to the *NucD* nucleosome position in response to hypotonic stress was observed (Figure 4F), reinforcing that *PAPAS* recruits the NuRD complex to rDNA, which shifts the promoter-bound nucleosome into the 'off'-position that precludes transcription initiation.

DISCUSSION

Our study has uncovered a pathway of lncRNA-dependent epigenetic regulation, which reinforces transcriptional repression by targeting a chromatin remodeling complex to a specific genomic site in response to hypotonic stress. Although we cannot formally rule out that upregulation of *PAPAS*, per se, negatively affects rRNA synthesis, our knockdown experiments clearly show the requirement of both *PAPAS* and NuRD for nucleosome repositioning upon hypotonicity. This finding extends recent studies showing that two lncRNAs, *SChLAP1* and *Myheart* (*Mhrt*), affect gene expression by interacting with

subunits of the chromatin remodeling complex SWI/SNF, thereby impairing its remodeling activity. Mechanistically, *SChLAP1* antagonizes the genome-wide localization and regulatory functions of the SWI/SNF chromatin-modifying complex by attenuating chromatin binding (Prensner et al., 2013). Similarly, the cardioprotective lncRNA *Mhrt* antagonizes the function of Brg1 by preventing SWI/SNF from recognizing its genomic DNA targets (Han et al., 2014). *PAPAS,* however, recruits the NuRD complex to rDNA in hypotonic conditions, leading to remodeling of the promoter-bound nucleosome and reinforcement of transcriptional repression. Notably, this mode of action is different from that of serum-deprived cells, where the upregulation of *PAPAS* recruits Suv4-20h2, which induces trimethylation of H4K20 and chromatin compaction (Bierhoff et al., 2014). H4K20me3-dependent chromatin compaction would be inefficient under hypotonic conditions, where an increased nuclear volume leads to passive "opening" of chromatin (Walter et al., 2013). Therefore, Nedd4-induced degradation of Suv4-20h2 might be an adaptive mechanism to hypotonicity that enables *PAPAS* to interact with NuRD. This mechanism is in accord with the stress-dependent nuclear functions of Nedd4/Rsp5p (Haitani et al., 2006) and its role in osmotic homeostasis by targeting several plasma membrane permeases and ion channels (Ingham et al., 2004). Nedd4 is inactivated upon quiescence while NuRD activity is low (Polo et al., 2010; Bae et al., 2015), favoring the *PAPAS*/Suv4-20h2 pathway. Proteasome activation in response to hypotonicity (Tao et al., 2002) facilitates Suv4-20h2 degradation, allowing NuRD-dependent chromatin remodeling at the rDNA promoter. As osmolarity can change quickly, reversible NuRD-dependent movement of the promoter-bound nucleosome into a transcription-refractory position may be superior to the long-term epigenetic silencing upon growth arrest. However, we need to stress that changes of chromatin structure usually do not cause, but accompany and reinforce, the primary mechanisms that cells use to adapt Pol I transcription to external signals. For instance, the c-*jun* N-terminal protein kinase (JNK) is rapidly induced upon hypoosmotic stress, and JNK downregulates Pol I transcription by phosphorylation of the basal transcription factor TIF-IA at threonine 200 (Hubert et al., 2004; Mayer et al., 2005). In animals, abnormal water intake or reduced excretion causes systemic hypotonic stress, often coupled with hyponatremia. Further studies, ideally using an animal model for "vasopressin escape" (Verbalis and Drutarosky, 1988; Ecelbarger et al., 1997; Zhang et al., 2003), will show whether, under these conditions, *PAPAS* is upregulated and the position of the rDNA promoter-bound nucleosome is altered in tissues. Together with global analyses of cellular transcriptomes and nucleosome positions, these studies will reveal whether lncRNA-dependent changes in nucleosomal architecture is a general mechanism under hypotonicity to safeguard cellular homeostasis.

EXPERIMENTAL PROCEDURES

Cell Culture, Transfection, and Retroviral Infection

NIH 3T3 and HEK293T cells were cultured in DMEM supplemented with 10% fetal calf serum (FCS). Hypotonic stress was applied by incubation in 30% DMEM/70% H_2O for 4 hr, if not stated otherwise. Isotonic control conditions with 30% DMEM/70% PBS buffer did not induce a stress response. In-vitro-synthesized boxC/D and *PAPAS*-boxC/D RNAs and siRNAs were transfected with Lipofectamine RNAiMAX, and plasmids were transfected with Lipofectamine 2000 (Life Technologies) into NIH 3T3 cells. Cells were analyzed 48 hr after siRNA transfection for knockdown and 24–36 hr after plasmid transfection for overexpression. HEK293T cells were transfected by calcium phosphate precipitation. For retroviral infections, pBabe-puro viruses were produced in ecotropic Phoenix cells. Plasmids are listed in the Supplemental Information.

ChIP

ChIP assays have been performed as described previously (Bierhoff et al., 2014). In all experiments, control ChIP assays with either unspecific immunoglobulin G (IgG) antibodies or no antibody were carried out in parallel, and the DNA enrichment with specific antibodies was at least 5-fold above the controls. The qPCR primers used to analyze ChIP DNA are included in Table S1.

RNA Analysis and In Vitro Transcription

RNA was isolated from cells with TRI Reagent (Sigma) and analyzed by qRT-PCR or northern blotting. For qRT-PCR, cDNA was synthesized using random hexamers or sequence-specific primers (see Table S1). For detection of pre-rRNA on northern blots, a radiolabeled riboprobe covering rDNA sequences from +155 to +1 was used. Synthetic *PAPAS* (−1/−205), pRNA (−205/−1), boxC/D, and *PAPAS*-boxC/D RNAs were generated by in vitro transcription with T7 RNA polymerase. Details are provided in the Supplemental Information.

Protein-Protein and RNA-Protein Interaction Analysis

For protein co-immunoprecipitation (coIP) and RNA immunoprecipitation (RIP) assays, cells expressing epitope-tagged proteins were lysed in IP buffer (20 mM Tris-HCl [pH 8.0], 200 mM NaCl, 1 mM EDTA, 1 mM EGTA, 0.5% Triton X-100, protease inhibitor cocktail [Roche]) for 1 hr at 4°C. Lysates were cleared by centrifugation, and proteins were precipitated using GFP-Trap (Chromotek) or M2 (Sigma) agarose beads. After washing, proteins were eluted in SDS sample buffer and analyzed by western blotting. Alternatively, RNA was eluted by Proteinase K digestion, isolated with TRI Reagent, and analyzed by qRT-PCR. For in vitro pull-down experiments, GFP-tagged proteins were expressed in HEK293T cells, immobilized on GFP-Trap agarose, and incubated with radiolabeled transcripts for 1 hr at room temperature. Captured RNA was eluted with formamide, run on 6% denaturating polyacrylamide gels and visualized by phosphorimaging. Ectopic Suv4-20h2 and CHD4 were pulled down from cell lysates with biotinylated transcripts immobilized on streptavidin-coupled Dynabeads (Life Technologies), followed by elution in SDS sample buffer and western blotting.

Nucleosome Positioning Assay

Nucleosome positions were analyzed according to Li et al. (2006) with slight modifications. For details, see the Supplemental Information.

Statistical Analysis

Data are reported as mean values from at least three biological replicates, with error bars denoting SD. Comparisons between two groups were performed using a paired two-tailed Student's t test.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, four figures, and two tables and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2016.01.073.

AUTHOR CONTRIBUTIONS

H.B. and I.G. conceived and guided the study; Z.Z., M.A.D., and H.B. carried out the experiments and analyzed the data; H.B. and I.G. wrote and edited the manuscript.

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