SUPPLEMENTARY INFORMATION

The sub-nucleolar localization of PHF6 defines its role in rDNA transcription and early processing events.

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SUPPLEMENTARY MATERIALS AND METHODS

PHF6 Knockdown

Stable PHF6 shRNA knockdown cell lines were generated using the GIPZ PHF6 shRNA Viral Particle Starter Kit (Open Biosystems, VGH5526-EG84295). Briefly, HEK 293T cells, seeded the day before transduction at 5 x 10^4 cells per well, were transduced for 6 hours at 37^o C in serum-free media containing 4 x 10^4 lentivirus particles and 8 μg/μL polybrene then allowed to recover in growth media (DMEM, 10% FBS, 1% penicillin-streptomycin) for 72 hours, prior to selection in 1.5 μg/μL puromycin. The following antisense sequences were used for PHF6 shRNA mediated knockdown: PHF6 shRNA #1 (5'-TCAAAGTCTCCAAATTCTG, catalogue # V2LHS_138602), PHF6 shRNA #2 (5'-TTTCCTCGTTTAATCTCCT, catalogue # V3LHS_350404), and PHF6 shRNA #3 (5'-AAAACTTTCTTCTAAATCA, catalogue # V3LHS_350403). PHF6 knockdown was confirmed both by qRT-PCR (Figure S4B) and Western blot (Figure S4C). HEK 293T cell lines stably infected with the empty GIPZ vector were used as a negative control.

Western blotting

Transformed HEK 293T cells were harvested on ice and lysed in radioimmunoprecipitation assay buffer (RIPA; 50 mM Tris HCl pH 7.8, 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulphate), resolved by SDS-PAGE (3-8% Tris-Acetate Novex gel, Life Technologies), and transferred onto a PVDF membrane (Millipore). Membranes were blocked in 1X TBST with 5% milk for 2 hours at room temperature, then incubated with primary antibodies for PHF6 and α-tubulin (see Table S1) for overnight at 4°C in 1X TBST with 5% BSA. Following three 1X TBST washes, membranes were incubated with the appropriate anti-rabbit HRP or anti-mouse HRP secondary antibody (see Table S1) in blocking solution for 45 minutes at room temperature.

Development of the membranes was achieved using enhanced chemiluminescence (GE Healthcare) and an SRX-101A (Raytech) film developer.

SUPPLEMENTARY LEGENDS TO FIGURES

Figure S1. PHF6 co-localizes with the nucleolar FC and partially co-localizes with the DFC.

(A) To demonstrate the reversibility of the DRB treatment, HEK 293T cells were treated with 25 μg/mL DRB for 2 hours and allowed to recover for 2 hours (DRB off). HEK 293T cells were also treated with 5 μg/mL of α-amanitin, an RNA pol II inhibitor, to confirm if the ActD-mediated nucleolar disassembly observed in Figure 2 was indeed specific to RNA Pol I inhibition. Cells were then fixed and labeled with antibodies to PHF6 (green) and either UBF, FBRL, or NCL (red), then visualized by confocal microscopy. Cells were counterstained with DAPI to visualize nuclei. (B) HEK 293T cells were treated with either 25 μg/mL DRB or 0.5 μg/mL Act D for 2 hours. Cells were then fixed and labeled with antibodies to PHF6 (green) and COILIN (red), a marker for Cajal bodies. Representative cell images are shown. This supplementary figure is an accompaniment to Figure 2.

Figure S2. The nucleolar localization of PHF6 is RNA-dependent.

(A, B) HEK 293T cells were digested with either DNase I (10 μg/mL) or RNase A (10 μg/mL) and compared with untreated cells by immunocytochemistry. Cells were immunofluorescently stained with antibodies to PHF6 (green) and counterstained with (A) Pyronin Y to label RNA, or (B) FBRL, a DFC marker. Treatment with RNase A results in the loss of PHF6 from the nucleolus, as indicated by the loss of co-localization between PHF6 and Pyronin Y. The efficacy of the RNase A treatment is confirmed by an accompanying reduction in Pyronin Y intensity. Conversely, nucleolar PHF6 expression is not affected following treatment with DNase I. Like PHF6, the nucleolar localization of FBRL is unaffected by DNase I digestion, yet severely reduced in the RNase A treatment. Representative cell images are shown. (C) Histone H3 (n=10) ChIP-qPCR positive control

for binding to the IGS (primer sets A-C, red), promoter (primer set D, yellow), and coding (primer sets E-H, green) sequences of the rDNA gene. This supplementary figure is an accompaniment to Figure 3.

Figure S3. Quantification of PHF6 in both overexpression and knockdown cell lines.

(A,B) PHF6 RNA levels (normalized to GAPDH) were quantified by qRT-PCR in both (A) overexpression (PHF6-NTAP, PHF6-CTAP; n=4) and (B) shRNA knockdown (PHF6 shRNA #1, PHF6 shRNA #2, PHF6 shRNA #3; n=6) model cell lines. PHF6 and GAPDH primer sequences are shown in Table S2. Bars represent standard error (*$p<0.05$, **$p<0.01$, two-tailed student's t-test). (C) PHF6 protein levels were quantified by Western blot using antibodies against PHF6 and α-tubulin (loading control). Note that the tagged PHF6 protein (MW 47 kDa) is detected above the endogenous PHF6 band (42 kDa) [OE = overexpression]. (D) Normalized enrichment following M2-Flag ChIP from a PHF6-CTAP overexpression cell line (n=2) and an empty vector cell line (n=2). Normalized enrichment (PHF6-CTAP enrichment over input/empty vector enrichment over input) is shown. Enrichments over input were calculated using the following equation: fold change = $2^{(\text{input mean Ct - ChIP mean Ct})}$.

Figure S4. Relative abundance of RNA transcripts quantified from the rDNA gene

(A) qRT-PCR products from Figure 4A were re-analyzed with normalization performed against pre-pRNA levels (primer set D; see Figure 3C) from the same RNA sample (n=8). (B) Quantification of non-coding IGS RNA (primer sets A-D) and coding rRNA (primer sets E-H) from the rDNA gene was performed for PHF6 shRNA knockdown cell lines (n=4) by qRT-PCR, as described for Figure 4A. The y-axes are logarithmic (base 10). Bars represent standard error (*$p<0.05$, **$p<0.01$, two-tailed student's t-test). This supplementary figure is an accompaniment to Figure 4.

Figure S1

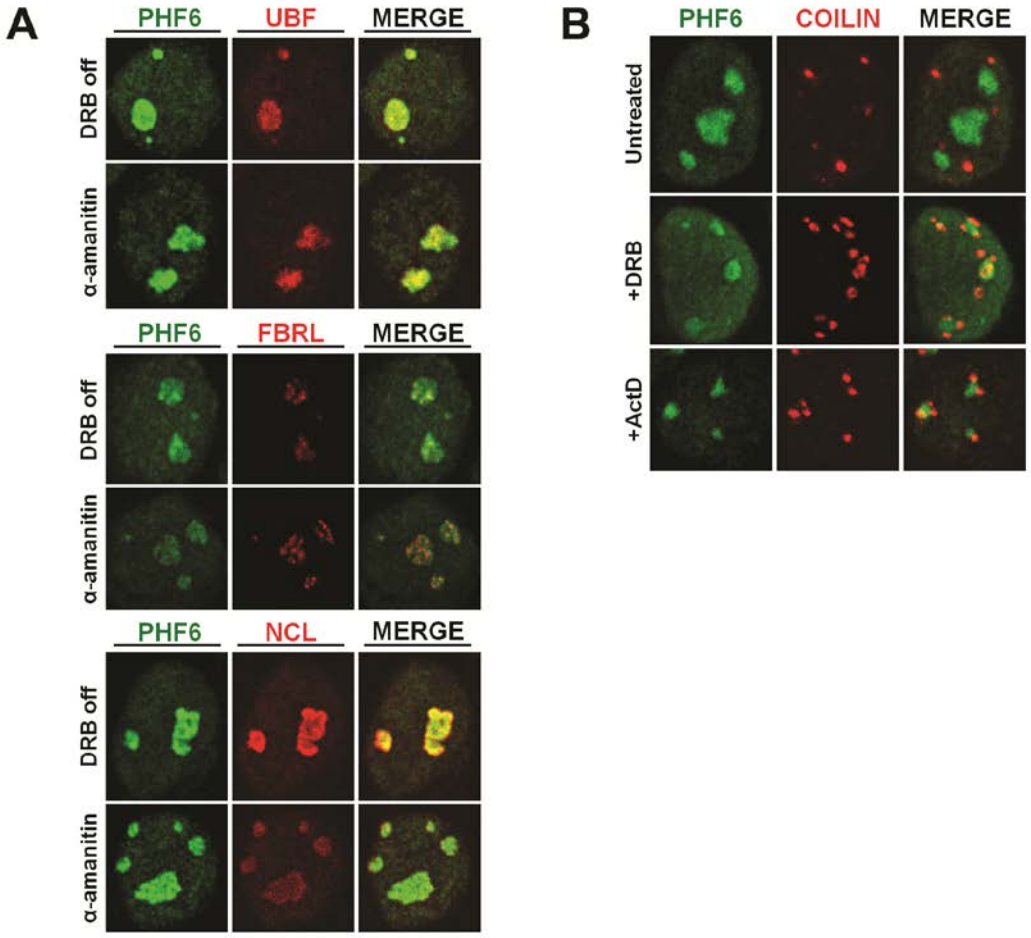
A
PHF6
UBF
MERGE
DRB off
α-amanitin
PHF6
FBRL
MERGE
DRB off
α-amanitin
PHF6
NCL
MERGE
DRB off
α-amanitin
B
PHF6
COILIN
MERGE
Untreated
+DRB
+ActD

Figure S2

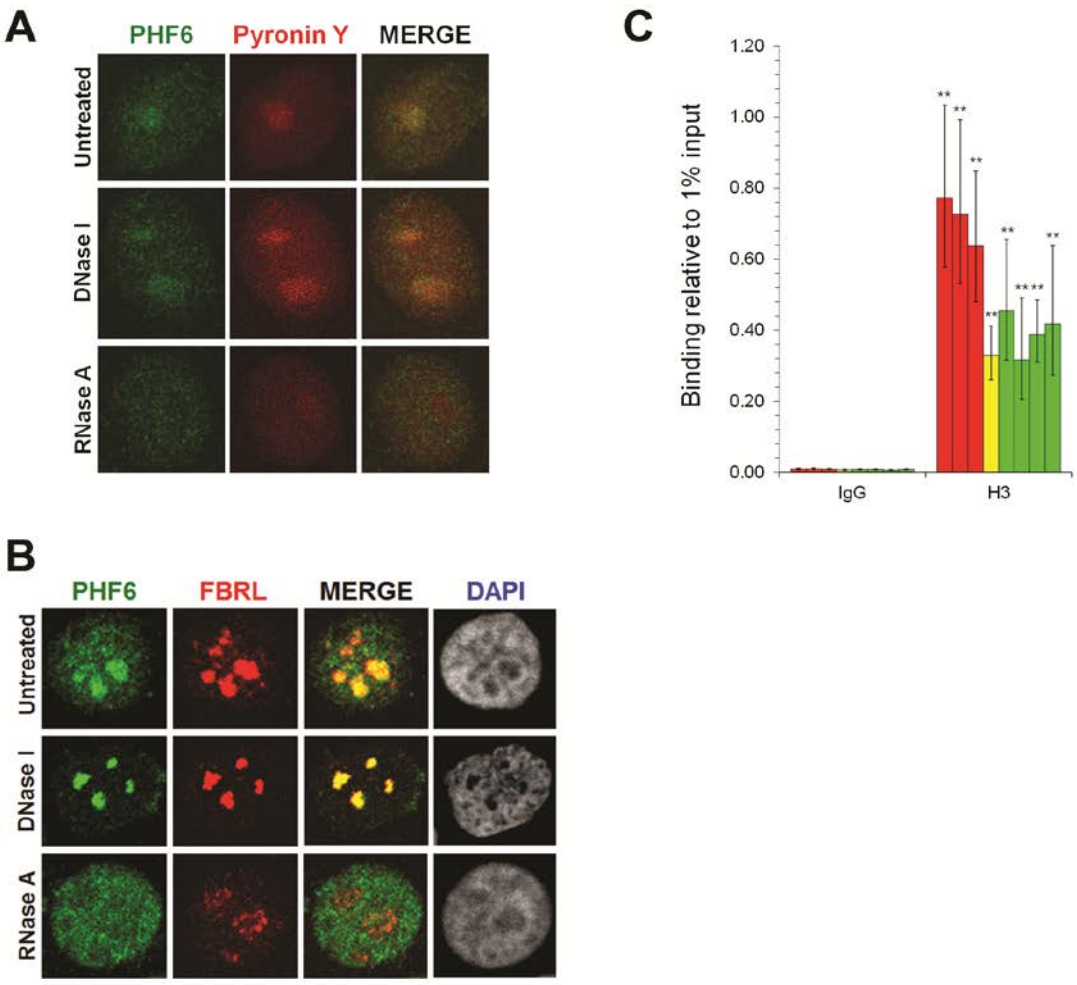
A
PHF6
Pyronin Y
MERGE
Untreated
DNase I
RNase A
B
PHF6
FBRL
MERGE
DAPI
Untreated
DNase I
RNase A
C
Binding relative to 1% input
1.20
1.00
0.80
0.60
0.40
0.20
0.00
IgG
H3

Figure S3

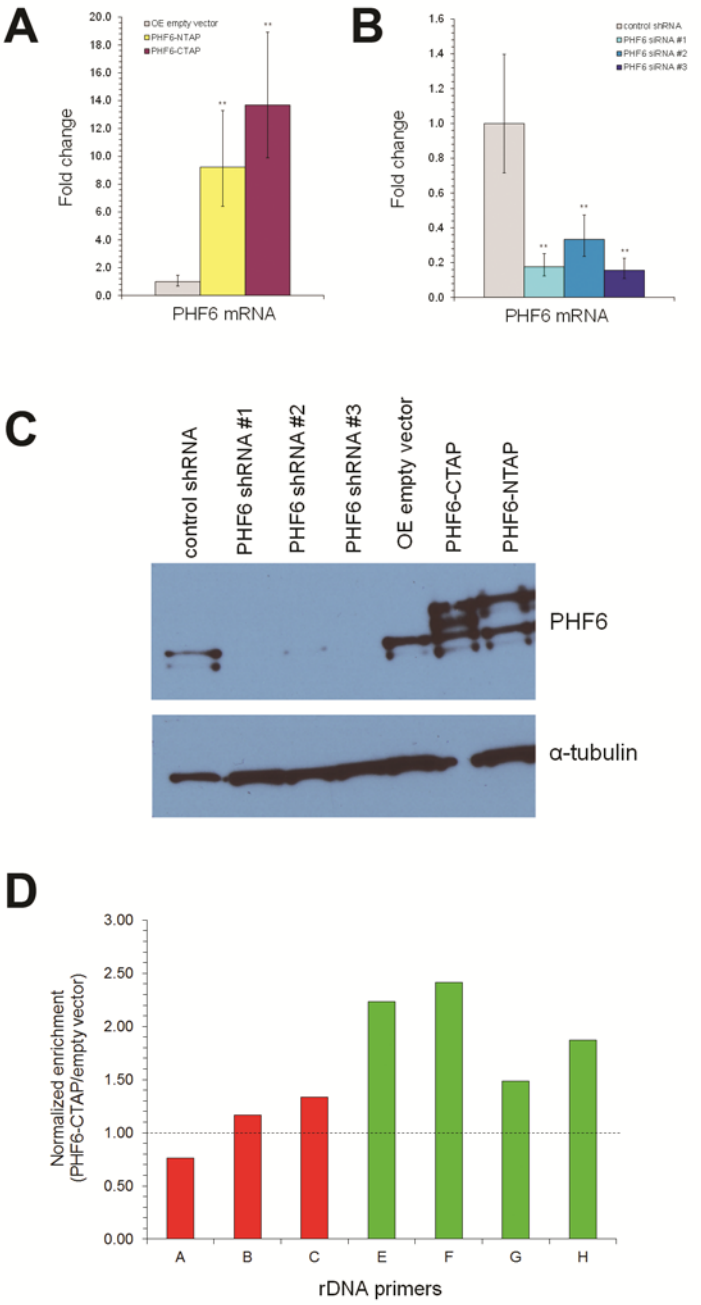
A
OE empty vector
PHF6-NTAP
PHF6-CTAP
Fold change
PHF6 mRNA
B
control shRNA
PHF6 siRNA #1
PHF6 siRNA #2
PHF6 siRNA #3
Fold change
PHF6 mRNA
C
control shRNA
PHF6 shRNA #1
PHF6 shRNA #2
PHF6 shRNA #3
OE empty vector
PHF6-CTAP
PHF6-NTAP
PHF6
α-tubulin
D
Normalized enrichment
(PHF6-CTAP/empty vector)
A
B
C
E
F
G
H
rDNA primers

Figure S4

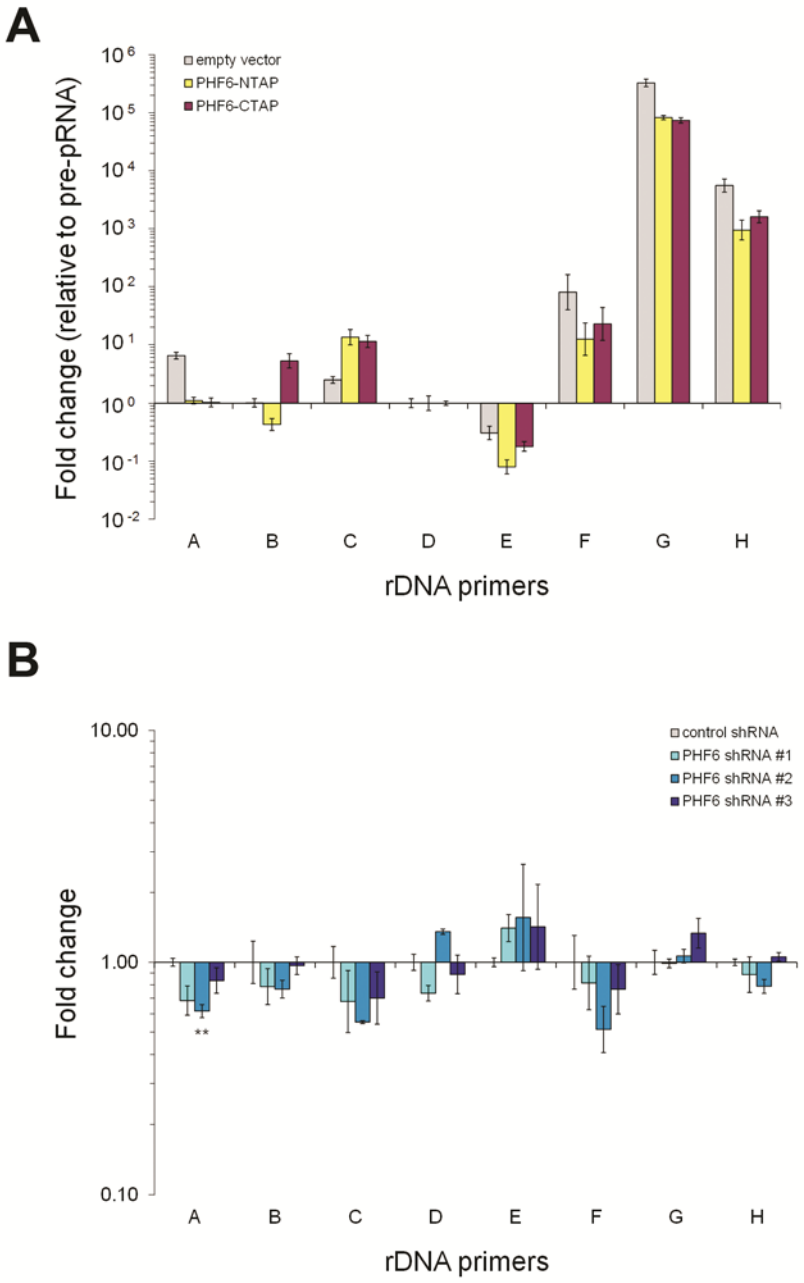
A
empty vector
PHF6-NTAP
PHF6-CTAP
Fold change (relative to pre-pRNA)
10^6
10^5
10^4
10^3
10^2
10^1
10^0
10^-1
10^-2
A
B
C
D
E
F
G
H
rDNA primers
B
control shRNA
PHF6 shRNA #1
PHF6 shRNA #2
PHF6 shRNA #3
Fold change
10.00
1.00
0.10
**
A
B
C
D
E
F
G
H
rDNA primers

Table S1. List of antibodies used for Western blotting (W), immunocytochemistry (ICC), and chromatin immunoprecipitation (ChIP).

Antibody	Source	Application
Primary antibodies		
PHF6 (rabbit)	Sigma (HPA001023)	W (1:5000), ICC (1:1000), ChIP
Coilin (mouse)	BD Biosciences (612074)	ICC (1:1000)
CTCF (rabbit)	Millipore (07-729)	ChIP
FBRL (mouse)	Abcam (ab4566)	ICC (1:1000)
Histone H3 (rabbit)	Abcam (ab1791)	ChIP
NCL (mouse)	Millipore (05-565)	ICC (1:250)
α-tubulin (mouse)	Sigma (T6199)	W (1:10000)
UBF (mouse)	Santa Cruz (sc-13125)	ICC (1:1000)
Secondary antibodies		
Anti-mouse HRP	Sigma (A5906)	W (1:10000)
Anti-rabbit HRP	Sigma (A4914)	W (1:10000)
Anti-mouse 488 Alexa Fluor	Life Technologies (A21202)	ICC (1:4000)
Anti-rabbit 594 Alexa Fluor	Life Technologies (A21207)	ICC (1:4000)

Table S2. Primer sequences for ChIP-qPCR and qRT-PCR.

Primer name*	Sequence	Annealing temp. (ºC)
rDNA forward primer A	5'- CCTTCCACGAGAGTGAGAAGC	57
rDNA reverse primer A	5'- TCGACCTCCCGAAATCGTAC	
rDNA forward primer B	5'- TGCTCGGATGCCCCGAGTGA	57
rDNA reverse primer B	5'- ACGTGTGTCCCGAGCTCCTGT	
rDNA forward primer C	5'- CCCCGGGAGACAGACCCTGG	57
rDNA reverse primer C	5'- CGAGGCGGAAGAGTCCACGC	
rDNA forward primer D	5'- AGAGGGGCTGCGTTTTCGGCC	70
rDNA reverse primer D	5'- CGAGACAGATCCGGCTGGCAG	
rDNA forward primer E	5'- GGTATATCTTTCGCTCCGAG	57
rDNA reverse primer E	5'- GACGACAGGTCGCCAGAGGA	
rDNA forward primer F	5'- TGTCAGGCGTTCTCGTCTC	57
rDNA reverse primer F	5'- GAGAGCACGACGTCACCAC	
rDNA forward primer G	5'- AGTCGGGTTGCTTGGGAATGC	57
rDNA reverse primer G	5'- CCCTTACGGTACTTGTTGACT	
rDNA forward primer H	5'- ACCTGGCGCTAAACCATTCGT	57
rDNA reverse primer H	5'- GGACAAACCCTTGTGTCGAGG	
GAPDH forward primer	5'-AAGGTGAAGGTCGGAGTCAAC	57
GAPDH reverse primer	5'-CAGGGATGATGTTCTGGAGA	
PHF6 forward primer	5'-GAAAACTGCACATAACTC	57
PHF6 reverse primer	5'-TGTGTGGAGACCTATC	

* Note: rDNA primer sets A-H were derived from Zhu et al. 2010.[33]