NDF is a transcription factor that stimulates elongation by RNA polymerase II

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RNA polymerase II (Pol II) elongation is a critical step in gene expression. Here we found that NDF, which was identified as a bilaterian nucleosome-destabilizing factor, is also a Pol II transcription factor that stimulates elongation with plain DNA templates in the absence of nucleosomes. NDF binds directly to Pol II and enhances elongation by a different mechanism than that used by transcription factor TFIIS. Moreover, yeast Pdp3, which is related to NDF, binds to Pol II and stimulates elongation. Thus, NDF is a Pol II binding transcription elongation factor that is localized over gene bodies and is conserved from yeast to humans.

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In eukaryotic cells, transcription by RNA polymerase II (Pol II) beyond the proximal promoter region is influenced by a number of factors that function by different mechanisms (for reviews, see Sims et al. 2004; Guo and Price 2013; Chen et al. 2018; Conaway and Conaway 2019; Cramer 2019; Lis 2019; Roeder 2019; Schier and Taatjes 2020). Some factors enable Pol II to overcome nucleosome-mediated inhibition, whereas others interact with Pol II and increase its ability to elongate transcripts. Because of their key role in gene expression, the identification and characterization of the factors that control Pol II elongation are of critical importance.

In our studies of chromatin dynamics, we identified and purified nucleosome-destabilizing factor NDF based on its ability to disrupt nucleosomes in a biochemical assay (Fei et al. 2018). We further found that NDF can facilitate Pol II transcription through a downstream nucleosome in vitro and is recruited to thousands of gene bodies upon transcriptional induction in mammalian cells.

NDF is present in most animals but has not been studied in simpler organisms such as yeast. In *Drosophila*, NDF (also known as CG4747) was identified as an MSL (male-specific lethal) complex-interacting protein that targets the complex to active gene bodies and is important for X-chromosome dosage compensation (Wang et al. 2013). In humans, NDF (also known as GLYR1, N-PAC, and NP60) was found as a protein that binds preferentially to H3K36me3 relative to unmethylated H3K36 (Vermeulen et al. 2010). Human NDF was also observed to stimulate H3K4me1 and H3K4me2 demethylation by LSD2/KDM1B (Fang et al. 2013); the region of human NDF that interacts with LSD2/KDM1B is not present in *Drosophila* NDF. NDF has a conserved PWWP motif, which binds to methylated lysines (Qin and Min 2014). In addition, NDF (GLYR1) is present at high levels in all (45 out of 45) tested tissues in humans (Uhlen et al. 2017).

Because NDF was identified as a nucleosome-destabilizing factor in biochemical assays with purified nucleosomes (Fei et al. 2018), there was no obvious direct connection between NDF and Pol II function. However, closer re-examination of our NDF transcription data suggested that purified NDF might enhance transcription by purified Pol II with plain (naked) DNA templates in the absence of nucleosomes. We therefore further investigated the unexpected possibility that NDF stimulates Pol II transcription.

Results and Discussion

NDF stimulates in vitro transcription by Pol II with calf thymus DNA

In initial experiments, we sought to determine whether NDF affects basal transcription by Pol II. To this end, we used a simple biochemical assay in which transcription by purified Pol II primarily initiates at nicks, gaps, and ends of genomic DNA in a non-sequence-specific manner. This assay was used widely in early transcription studies, such as for the purification of the Pol II elongation factor TFIIS (also known as S-II) (for example, see Sekimizu et al. 1976; Reinberg and Roeder 1987). In our work, we performed reactions with purified yeast Pol II (yPol II) and calf thymus DNA in the absence or presence of NDF and measured the incorporation of radiolabeled CTP into RNA (Supplemental Fig. S1A).

These studies revealed that human NDF (hNDF) as well as *Drosophila* NDF (dNDF) can stimulate nonspecific Pol II transcription with plain (naked) DNA. Transcription levels were observed to increase with NDF concentration (with saturation at ~100 nM hNDF and ~200 nM dNDF) (Fig. 1A,B) as well as to be linear over the first 15 min (Fig. 1C,D). As controls, we found that the preparations of hNDF and dNDF did not contain polymerase activity and that the Pol II lacked Pol I and Pol III activity (Supplemental Fig. S1B). In addition, these experiments were performed at a Pol II concentration in the linear concentration activity range (Supplemental Fig. S1C).

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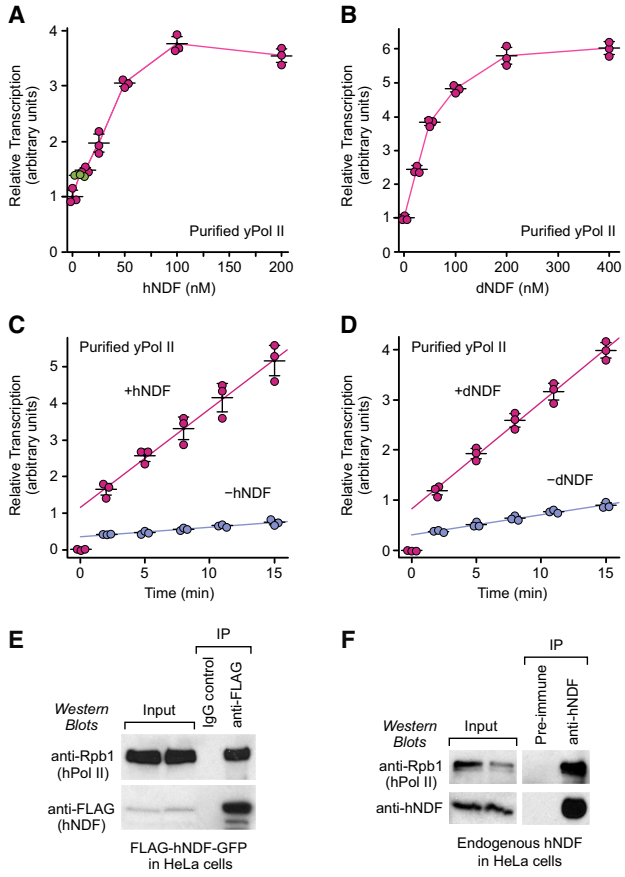

Figure 1. NDF stimulates nonspecific in vitro transcription by purified yeast Pol II (yPol II) and binds to human Pol II (hPol II) in cell extracts. (*A*,*B*) Stimulation of in vitro Pol II transcription by hNDF and dNDF. (*C*,*D*) Time course of transcription in the presence (red dots) or absence (blue dots) of hNDF and dNDF. For *A*–*D*, reactions were carried out as depicted in Supplemental Figure S1A, and the primary data are in Supplemental Table S1. Three replicate reactions were performed for each condition. Each cluster of dots corresponds to a single reaction condition (in *A*, the 6.25 nM hNDF data points are colored green to distinguish them from the red 12.5 nM hNDF data points). The standard deviation (error bars) and mean (central horizontal line) are indicated. The lines connect the mean values. (*E*) Flag-hNDF-GFP coimmunoprecipitates with endogenous hPol II In HeLa cells. Lysates from cells containing Flag-hNDF-GFP were incubated with Flag M2 antibodies or IgG control. The immunoprecipitated proteins were subjected to Western blot analysis with anti-Flag and anti-Rpb1 (Pol II) antibodies. (*F*) Coimmunoprecipitation of endogenous hNDF and hPol II. HeLa whole-cell lysates were incubated with anti-hNDF polyclonal antibodies (or preimmune serum as the control) and Dynabeads protein A. The beads were washed, and the associated proteins were subjected to Western blot analysis.

Yeast Pdp3 protein is related to NDF and stimulates transcription

The ability of both dNDF and hNDF to stimulate transcription by yeast Pol II suggested that there might be NDF-related factors in yeast. We investigated this possibility and identified *Saccharomyces cerevisiae* Pdp3 (yPdp3) as being closely related to hNDF (Supplemental Fig. S2). yPdp3 is a PWWP domain-containing protein (Gilbert et al. 2014) with extensive sequence similarity (~25% identity; ~39% similarity) to the N-terminal region of hNDF. yPdp3 lacks the catalytically inactive dehydrogenase domain at the C terminus of hNDF. Notably, the similarity between yPdp3 and hNDF extends throughout the entire yPdp3 protein and is not restricted to the PWWP domain. Moreover, like bilaterian NDF, yPdp3 is localized over gene bodies (Flury et al. 2017).

To test the biochemical activities of yPdp3, we purified the protein (Supplemental Fig. S3A) and found that it is able to stimulate in vitro transcription by yPol II with the calf thymus DNA assay (Supplemental Fig. S3B,C). In addition, yPdp3 did not exhibit synergism with hNDF in the stimulation of transcription (Supplemental Fig. S3D). This finding is consistent with yPdp3 and hNDF activating transcription by a similar mechanism. Hence, yPdp3 is related to NDF and also stimulates transcription by Pol II.

hNDF and yPdp3 bind directly to Pol II

Because NDF and yPdp3 stimulate Pol II transcription, we tested their ability to interact with Pol II. First, in HeLa cells that stably express Flag-hNDF-GFP, we found that the hNDF fusion protein coimmunoprecipitates with the endogenous Pol II (Fig. 1E). Second, in wild-type HeLa cells, we saw that the endogenous hNDF coimmunoprecipitates with the endogenous Pol II (Fig. 1F). Third, we carried out sucrose gradient sedimentation analyses with purified yeast Pol II and purified hNDF and observed that Pol II binds directly to NDF (Supplemental Fig. S4). Fourth, we observed that purified hNDF interacts with purified hPol II (Supplemental Fig. S5A). Fifth, we found that purified yPdp3 associates with purified yPol II (Supplemental Fig. S5B). Consistent with this finding, it was observed that the isolation of TAP-tagged yPdp3 from yeast extracts resulted in the copurification of the Rpb2 and Rpb4 subunits of yeast Pol II (Gilbert et al. 2014). These results collectively indicate that hNDF as well as yPdp3 bind directly to Pol II.

NDF as well as Pdp3 can stimulate transcription elongation by Pol II

Next, we more rigorously investigated the effect of hNDF upon Pol II transcription with well-defined DNA templates. Because NDF is localized over the transcribed regions of active genes in *Drosophila* (Wang et al. 2013) and in humans (Vermeulen et al. 2010; Fei et al. 2018), we tested whether hNDF affects transcriptional elongation with purified human Pol II (hPol II).

In these experiments, we assembled Pol II elongation complexes with hPol II and examined the effect of hNDF on elongation through two different downstream sequences. To this end, we assembled functional transcription elongation complexes by using the method that was developed by Kashlev and colleagues (Sidorenkov et al. 1998; Kireeva et al. 2000; Komissarova et al. 2003). First, we hybridized a short 5′-labeled primer RNA to the template DNA strand, and then added purified hPol II to reconstitute the catalytically active elongation complex. Next, we annealed a 5′-biotinylated nontemplate DNA strand to give the elongation complex, as shown in the top left diagram in Figure 2A. We then ligated different downstream sequences to the complex and analyzed the ability of hNDF to stimulate hPol II elongation through these sequences (Fig. 2A).

With the *Xenopus borealis* 5S rDNA as the downstream sequence, we found that hNDF enhances the formation of

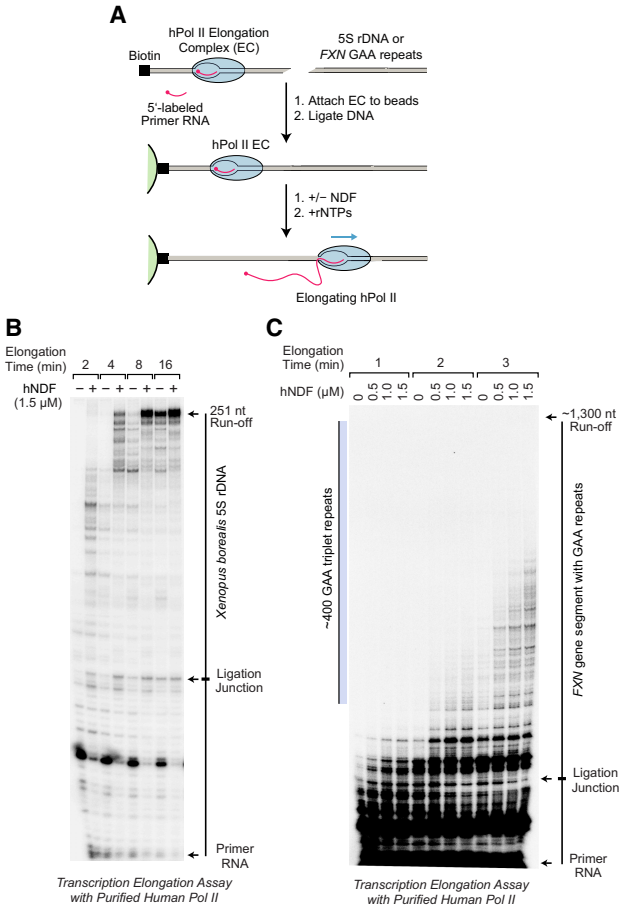

Figure 2. hNDF stimulates transcription elongation by purified human Pol II. (*A*) Diagram of the assay with purified Pol II elongation complexes. The transcripts were analyzed by denaturing 8% polyacrylamide–urea gel electrophoresis. (*B*) Transcription elongation reactions with purified HeLa Pol II and the *Xenopus borealis* 5S rDNA template. Reactions were performed in the presence or absence of purified hNDF for the indicated times. (*C*) Transcription elongation reactions with purified HeLa Pol II and a patient-derived human *FXN* gene segment containing ~400 GAA trinucleotide repeats. The estimated location of the GAA repeats is shown. The concentrations of purified hNDF protein and reaction times are indicated.

full-length runoff transcripts (Fig. 2B). Hence, beginning with a productive hPol II elongation complex, hNDF is able to stimulate the elongation of Pol II in the production of full-length runoff transcripts.

We additionally tested transcription into the ~400 GAA triplet repeats in the human *FXN* gene. The expanded GAA triplets in the first intron of the *FXN* gene impede Pol II elongation and thus appear to cause Friedreich's ataxia, which is caused by a deficiency in the levels of the FXN protein (Bidichandani et al. 1998; Punga and Bühler 2010; Li et al. 2015). Transcription into the downstream *FXN* sequences showed that hNDF substantially increases the amount of elongation of hPol II into the GAA triplet repeats (Fig. 2C). We also found that yPdp3 stimulates transcription elongation into downstream *FXN* sequences with GAA repeats (Supplemental Fig. S6).

These findings indicate that NDF as well as Pdp3 can stimulate the elongation of transcription by Pol II with defined DNA templates. NDF was identified based on its ability to destabilize nucleosomes and was found to be able to facilitate Pol II transcription through a nucleosome (Fei et al. 2018). Here, we show that hNDF as well as the related yPdp3 protein can stimulate Pol II elongation with plain (naked) DNA in the absence of nucleosomes (Figs. 1, 2). Thus, the enhancement of Pol II elongation by NDF/Pdp3 occurs independently of nucleosome-mediated inhibition of transcription.

NDF enhances Pol II elongation by a different mechanism than that used by TFIIS

We then examined whether the transcript-elongating function of NDF is similar to that of TFIIS (also known as S-II), which rescues arrested and backtracked Pol II elongation complexes by stimulating the polymerase-mediated cleavage of the 3′ end of the nascent transcript (for reviews, see Wind and Reines 2000; Sims et al. 2004; Nudler 2012; Guo and Price 2013; Conaway and Conaway 2019; Cramer 2019; Schier and Taatjes 2020). The TFIIS-stimulated transcript cleavage enables backtracked Pol II to be properly aligned with the 3′ end of the transcript so that the polymerase can resume transcription elongation.

To test the ability of NDF to rescue arrested Pol II, we performed Pol II elongation assays with a downstream 9A pause site that strongly arrests Pol II elongation (Fig. 3A; Sigurdsson et al. 2010). Unlike TFIIS (Sigurdsson et al. 2010), NDF has only a slight effect on the transcription of Pol II through the 9A pause site (Fig. 3B). To investigate whether NDF can rescue backtracked/arrested Pol II and resume elongation, we added NDF to preformed backtracked/arrested Pol II complexes and chased with rNTPs (Fig. 3C). We found that NDF, in contrast to TFIIS, is not able to rescue purified arrested Pol II elongation complexes. We further observed that NDF, unlike TFIIS, is not able to stimulate the cleavage of the nascent transcripts by stalled and backtracked Pol II (Fig. 3D). These findings indicate that NDF and TFIIS stimulate Pol II elongation by different mechanisms. This conclusion is also supported by the observation that there is synergy between TFIIS and NDF in the stimulation of Pol II transcription with the calf thymus DNA template system (Supplemental Fig. S7).

NDF has a small but distinct effect on ongoing transcription in cells

Because NDF functions as a Pol II elongation factor in biochemical assays, we investigated whether it participates in ongoing transcription in cells. Although there are other Pol II elongation factors that could potentially compensate for the loss of NDF, we nevertheless felt that it would be useful to examine whether NDF contributes to transcription in cells. Also, in previous work, we found that the loss of NDF results in a decrease in steady-state transcripts by RNA-seq as well as a decrease in run-on transcription in nuclei by GRO-seq (Fei et al. 2018). To assess whether NDF has an effect on ongoing transcription in cells, we used the Bru-seq method (Paulsen et al. 2013). This technique involves the transient treatment of cells with 5-bromouridine, which is a relatively nontoxic uridine analog that is incorporated into nascent transcripts.

We therefore performed Bru-seq with wild-type (WT) and NDF knockout (KO) HeLa cells. These experiments showed that genes with high NDF occupancy levels

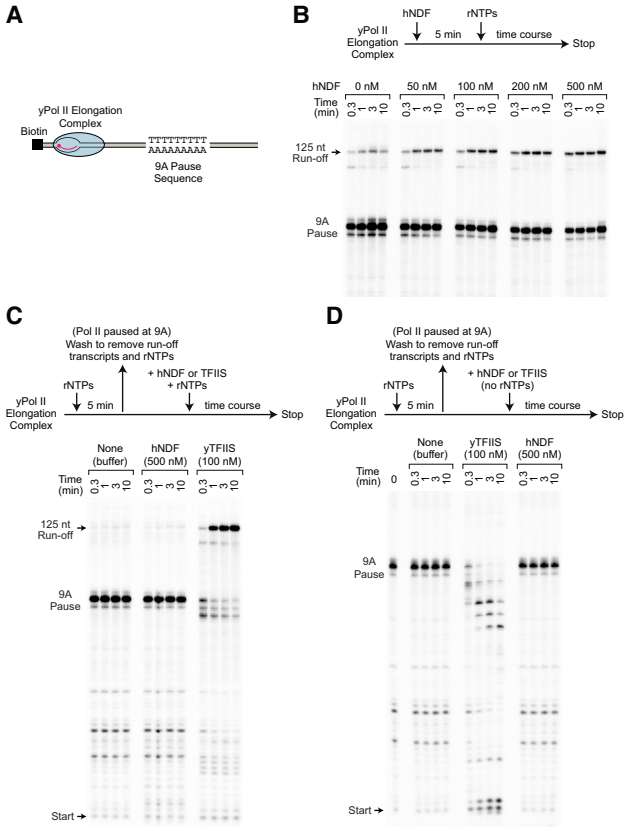

Figure 3. NDF-mediated Pol II stimulation is distinct from TFIIS-mediated rescue of backtracked Pol II. (*A*) Diagram of the Pol II elongation system used in these experiments. The downstream 9A sequence is in the template strand and induces Pol II pausing and backtracking. The 9A paused transcripts and the full-length runoff transcripts were resolved by denaturing 8% polyacrylamide–urea gel electrophoresis. (*B*) NDF has little effect on the transcription of Pol II through the 9A pause site. (*C*) TFIIS, but not NDF, can rescue purified arrested Pol II elongation complexes. The arrested Pol II complexes were treated with hNDF or yTFIIS in the presence of rNTPs for the indicated times. Cleaved and extended transcripts can be seen with TFIIS but not with NDF. (*D*) Unlike TFIIS, NDF cannot stimulate the RNA endonuclease activity of Pol II. Purified arrested Pol II elongation complexes were treated with hNDF or yTFIIS in the absence of rNTPs. The cleavage of the transcripts can be seen with TFIIS but not with NDF.

generally exhibit a small but distinct decrease in ongoing transcription in the KO cells relative to the WT cells (Fig. 4A; Supplemental Fig. S8A). We displayed the genes in four clusters, which are defined in the legend for Figure 4. Notably, cluster 1 contains genes with strong signals for RNA-seq, NDF ChIP-seq, and H3K36me3 ChIP-seq in WT HeLa cells. We did not observe a distinct correlation between the Bru-seq NDF KO/WT ratio and RNA-seq signal strength (Fig. 4B; Supplemental Fig. S8B). Hence, the Bru-seq data suggest that NDF contributes to ongoing transcription in cells, such as in the cluster 1 genes with high NDF occupancy levels. However, the loss of NDF does not result in a strong decrease of transcription in cells. In addition, the observed effects could be due to the function of NDF in Pol II stimulation and/or nucleosome disruption.

We further tested the effect of the loss of NDF on ongoing transcription by carrying out Bru-seq analyses in human SW480 cells (Supplemental Fig. S9). As in HeLa cells, we observed that the loss of NDF in SW480 cells results in a small but distinct decrease in transcription at genes that are associated with high levels of NDF (Supplemental Fig. S9B). To determine whether the decrease in transcriptional activity was due to the loss of NDF, we expressed recombinant NDF in the NDF knockout (KO) cells to give KO + NDF rescue cells (Supplemental Fig. S9A). We then carried out Bru-seq analyses of the KO + NDF rescue cells versus wild-type cells and observed that the expression of NDF in the KO cells resulted in the restoration of the loss in transcription at the high NDF genes (Supplemental Fig. S9C). These data support the conclusion that NDF contributes to ongoing transcription at NDF-associated genes in human cells.

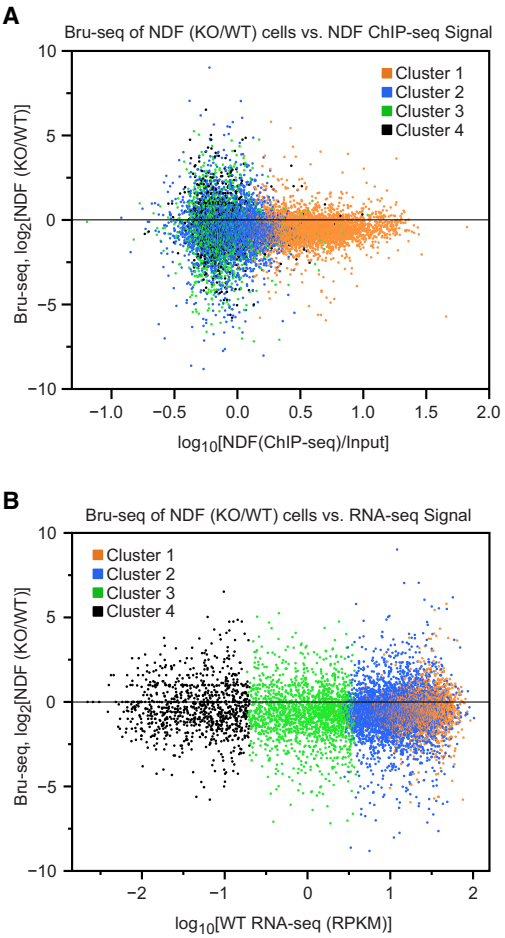

Figure 4. NDF has a small but distinct effect on ongoing Pol II transcription in cells. Transcription in wild-type (WT) versus NDF knockout (KO) HeLa cells was assessed by incubation of the cells for 10 min with 5-bromouridine (Bru-seq analysis) (Paulsen et al. 2013). The gene clusters were generated by k-means clustering of NDF ChIP-seq, H3K36me3 ChIP-seq, and RNA-seq data obtained with wild-type HeLa cells (Fei et al. 2018). Cluster 1 contains active genes that have high levels of NDF and H3K36me3. Cluster 2 has active genes with medium to low levels of NDF and H3K36me3. Clusters 3 and 4 contain genes with low or very low levels of activity, respectively. (*A*) NDF has a small but distinct effect on transcription in genes that contain high levels of NDF in WT cells. The $\log_2$ of the Bru-seq signal in KO cells versus WT cells is plotted against $\log_{10}$ of the NDF ChIP-seq signal in WT cells. (*B*) Comparison of the Bru-seq signal in KO versus WT cells with the RNA-seq signal in WT cells.

Because there are many different factors that participate in the Pol II elongation process, it is likely that the small effect observed upon loss of NDF is due to compensation by the other Pol II elongation factors. In this regard, it is relevant to note that modest effects on transcription have been observed upon loss or inhibition of other Pol II elongation factors such as TFIIS, Elongin, and ELL (for example, see Gopalan et al. 2018; Sheridan et al. 2019; Ardehali et al. 2021; Wang et al. 2021).

NDF is a Pol II elongation factor that is conserved from yeast to humans

NDF was purified and identified as a protein that destabilizes nucleosomes (Fei et al. 2018). Consistent with this function, NDF was found to be able to facilitate Pol II transcription through a nucleosome. Here we found, unexpectedly, that NDF is a Pol II transcription factor that stimulates transcript elongation with plain DNA templates in a nucleosome-independent manner. Moreover, yeast Pdp3, which is related to NDF, binds directly to Pol II, stimulates transcription elongation, and is localized to gene bodies. Thus, NDF is an ancient protein that is conserved from yeast to humans.

In humans, hNDF, which is encoded by the *GLYR1* gene, is present at high levels in all (45 out of 45) tested tissues (Uhlen et al. 2017). This property suggests that hNDF has a widespread biological function. Upon transcriptional induction, NDF is recruited to the transcribed regions of thousands of genes, but not all induced genes, in mammalian cells (Fei et al. 2018). Its conserved PWWP motif, which binds to methylated lysines (Qin and Min 2014), is probably important for the association of NDF with its target genes, but it should also be noted that NDF is not enriched at many genes that have moderate to high levels of H3K36me3 (Fei et al. 2018). It thus seems likely that other factors, such as those associated with the transcription process, additionally contribute to the recruitment of NDF to genes during transcription. In this regard, the direct interaction between NDF and Pol II may contribute to the recruitment of NDF to gene bodies.

There are many different transcription factors that bind directly to Pol II and facilitate the elongation process (for reviews, see Sims et al. 2004; Guo and Price 2013; Chen et al. 2018; Conaway and Conaway 2019; Cramer 2019; Lis 2019; Roeder 2019; Schier and Taatjes 2020). These Pol II binding elongation factors can be placed into two general categories. The first group comprises TFIIS and CSB, which rescue arrested and backtracked Pol II, and second group includes TFIIF, ELL, Elongin, and Spt4–Spt5, which suppress transient Pol II pausing. ELL, Elongin, and Spt4–Spt5 are also localized over gene bodies. Given that NDF functions differently than TFIIS and is localized over gene bodies, it appears that NDF and Pdp3 may act similarly to ELL, Elongin, and Spt4–Spt5. With this working hypothesis, it will be important to carry out structural, biochemical, and genetic experiments on NDF and Pdp3 that will shed additional light on their mechanisms and biological functions.

Materials and methods

Antibodies

Rabbit polyclonal antisera against hNDF were described in Fei et al. (2018). Commercial antibodies were as follows: anti-BrdU (5 µL per sample for nascent RNA sequencing; BD Biosciences 555627) and anti-Pol II (rabbit polyclonal antibodies raised against amino acids 1–224 of the largest subunit of human Pol II; 1:1000 dilution for Western blots; Santa Cruz Biotechnology sc-9001). Horseradish peroxidase (HRP)-conjugated protein A was obtained from Thermo Fisher (101023).

Cell culture

HeLa cells were cultured by using Dulbecco's modified Eagle's medium (DMEM) containing 10% (v/v) fetal bovine serum (FBS; Gibco), 100 U/mL penicillin, and 0.1 mg/mL streptomycin. Cells were maintained in a humidified incubator atmosphere at 37°C with 5% CO_2. NDF knockout (KO) HeLa cells were described in Fei et al. (2018).

Nucleic acids

For transcription elongation experiments, template strand (TS), nontemplate strand (NTS), RNA primer, and 5S rDNA sequences were used as described (Xu et al. 2017; Fei et al. 2018). The Friedreich's ataxia (*FXN*) gene fragment containing ~400 GAA trinucleotide repeats was amplified (forward primer: 5′-ATACCGGATCCGGGATTGGTTGCCAGTGCTTAAAAGTTAG-3′, BamHI site is underlined; reverse primer: 5′-GGTATGGTACCGATCTAAGGACCATCATGGCCACACTTGCC-3′, KpnI site is underlined) from DNA obtained from GM16223 Friedreich's ataxia patient cells (Coriell Institute for Medical Research) by using the PCR conditions described in Campuzano et al. (1996) and subcloned into the BamHI and KpnI sites of pBS (BlueScribe, Stratagene) to give the pFXN-GAA plasmid. The pause rescue experiments with the site-specific 9A pause site were performed as previously described (Xu et al. 2017). The oligonucleotides were as follows: primer RNA (5′-AUCGAGAGGA-3′), template strand DNA (5′-CAGACTCTAACCACACATCACTTACCCTACATACACCACACACCACACCGAGAAAAAAAAAATTACCCCTTCACCCTCACTGCCCCACATCATCACTTACCTGGATACACCCTTACTCCTCTCGATACCTCACCACCTTACCTACCACCCAC-3′), and biotin-labeled nontemplate strand DNA (5′-biotin-TTTGTGGGTGGTAGGTAAGGTGGTGAGGTATCGAGAGGAGTAAGGGTGTATCCAGGTAAGTGATGATGTGGGGCAGTGAGGGTGAAGGGGTAATTTTTTTTTCTCGGTGTGGTGTGTGGTGTATGTAGGGTAAGTGATGTGTGGTTAGAGTCTG-3′).

Additional methods are included in the Supplemental Material. The genome-wide data have been deposited at the Gene Expression Omnibus (GEO; accession no. GSE185464), and will be released upon acceptance of the paper for publication. All experiments were performed independently at least twice to ensure reproducibility of the data.

Competing interest statement

The authors declare no competing interests.

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Author contributions: J.F. and J.T.K. conceived the project and oversaw the overall execution of this work. J.F. performed the initial studies of NDF-mediated stimulation of Pol II transcription (related to Fig. 1; Supplemental Fig. S1) as well the experiments shown in Figures 2 and 4 and Supplemental Figures S4, S5A, S8, and S9. J.X. and D.W. oversaw and carried out the comparison of NDF and TFIIS in transcription elongation (Fig. 3). G.A.K. performed the analysis of the effect of NDF and yPdp3 on Pol II transcription with the calf thymus DNA assay (Fig. 1; Supplemental Figs. S1, S3, S7; Supplemental Table S1). Z.L. performed the experiment in Supplemental Figure S6, and K.X. carried out the experiment in Supplemental Figure S5B. J.F. and J.T.K. were primarily responsible for writing the manuscript and received substantial contributions from all other authors in the preparation of figures and the writing and editing of the text.

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