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RNA Polymerase II Transcription Attenuation at the Yeast DNA Repair Gene, DEF1, Involves Sen1-Dependent and Polyadenylation Site-Dependent Termination

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ABSTRACT Termination of RNA Polymerase II (Pol II) activity serves a vital cellular role by separating ubiquitous transcription units and influencing RNA fate and function. In the yeast Saccharomyces cerevisiae, Pol II termination is carried out by cleavage and polyadenylation factor (CPF-CF) and Nrd1-Nab3-Sen1 (NNS) complexes, which operate primarily at mRNA and non-coding RNA genes, respectively. Premature Pol II termination (attenuation) contributes to gene regulation, but there is limited knowledge of its prevalence and biological significance. In particular, it is unclear how much crosstalk occurs between CPF-CF and NNS complexes and how Pol II attenuation is modulated during stress adaptation. In this study, we have identified an attenuator in the DEF1 DNA repair gene, which includes a portion of the 5′-untranslated region (UTR) and upstream open reading frame (ORF). Using a plasmid-based reporter gene system, we conducted a genetic screen of 14 termination mutants and their ability to confer Pol II read-through defects. The DEF1 attenuator behaved as a hybrid terminator, relying heavily on CPF-CF and Sen1 but without Nrd1 and Nab3 involvement. Our genetic selection identified 22 cis-acting point mutations that clustered into four regions, including a polyadenylation site efficiency element that genetically interacts with its cognate binding-protein Hrp1. Outside of the reporter gene context, a DEF1 attenuator mutant increased mRNA and protein expression, exacerbating the toxicity of a constitutively active Def1 protein. Overall, our data support a biologically significant role for transcription attenuation in regulating DEF1 expression, which can be modulated during the DNA damage response.

KEYWORDS RNA polymerase II termination attenuation Def1 cleavage and polyadenylation complex (CPF-CF) Nrd1/Nab3/Sen1 complex (NNS)

RNA Polymerase II (Pol II) transcribes a wide assortment of transcripts in eukaryotes, including all protein-coding mRNAs and most non-coding RNAs. The high density of Pol II across the genome must be

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confined to prevent interference with surrounding protein:DNA transactions (Jensen et al. 2013). Genomic partitioning is achieved in part by transcription termination, which releases Pol II and its RNA product from the DNA template. The biological significance of termination is not limited to defining transcriptional boundaries. Due to its connection with 3′-end processing, stability, and export, Pol II termination has the ability to influence RNA fate and function (Kuehner et al. 2011; Mischo and Proudfoot 2013; Arndt and Reines 2015; Porrua and Libri 2015). In addition, there is an expanding collection of genes regulated by premature transcription termination (i.e., attenuation), some of which exhibit altered expression during cell metabolism and stress. The significance of Pol II termination has also been revealed in human pathology, including its roles in HIV latency, herpes viral infection, and renal cell carcinoma (Natarajan et al. 2013; Rutkowski et al. 2015; Grosso et al. 2015; Loya and Reines 2016).
Pol II termination is best understood in the model eukaryote S. cerevisiae, which is especially dependent on the process due to its compact genome (Goffeau et al. 1996). In yeast, Pol II termination occurs via two major pathways: cleavage and polyadenylation factor (CPF-CF) termination and Nrd1-Nab3-Sen1 (NNS) termination (Kuehner et al. 2011; Mischo and Proudfoot 2013; Arndt and Reines 2013; Porrua and Libri 2015). Pol II termination of most mRNAs occurs via CPF-CF termination, during which recognition of a polyadenylation (pa) site contributes to 3’-end processing and Pol II eviction, perhaps via allosteric changes in the elongation complex. Pol II termination of most noncoding RNAs occurs via NNS termination. Noncoding RNAs, including some small nuclear RNAs (snRNAs) and small nucleolar RNAs (snoRNAs), can be processed by endonucleolytic cleavage and exonucleolytic trimming, resulting in stable and abundant products (Bernstein and Toth 2012). Noncoding RNAs can also behave as cryptic unstable transcripts (CUTs) due to NNS-mediated degradation (Schmidt and Butler 2013; Tudek et al. 2016). While CPF-CF and NNS termination pathways exhibit distinct elements and protein factors associated with the Pol II Rpb1 C-terminal domain (CTD), which is modified in accordance with Pol II distance from a transcription start site (TSS) (Rondon et al. 2008). There is evidence to support direct roles for a Pol II CTD-binding protein (Pcf11), an RNA exonuclease (Rat1), and an RNA/DNA helicase (Sen1) in the actual Pol II release step, but the termination mechanism remains unresolved (Kuehner et al. 2011; Mischo and Proudfoot 2013; Arndt and Reines 2013; Porrua and Libri 2015).

While CPF-CF and NNS termination pathways exhibit distinct behavior, they display substantial overlap among factor requirements, and they may have evolved independently to recognize highly similar sequence elements (Porrua et al. 2012). NNS termination typically occurs <1 kb from a TSS due to Nrd1-mediated recognition of Ser5-phosphorylated CTD, which is predominant when Pol II is proximal to the promoter (Rondon et al. 2008). CPF-CF termination typically occurs >1 kb from a TSS due to Pcf11-mediated recognition of Ser2-phosphorylated CTD, which is predominant when Pol II is distal to the promoter. However, exceptions have been identified that circumvent these trends. NNS terminators can be recognized outside of their normal context and distance threshold, in some cases acting as a fail-safe mechanism to prevent transcription interference (Steinmetz et al. 2006a; Gudipati et al. 2008; Ghazal et al. 2009; Rondon et al. 2009; Porrua et al. 2012). Likewise, NNS terminators can be dependent on CPF-CF components (Fatica et al. 2000; Morlando et al. 2002; Dheur et al. 2003; Kim et al. 2006; Pearson and Moore 2014). The basis of Pol II flexibility in terminator recognition remains unclear, as does the extent of crosstalk and function between CPF-CF and NNS components.

In addition to Pol II termination occurring downstream of genes, premature Pol II termination (attenuation) can regulate mRNA gene expression. Attenuation has long been recognized as a widespread mechanism of bacterial gene regulation (Naville and Gautheret 2010), but the extent of its activity and biological significance has been studied more recently in eukaryotes (Colin et al. 2011; Contreras et al. 2013). Genome-wide analysis of CUTs and NNS factors suggest that 5–10% of yeast mRNA genes may be regulated by attenuation (Neil et al. 2009; Webb et al. 2014). The NRD1 gene was identified as an early target of attenuation in yeast, whereby Nrd1 autoregulates its mRNA expression as part of the NNS termination complex (Arigo et al. 2006). Similar autoregulatory schemes appear to operate for RNA processing factor genes HRP1 and PCF11 (Kuehner and Brow 2008; Creamer et al. 2011). Attenuator recognition and bypass has been linked to changes in cell metabolism and stress response genes, but in most cases the signaling mechanism is unknown. Examples of mRNA gene attenuation targets include IMD2 and URA2 (nucleotide biosynthesis), FKS2 (cell wall damage), CLN3 (glucose starvation), GPH1 (glycogen metabolism), and GLT1 (nitrogen metabolism) (Jenks et al. 2008; Kuehner and Brow 2008; Thiebaut et al. 2008; Kwapisz et al. 2008; Kim and Levin 2011; Darby et al. 2012; Chen et al. 2017; Merran and Corden 2017).
Construction of pRS426-DEF1 plasmid and A-1G and C1590A mutants
PCR using Q5 Hot Start High-Fidelity 2X Master Mix (NEB) was performed to amplify the DEF1 promoter, 5’-UTR, ORF, and 3’-UTR (-428 to +2596 relative to the +1 ATG) from yeast strain BY4742 genomic DNA and cloned into the NotI restriction site of the pRS426 (URA3) vector. The A-1G and C1590A point mutations were generated using a Quik-Change Lightning Site-Directed Mutagenesis Kit (Agilent).

Construction of pRS314-HRP1 plasmid and the hrp1-5 mutant
PCR using Q5 Hot Start High-Fidelity 2X Master Mix (NEB) was performed to amplify the HRP1 promoter, 5’-UTR, ORF, and 3’-UTR (-500 to +1848 relative to the +1 ATG) from yeast strain BY4742 genomic DNA and cloned into the NotI/XhoI restriction site of the pRS314 (TRP1) vector. The L20SS point mutation (hrp1-5) was generated using a Quik-Change Lightning Site-Directed Mutagenesis Kit (Agilent).

Spot Test Assays
Yeast bearing the reporter plasmids of interest were grown overnight in a shaking incubator (at 25°C or 30°C) in appropriate selective liquid media. The absorbance at OD_{600} was measured for each strain using a spectrophotometer (Implen Dluphotometer) and cultures were diluted to OD_{600} = 1.0. Each diluted culture (200 µL) was transferred to a 96-well plate, followed by 10-fold serial dilutions (x4) into adjacent wells. All samples were mixed and spotted using a 96-well replica plater (Sigma) on appropriate plate media. The plates were air-dried and incubated for 3-7 days.

Yeast Doubling-Time Growth Assays
The BY4742 def1Δ strain bearing the pRS426-DEF1 plasmids of interest were grown overnight in a shaking 30°C incubator in -Ura media. The absorbance at OD_{600} was measured for each strain using a spectrophotometer (Implen Dluphotometer) and cultures were diluted to OD_{600} = 0.15. Each diluted culture (200 µL) was transferred to a 96-well plate and then split into separate cultures, with half of the culture shifted to 39°C and the other half remaining at 30°C for 2.5 hr. The absorbance at OD_{600} was measured every half hour, and doubling times were calculated using the following formula: Doubling time = (Time Duration x log(2)) / (log (Initial Concentration) - log (Final Concentration)).

Yeast β-galactosidase Microplate Plate Assay
Yeast bearing the reporter plasmids of interest were grown overnight in a shaking incubator (30°C for most strains or 25°C for nab3-11 and hrp1-5) in appropriate selective liquid media. The cultures were diluted 10-fold and absorbance at OD_{600} was measured for each strain using a plate reader (SpectraMax 190). Cultures were diluted to OD_{600} = 0.15, grown at 30°C for 2 hr (recovery period), and then shifted to a 39°C thermo-shaker (recovery), and shifted to a 30°C thermo-shaker (recovery). Total RNA was purified from yeast cells by measuring the absorbance at OD_{600} of the desired culture and harvesting 1.5 ODs of cells. The Master Pure Yeast RNA Purification Kit (Epicentre) was used to purify total RNA (including 1 hr DNase I treatment), and RNA quality and yield was determined using a NanoDrop spectrophotometer (Thermo-Fisher). To convert the purified RNA to cDNA, RT-PCR was performed using a OneTaq RT-PCR kit (NEB). Pairwise reactions were set up including 1 ug of total RNA and random primer (mix of hexamer and d(T)_{23}VN primers). A negative control received no reverse transcriptase (RT) to ensure that the final signal was RNA-dependent and not derived from chromosomal DNA template. Amplification of CUP1, lacZ, DEF1, and 18S RNA was performed using OneTaq DNA polymerase in a 25 µL reaction with 2 µL of diluted cDNA template, as directed by the manufacturer. The amplification cycle number ranged from 12-25 cycles depending on the linearity and intensity of the PCR product signal. The PCR products (10 µL) were loaded into a 2% agarose gel stained with SYBR Safe (Invitrogen). The gel band intensity and ratios of total, read-through, and attenuated transcripts were measured using the Gel Doc EZ System (BioRad) and ImageStudio software (LICOR).

Western blot analysis of Def1 protein levels
The BY4741 def1Δ strains containing pRS426-DEF1 or the def1 mutants were grown overnight at 30°C, diluted back to OD_{600} = 0.4, grown for 4 hr in a 30°C shaking incubator (recovery), and shifted to a 39°C shaking incubator (non-permissive temperature) for 0, 1, or 2 hr. The cells were harvested (10 OD units), washed in 20% TCA, and stored at -20°C. Whole cell protein extracts were prepared using a TCA method (Keogh et al. 2006). Briefly, cell pellets were resuspended in 250 µL of 20% TCA and lysed using glass beads and a vortexer in a 4°C room (3 x 1 min) with 1 min pauses on ice between runs. The supernatant was transferred to a new tube using a gel-loading tip to avoid the beads. 700 µL of 5% TCA was added to the supernatant (1.25 mL final) and inverted to mix. The sample was microcentrifuged at 13,300 RPM for 10 min at 4°C. The supernatant was discarded and the pellet was washed with 750 µL of 100% ice-cold ethanol. The wash buffer was discarded, and the pellet was resuspended in 40 µL of 1 M Tris Cl, pH 8.0.
additional 80 µL of 2X SDS reducing sample buffer (66 mM Tris-HCl pH 6.8, 26% (w/v) glycerol, 2.1% SDS, 0.01% bromophenol blue) was added, and the sample was heated to 95°C for 5 min, microcentrifuged at top speed for 5 min., and the supernatant was used for Western blot analysis. Western blots were incubated with rabbit polyclonal anti-Def1 antibody (1:5,000; kind gift of Sveistrup lab) and mouse anti-α actin antibody (1:5,000, Abcam) prior to incubation with an anti-rabbit or anti-mouse HRP-conjugated secondary antibody (1:15,000; Jackson Immunoresearch). Target proteins were detected via chemiluminescence using Clarity ECL Western blotting substrate (BioRad) and a C-Digit blower scanner with Image Studio software (LICOR).

Data Availability
Strains, plasmids, and primer sequences are available upon request. The authors affirm that all data necessary for confirming the conclusions of the article are present within the article, figures, and tables. Supplemental material available at Figshare: https://doi.org/10.25387/g3.6167696.

RESULTS

DEF1 promoter-proximal region resembles a transcriptional attenuator
Our labs have previously identified DEF1 as a gene with both promoter-proximal (pA1) and promoter-distal (pA2) polyadenylation sites (Graber et al. 2013). This arrangement of signals for DEF1 3’-end processing results in multiple mRNA isoforms due to alternative poly(A) (pA) site usage. In the absence of stress, 70% of DEF1 transcripts terminate near pA1 (< 50 nt downstream of the start codon), resulting in a mixture of short RNAs (~150 nt) that are not capable of being translated into Def1 protein. Upon exposure to the DNA damaging agent 4-NQO, pA1 usage is reduced to ~30%, resulting in ~70% of polyadenylation occurring at pA2, which is ~2.5 kb downstream of the TSS (Figure 1A). The switch in pA usage likely contributes to the ~twofold increase in full-length DEF1 mRNA produced upon exposure to 4-NQO. Aside from the observed changes in pA/terminator usage, DEF1 bears additional hallmarks of attenuator regulation. The promoters-proximal DEF1 transcripts consist of both a polyadenylated fraction and a CUT fraction (Neil et al. 2009; Oszolak et al. 2010; Graber et al. 2013), which is consistent with the CPF-CF and/or NNS termination pathway operating in this region. In addition, the DEF1 5’-UTR is highly conserved among yeast species (Figure 1B), suggesting that the RNA takes on an important regulatory function, as observed for IMD2 and HRP1 attenuators (Kuehner and Brow 2008).

DEF1 promoter-proximal pA site is sufficient to confer Pol II attenuation in a reporter plasmid
To better understand the signals and factors involved in recognition and read-through of the DEF1 promoter-proximal pA site, we fused the DEF1 promoter, 5’-UTR, and pA1 region (pDEF1-pA1) from -253 to +93 (relative to +1 start codon) to the CYC1 reporter gene, which confers resistance to copper-containing media in a cup1Δ strain background (Kuehner and Brow 2008). The pDEF1-pA1-CUP1 strain was copper sensitive but failed to exhibit copper resistance in any of the termination factor mutants tested (data not shown). The unexpected behavior of pDEF1-pA1-CUP1 was likely due to misfolding of the Def1-Cup1 fusion protein upon addition of DEF1 encoded amino acids (+1 to +93). A fusion of CUP1 to the DEF1 promoter and 5’-UTR in the absence of the upstream DEF1 ORF (-253 to -1) resulted in only modest sensitivity on 0.8 mM copper (data not shown).

To test the activity of the DEF1 pA1 site in a context where the ORF region is not translated into protein, we cloned the DEF1 attenuator into the intron of the pGAC24-CUP1 reporter gene plasmid (Figure 2A) (Lesser and Guthrie 1993; Steinmetz and Brow 1996). The DEF1 attenuator included the DEF1 5’-UTR and the pA1 within the upstream ORF (+187 to +93) but did not include the upstream TATA box promoter element. In the absence of a transcriptional terminator insert (No Term.), the cup1Δ strain was copper-resistant as expected (Figure 2B). Insertion of the DEF1 pA1 into the pGAC24 reporter was sufficient to confer copper sensitivity to 0.3 mM copper (Figure 2B). The DEF1 pA1 insert resulted in predominant production (71%) of short RT-PCR products, consistent with transcription attenuation (Figure 2C). Mutations in the NNS protein Sen1 and the CPF-CF proteins Ssu72 and Hrp1 conferred DEF1-CUP1 resistance to 0.3 mM copper, while a mutation in the NNS Nrd1 protein had no effect (Figure 2B). For comparison, we analyzed transcription termination activity from the mRNA gene CYC1, which contains a hybrid CPF-CF-NNS-dependent terminator (+448 to +528 relative to +1 ATG start codon), and the snRNA gene SNR13, which contains an NNS-dependent terminator (+125 to +232 relative to +1 TSS). The CYC1 gene contains a traditional mRNA 3’-end processing site but is somewhat NNS-dependent, at least in part because it is a short gene containing a pA site located <1 kb from the promoter (Steinmetz et al. 2006a). The sen1, ssu72, and hrp1 mutants conferred resistance to 0.3 mM copper for CYC1-CUP1, and the sen1, nrd1, and ssu72 mutants conferred resistance to 0.2 mM copper for SNR13-CUP1 (Figure 2B). These data for the control terminators were mostly consistent with previous reports (Steinmetz et al. 2001; Steinmetz and Brow 2003; Steinmetz et al. 2006a; Chen et al. 2017).

To extend the versatility of the reporter gene and improve quantitation of read-through defects, we replaced the CUP1 gene with the lacZ gene, which allows β-galactosidase activity to be used as a readout of transcriptional activity. In addition, the lacZ reporter in the pGAC24 plasmid is under control of a constitutive promoter instead of a previously described GAL-inducible lacZ reporter (Hyman et al. 1991). The ability to maintain the pGAC24-DEF1-lacZ reporter in selective glucose media avoids complications arising from the galactose-sensitivity of some termination mutants (data not shown). In the absence of a terminator (No Term.), the reporter produced ~4,500 units of β-gal activity (Figure 2D). The DEF1-lacZ reporter produced ~800 β-gal units, which is a ~fivefold reduction compared to the no terminator control. The CYC1-lacZ reporter produced ~1500 β-gal units (threefold reduction), and the SNR13-lacZ reporter exhibited ~40 units of β-gal activity (120-fold reduction). Overall the DEF1-lacZ reporter exhibited an intermediate level of termination activity between the stronger SNR13 terminator and the weaker CYC1 terminator (Figure 2D).

Mutations in both NNS and CPF-CF pathways confer Pol II read-through of DEF1 attenuator
To investigate the trans-acting requirements of DEF1 attenuation, we measured β-gal reporter activity in response to a variety of Pol II termination mutants. The mutations targeted members of CFI (Pcf11, Rna14, Rna15, Hrp1), CPF (Cth2, Glc7, Ssu72), and NNS (Nrd1, Nab3, Sen1). Our genetic screen also included Ctk1 and Paf1, which promote Pol II modification and recruitment of termination factors (Bowman and Kelly 2014; Van Oss et al. 2017). We first classified the effect of mutants on the control terminators to determine their relative impact on NNS vs. CPF-CF pathways in our reporter system. Pol II terminator read-through activity of SNR13-lacZ, but not CYC1-lacZ, was increased (>twofold) by the paf1Δ, nrd1-5, nab3-11, pcf11-2, pcf11-9, and pcf11-13 mutants, indicating defects in NNS-dependent termination (Figure 3A-F). In contrast, the reporter activity of CYC1-lacZ, but not SNR13-lacZ, was increased (>twofold)
by the hrp1-1 and cft2-5001 mutants, indicating defects in CPF-CF termination (Figure 3G, H). Interestingly, the reporter activity of DEF1-lacZ increased (~twofold) in the paf1Δ, hrp1-1, and cft2-1 mutants (Figure 3A, G, H), indicating a dependence on both NNS- and CPF-CF termination pathways for attenuator recognition. We identified an additional class of mutants (rna14-5, rna15-58, glc7-12, ssu72-2, sen1-1, ctk1Δ) that conferred read-through defects for both SNR13-lacZ and CYC1-lacZ control reporters (Figure 4A-F). All of these mutants likewise increased DEF1-lacZ activity, confirming that the DEF1 attenuator behaves as a hybrid Sen1- and CPF-CF-dependent terminator.

To confirm that the β-gal activity of the reporter genes was due to increased Pol II read-through of the transcriptional terminator, we analyzed RNA from the DEF1-lacZ reporter in sen1-1 and rna15-58 strains using RT-PCR to detect attenuated and read-through mRNA (Figure 4G). In wild-type strains, the preponderance of RNA (96%) corresponded to attenuated RNA. The higher efficiency of attenuator activity in DEF1-lacZ compared to DEF1-CUP1 (compare Figure 4G to Figure 2C) may reflect differences in Pol II elongation through the reporter genes. The sen1-1 and rna15-58 mutants increased the level of read-through mRNA ~3 fold, which is consistent with the trend observed in the β-gal assay. These data validate the use of β-gal activity from the DEF1-lacZ reporter as a proxy for Pol II attenuator recognition and read-through at the RNA level.

To more quantitatively compare the DEF1 terminator to control terminators, we created a read-through index to rank the level of β-gal activity in mutant vs. wild-type strains. Based on the range of the data, we assigned the mt/WT ratio into 3 categories: little/no effect, ~twofold; intermediate effect, 2-10 fold; and strong effect, >10-fold (Figure 5). Ranked in order, the mutants most defective for DEF1 attenuator recognition were rna14-5 and rna15-58, and those with an intermediate effect were glc7-12, ssu72-2, hrp1-1, sen1-1, ctk1Δ, paf1Δ, and cft2-5001. The DEF1 attenuator behaved more similarly to the CYC1 terminator than the SNR13 terminator, with DEF1 matching CYC1 in 10/12 cases that allowed for direct comparison within the index. These results indicate
that the DEF1 attenuator exhibits hybrid characteristics but is more heavily influenced by the CPF-CF termination pathway.

**DEF1 attenuator consists of multiple cis-acting elements spanning the ORF start codon**

In order to define the cis-acting sequence elements that promote DEF1 attenuator recognition, we randomly mutagenized the DEF1-CUP1 reporter and utilized a genetic selection to identify copper resistant colonies. We identified 22 attenuator point mutations within a 78 bp region (-31 to +47 relative to DEF1 +1 ATG start codon) (Figure 6A). The most frequently identified mutants were A−1G (n = 8), T+6C (n = 5), A+43G (n = 3), A+40G (n = 2), and T−20C (n = 2). The remaining mutants were each identified once in the genetic selection. To compare the relative level of copper resistance, we conducted growth assays for
six of the mutants and analyzed the ratio of attenuated:read-through transcripts via RT-PCR. The spot test and RT-PCR assays exhibited strong agreement with respect to attenuator activity. The WT DEF1 attenuator resulted in no growth (−) on 0.6 mM copper and 78% usage of the attenuator (Figure 6B, C). The A-1G and T+6 mutants were most defective for attenuator recognition, resulting in strong copper-resistant growth (+++) and decreased attenuator usage (6−15%). The A+43G, A+16G, and A-5G mutants were of intermediate strength, and the T-31C mutant had the weakest effect of the mutants tested.

We arranged the mutants into four regions (I−IV) based on their clustering pattern and similarity to known 3′-end processing elements (Figure 6A) (Tian and Graber 2011). The most commonly used DEF1 pA site in our previously reported RNA-Seq data was A+43 (Graber et al. 2013). Our identification of A+43G as a read-through mutant validates our genetic approach and defines region IV as a putative cleavage site (CS). Region II of the DEF1 attenuator contains a consensus match to the yeast efficiency element (EE), and 5/22 unique DNA point mutations targeted the TATATA sequence. The mutations within

Figure 3 Trans-acting mutants paf1, hrp1, and cft2 result in Pol II read-through (>twofold) of the DEF1 attenuator, representing a contribution of both NNS- and CPF-CF-dependent termination activity. (A)-(H) The DEF1-lacZ reporter and the control reporters CYC1-lacZ (CPF-CF-NNS hybrid terminator) and SNR13-lacZ (NNS terminator) were transformed into the indicated strains above. Cultures were grown at permissive temperature (25°C or 30°C) and diluted prior to shifting to 30°C for 2 hr and 37°C (non-permissive) for 2 hr. β-galactosidase activity was measured following cell lysis and incubation with ONPG substrate, using absorption at OD600 for cell density and OD420 for β-gal production. Experiments were performed in biological triplicate, and errors bars show standard deviation.
the proposed EE include A-1G, which was the strongest and most-commonly identified read-through mutant. In yeast, the UAUAUA element serves as an RNA binding site for Hrp1 (Chen and Hyman 1998; Valentini et al. 1999). Based on its position between the EE and CS, region III of the DEF1 attenuator is likely to contain a positioning element (PE). Region III contains a partial match (AATTTA) to the consensus PE (AATAAA), and the A+11G and A+16G mutants alter this proposed PE. The PE serves as an RNA-binding site for Rna15 (Gross and Moore 2001). The mutations we have identified in the EE and PE are consistent with hrp1-1 and rna15-58 mutants being defective for DEF1 attenuator recognition (Figure 3G, 4B).

HRP1 overexpression suppresses read-through defects of cis-acting DEF1 attenuator mutants

Our identification of a pA site efficiency element (EE) and our characterization of the hrp1-1 mutant suggested that Hrp1 may recognize the DEF1 attenuator. To test whether Hrp1 binds to the DEF1 EE or other regions of the attenuator, we overexpressed HRP1 by
transforming cis-acting mutant strains with a plasmid version of HRP1 in addition to the chromosome. We chose a low-copy (CEN) HRP1 plasmid since we have previously observed that high-copy HRP1 expression from a 2μ plasmid is toxic (data not shown). We predicted that HRP1 overexpression would enhance attenuator recognition, reduce CUP1 reporter expression, and therefore increase copper sensitivity. As expected, the CUP1 reporter lacking a terminator (No Term.) was copper-resistant, the wild-type DEF1-CUP1 reporter was copper-sensitive, and cis-acting attenuator mutants conferred various degrees of copper resistance in the presence of the pRS314 empty vector control (Figure 7A). The A-1G, T-6C, and T-4C mutants were more copper-sensitive with copper resistance in the presence of the pRS314 empty vector control, consistent with improved binding of Hrp1 to the mutant region I and/or that region I influences Hrp1 binding at region II. The effect of HRP1 overexpression was allele-specific, with little-to-no genetic interaction observed between pRS314-HRP1 and the DEF1 attenuator mutants T+6C, A+16G, or A+43G in regions III and IV.

To confirm that increased copper-sensitivity was due to enhanced Pol II termination, we analyzed the ratio of attenuated:read-through transcripts by RT-PCR. As expected for enhanced attenuator recognition, HRP1 overexpression increased attenuated RNA ~twofold, resulting in less read-through mRNA compared to the empty vector control (Figure 7B). Taken together, our genetic analysis of the DEF1 attenuator indicates that region II contains the EE and region IV contains the CS (Figure 7C). Region III is likely to contain the PE, and the role of region I is unclear but its activity is influenced by Hrp1.

### Attenuator mutant results in DEF1 overexpression and exacerbates toxicity of constitutively active Def1

Thus far, our characterization of the DEF1 attenuator utilized a reporter gene construct, with the terminator positioned within an ACT1 intron and under transcriptional control of a constitutive TDH3 promoter. To study the DEF1 attenuator in a more natural context, we cloned the full-length DEF1 gene (promoter, 5′-UTR, ORF, 3′-UTR) into a high-copy 2μ plasmid (pRS426) and transformed it into a delΔ strain, where the plasmid was the sole source of DEF1. We designed measured DEF1 attenuator activity based on the level of mRNA accumulation, using primers that amplified read-through RT-PCR products extending beyond pA1 (Figure 8A).

Given that Def1 protein expression exhibits post-translational regulation (Wilson et al. 2013; David 2013), we sought to establish biological significance for our observed transcriptional regulation. To test the biological significance of the DEF1 attenuator in isolation from post-translational regulation we created a def1 C1590A allele, which introduces a mutation (C1590A) that results in a premature stop codon (TA$	ext{C}ightarrow$TA$\text{A}$) (Figure 8A). The truncated version of Def1 (pr-Def1) produced from def1 C1590A mimics a UV-dependent processing event (Wilson et al. 2013). At elevated temperatures, the pr-Def1 protein becomes activated via nuclear localization, triggers Pol II ubiquitination and degradation, and is toxic to cells. We predicted that attenuator mutations would elevate levels of DEF1 mRNA and pr-Def1 protein, resulting in greater cell toxicity.

<table>
<thead>
<tr>
<th>mutant strain</th>
<th>Pol II Termination Pathway</th>
<th>$\text{CYC1-lacZ}_{\text{m}/\text{WT}}$ ratio</th>
<th>$\text{DEF1-lacZ}_{\text{m}/\text{WT}}$ ratio</th>
<th>$\text{SNR13-lacZ}_{\text{m}/\text{WT}}$ ratio</th>
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<td>NNS</td>
<td>1.3</td>
<td>4.3</td>
<td>9.2</td>
<td>SNR13</td>
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**Figure 5** Summary of trans-acting mutant effects on DEF1 attenuator indicates similar behavior to CYC1 hybrid CPF-CF-NNS terminator. The relative level of terminator read-through in mutant/WT was calculated from lacZ assays in Figs. 3 and 4 and summarized in the table (little/no effect: ~twofold (pink); intermediate: 2-10 fold (yellow); strong: >10-fold (green)). Pol II termination pathways for each factor were assigned based on sensitivity of mutants to a known NNS-dependent terminator (SNR13) or hybrid CPF-CF-NNS-dependent terminator (CYC1). The asterisk indicates cases in which the kinetic slope of lacZ activity from the WT SNR13-lacZ reporter was undetectable, and an approximate value was utilized that was comparable to other experiments (40 B-gal units).
We analyzed the growth of yeast strains containing an attenuator mutation (def1 A-1G) in the context of pr-Def1 expression (def1 C1590A). At the permissive temperature of 30°C, all strains grew at a similar rate and density (Figure 8C). As expected, the def1 C1590A mutation resulted in heat-sensitivity at 37°C and 39°C, similar to the def1 D strain with an empty vector (Wilson et al. 2013). Transformation of def1 D with pRS426-DEF1 restored growth to wild-type levels, consistent with full complementation by the plasmid-based DEF1 allele. The def1 A-1G attenuator mutant grew similarly to wild-type, presumably because transcriptional overexpression was masked by post-translational protein control. Interestingly, the def1 A-1G/C1590A double mutant exhibited a synthetic sick phenotype at the non-permissive temperature of 37°C. To better quantify this genetic interaction, we determined the growth rate of strains in liquid culture. The pRS426-DEF1 strain had a doubling time of 2.2 hr and 2.5 hr, at 30°C and 39°C respectively. In contrast, the def1 C1590A mutant had a doubling time of 3.0 hr and 5.3 hr, consistent with a heat-sensitive defect. The def1 A-1G/C1590A mutant had a doubling time of 3.0 hr and 7.9 hr, exhibiting a more severe growth defect at 39°C than the def1 C1590A single mutant.

To confirm that def1 A-1G enhanced def1 C1590A toxicity due to pr-Def1 overexpression, we quantified mRNA and protein levels. The def1 A-1G mutation increased mRNA expression by 1.6 compared to
def1 C1590 alone (Figure 8D). This mRNA overexpression correlated with ~twofold more Def1 protein in def1 A-1G/C1590A compared to def1 C1590A (Figure 8E). Overall, these data are consistent with Pol II attenuation contributing to DEF1 regulation in yeast and serving an important biological function.

**DISCUSSION**

Termination is one of the least understood aspects of Pol II transcription, and a further knowledge gap exists for regulation by premature termination (attenuation). In this study we conducted a thorough characterization of an attenuator in the DEF1 DNA repair gene, which bears unique features unseen in previous attenuator studies. The DEF1 attenuator relies on a hybrid of termination factors for efficient recognition, with a bias for the CPF-CF pathway vs. the NNS pathway. We have identified nine termination factors and four RNA sequence elements that contribute to attenuator activity, including the Hrp1 RNA-binding protein, a putative pA site efficiency element, and the region around the pA site itself. Furthermore, we have shown that disruption of the attenuator is biologically significant, supporting a new role for transcription attenuation in regulating a DNA damage response gene.

**The DEF1 attenuator exhibits a unique hybrid attenuator biased toward CPF-CF termination**

Our mutational analysis indicates that DEF1 attenuator recognition involves both CPF-CF and NNS termination pathways, but it is more reliant on CPF-CF recognizing a traditional pA site (Figure 6A; regions II, III, and IV). The importance of DEF1 attenuator region I is unclear, but it may influence Hrp1 binding to region II, perhaps by forming a secondary structure. The DEF1 attenuator exhibits Sen1-dependence but little-to-no dependence on Nrd1 or Nab3 despite sequence
similarity to consensus Nrd1 (GUAA, GUAG) and Nab3 RNA-binding sites (UCUU) (Steinmetz and Brow 1998; Carroll et al. 2004; Creamer et al. 2011; Porrua et al. 2012). The very limited role for Nrd1 and Nab3 at the DEF1 attenuator is a contrast to what has been observed for most other attenuators, including NRD1, IMD2, URA2, FKS2, CLN3, GPH1, and GLT1, which exhibit strong dependence on Nrd1, Nab3, or both (Arigo et al. 2006; Jenks et al. 2008; Kuehner and Brow 2008; Thiebaut et al. 2008; Kim and Levin 2011; Darby et al. 2012; Chen et al. 2017; Steinmetz and Brow 1998; Carroll et al. 2004; Creamer et al. 2011; Porrua et al. 2012).

Figure 8 The def1 A-1G attenuator mutant increases Def1 mRNA and protein and reduces cell viability when overexpressing pr-Def1. (A) Schematic of the DEF1 gene (not to scale). The relevant pA sites, efficiency element (EE), mutations (A-1G, C1590A), and RT-PCR primers are indicated. (B) Yeast strains containing chromosomal (CHR) DEF1 or def1Δ were transformed with empty vector (pRS426), WT DEF1 (pRS426-DEF1), or mutant (pRS426-DEF1, A-1G) plasmids. Total RNA was isolated, and Def1 read-through mRNA was detected via RT-PCR (using blue primers indicated in (A). The signal intensity of the DEF1 mRNA bands was normalized to the 18S loading control and then the def1Δ pRS426-DEF1 sample. RT: Reverse Transcriptase. (C) Yeast strains containing WT chromosomal DEF1 or def1Δ were transformed with empty vector (pRS426), WT DEF1 (pRS426-DEF1), or attenuator mutant (pRS426-DEF1-A-1G), pr-Def1 mutant (pRS426-DEF1-C1590A), or double mutant plasmids. Strains were spotted on -Ura plates and growth was assessed after 1 week at the indicated temperatures. (D) Total RNA was collected from strains in (C) containing def1 mutants C1590A and C1590A/A-1G in a def1Δ strain grown at 30°C. DEF1 read-through mRNA was detected via RT-PCR and quantified as in (B). (E) Western blot of extracts from strains in (C) following growth at 30°C and a temperature shift to 39°C for 0, 1, or 2 hr. Def1 protein levels were normalized to the actin loading control, and signal from def1 C1590A/A-1G was normalized to def1 C1590A at consistent time points.
Merran and Corden 2017). The lack of Nrd1/Nab3 involvement that we observe is consistent with DEF1 expression levels not increasing upon Nrd1/Nab3 depletion and Nrd1/Nab3 failing to crosslink to DEF1 during in-vivo crosslinking studies (Jamonnak et al. 2011; Merran and Corden 2017).

DEF1 attenuator recognition is dependent on CFI component Hrp1 rather than Nrd1 or Nab3, which is somewhat surprising given its promoter-proximal location. In the reporter system, the TSS to EE distance is 365 bp, but this distance is only ~100 bp in the natural DEF1 context, and we observe a termination defect in both cases with the def1 A-1G mutant. At individual genes, Hrp1 has been shown to crosslink to coding regions ~twofold better than the promoter, and in some cases Hrp1 shows the strongest occupancy at the pA site near the 3’-ends of genes (Komarnitsky et al. 2000; Mayer et al. 2010). However, Hrp1 is proposed to bind within its own 5’-UTR as a mean of autor-regulation, suggesting that it can act near gene promoters (Steinmetz et al. 2006b; Kuehner and Brow 2008; Chen et al. 2017). Furthermore, genome-wide crosslinking indicated that the majority of Hrp1 is bound to promoter-proximal regions of mRNAs (Tuck and Tollervey 2013), and transcriptome analysis in an hrp1 mutant revealed sn/snoRNA termination defects on approximately one-third of the sn/snoRNA genes (Chen et al. 2017). Overall, these data indicate that Hrp1 acts more generally as both a CPF-CF and an NNS termination factor.

Another unique feature of the DEF1 attenuator is its seeming lack of dependence on Pcf11. None of the pcf11 mutants altered DEF1 attenuator recognition despite the termination defects they exhibited for the SNR13 control terminator. At other gene targets, the pcf11-2 mutation impairs mRNA cleavage, the pcf11-13 mutation impairs Pol II CTD-binding, and pcf11-9 impairs both cleavage and Pol II CTD-binding. Accordingly, these mutants disrupt CPF-CF, NNS, or both termination pathways (Amrani et al. 1997; Sadowski et al. 2003; Kim et al. 2006; Grzegorczyk et al. 2015). The termination defect of pcf11-13 corresponds with failed release of Nrd1, reduced Ser2 CTD phosphorylation, and lack of Sen1 recruitment (Grzegorczyk et al. 2015). Seemingly the DEF1 attenuator does not require this Pcf11 function to elicit termination, perhaps because the Nrd1 and Nab3 proteins are not required either.

We have confirmed that DEF1 attenuator recognition requires Hrp1, Rna14, Rna15, Ssu72, Cik1, Glc7, Ctf2, Paf1, and Sen1. Like Hrp1, several of these proteins may contribute to DEF1 pA1 site recognition. Rna15 and Rna14 are also members of CFI, and while Rna15 recognizes pA site positioning elements, Rna14 is capable of bridging Rna15 and Hrp1 in a CFI complex (Gross and Moore 2001; Barnwal et al. 2012). Ctf2 is a component of the core CPF that binds the CYC1 pA site in vitro and crosslinks near pA sites in vivo, as well as interacting with the Pol II CTD (Dichtl and Keller 2001; Kyiburz et al. 2003; Baeven et al. 2014).

In lieu of direct DEF1 pA1 site recognition, some proteins may promote recruitment of termination factors. Ctk1 is a kinase that phosphorylates Ser2 residues of the Pol II CTD, an event that can occur relatively early in transcription and perhaps lead to promoter-proximal termination via recruitment of Sen1 (Mayer et al. 2010; Chinchilla et al. 2012; Lenstra et al. 2013). Glc7 and Ssu72 are components of the APT sub-complex of CPF, also termed the phosphatase module, which are required for termination of both mRNA and noncoding RNA genes (Mayfield et al. 2016; Casañal et al. 2017). Removal of Ser5-P from the Pol II CTD occurs via Ssu72, which is present at both the 3′-end of genes as well as promoter regions (Singh and Hampsey 2007; Zhang et al. 2012). Defects in Ssu72 increase Ser5-P, which may disrupt Ser2-P accumulation and Sen1:Pol II association. Glc7 promotes the removal of Tyr1-P from the Pol II CTD, and failure to remove Tyr1-P prevents recruitment of termination factors, including Nrd1, Pcf11, and Rti103 (Mayer et al. 2012; Schreieck et al., 2014). Paf1 has been implicated in both CPF-CF and NNS termination, and paf1 mutants exhibit altered histone modification and reduced Ser2-P levels (Sheldon et al. 2005; Nordick et al. 2008; Tomson et al. 2011; Terzi et al. 2011). There is also precedence for Paf1 helping to recruit CPF to Ser5-P CTD, consistent with Paf1:Pol II enrichment on transcripts containing CPF proteins Ctf2 and Mep1 (Nordick et al. 2008; Fischl et al. 2017).

Sen1 may contribute most directly to termination at the DEF1 attenuator via ATP-dependent RNA translocation and destabilization of paused Pol II, perhaps via melting of the RNA:DNA active site hybrid (Kim et al. 1999; Porrúa and Libri 2013; Martin-Tumasz and Brow 2015; Han et al. 2017; Leonaitė et al. 2017). Given the limited role of Nrd1 and Nab3 in DEF1 attenuation, Sen1 recruitment could occur instead through CPF component Glc7 or direct interaction with Ser2-P CTD, or connecting to CFI protein Hrp1 via CFI-CPF cross-factor interactions (Preker et al. 1995; Ohnacker et al. 2000; Kyburz et al. 2003; Holbein et al. 2011; Ghazy et al. 2012; Chinchilla et al. 2012).

Regulation of DEF1 by dual transcriptional and post-translational mechanisms

Our evidence suggests that Pol II transcriptional attenuation contributes to biologically meaningful DEF1 regulation in addition to a previously described post-translational mechanism (Wilson et al. 2013). Constitutive expression of a truncated protein that mimics Def1 activation is lethal to cells (Wilson et al. 2013), consistent with tight control of Def1 expression being vital for cell survival. DEF1 expression and function contributes to several biological processes, including rescue of stalled Pol II, nucleotide excision repair, telomere maintenance, translesion synthesis, and Pol II initiation (Woudstra et al. 2002; Chen et al. 2005; Daraba et al. 2014; Damodaren et al. 2017). In addition, Def1 contributes resistance to DNA damage stress, salt stress, and heat shock stress (Woudstra et al. 2002; Vanaclocha-Pedros et al. 2015; Damodaren et al. 2017). Given that both Def1 depletion and overexpression can be lethal, cells likely evolved multiple regulatory mechanisms to maintain DEF1 homeostasis. An advantage of Pol II attenuator read-through vs. upregulation of initiation is that it could provide a more rapid response to an environmental stressor, akin to release of paused Pol II (Adelman and Lis 2012).

There is limited evidence to explain the mechanism by which Pol II attenuator read-through occurs for stress response genes. In the case of IMD2, depletion of the guanine nucleotide pool causes Pol II to shift from an upstream guanine TSS to a downstream adenine TSS, bypassing an NNS-dependent attenuator and allowing synthesis of full-length mRNA (Steinmetz et al. 2006b; Jenks et al. 2008; Kuehner and Brow 2008). The Levin and Manley labs have described an alternative mechanism for regulation of the FKS2 attenuator. Cell wall damage activates the MAP kinase Mpk1, which associates with Pol II and Paf1, phosphorylates Tyr1 of the Pol II CTD, restricts Nrd1 recruitment, and allows Pol II to bypass an attenuator and terminate at a downstream pA site (Kim and Levin 2011; Yurko et al. 2017). We have identified several characteristics that are unique to DEF1 vs. IMD2 and FKS2 attenuators, perhaps reflecting a novel mechanism for Pol II recognition and read-through. DEF1 will be a useful model to expand our understanding of the signaling mechanisms that modulate Pol II termination, particularly during stress adaptation to DNA damage. It will also be of interest to explore connections between yeast Pol II attenuator function and alternative polyadenylation (APA) in metazoans. Splicing plays a dominant role in usage of promoter-proximal intronic pA sites, but upstream exonic pA sites remain relatively unexplored (Luo et al. 2013; Tian and Manley 2017).

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