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Stimulation of RNA Polymerase II ubiquitination and degradation by yeast mRNA 3′-end processing factors is a conserved DNA damage response in eukaryotes

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ABSTRACT

The quality and retrieval of genetic information is imperative to the survival and reproduction of all living cells. Ultraviolet (UV) light induces lesions that obstruct DNA access during transcription, replication, and repair. Failure to remove UV-induced lesions can abrogate gene expression and cell division, resulting in permanent DNA mutations. To defend against UV damage, cells utilize transcription-coupled nucleotide excision repair (TC-NER) to quickly target lesions within active genes. In cases of long-term genotoxic stress, a slower alternative pathway promotes degradation of RNA Polymerase II (Pol II) to allow for global genomic nucleotide excision repair (GG-NER). The crosstalk between TC-NER and GG-NER pathways and the extent of their coordination with other nuclear events has remained elusive. We aimed to identify functional links between the DNA damage response (DDR) and the mRNA 3′-end processing complex. Our labs have previously shown that UV-induced inhibition of mRNA processing is a conserved DDR between yeast and mammalian cells. Here we have identified mutations in the yeast mRNA 3′-end processing cleavage factor IA (CFIA) and cleavage and polyadenylation factor (CPF) that confer sensitivity to UV-type DNA damage. In the absence of TC-NER, CFIA and CPF mutants show reduced UV tolerance and an increased frequency of UV-induced genomic mutations, consistent with a role for RNA processing factors in an alternative DNA repair pathway. CFIA and CPF mutants impaired the ubiquitination and degradation of Pol II following DNA damage, but the co-transcriptional recruitment of Pol II degradation factors Elc1 and Def1 was undiminished. Overall these data are consistent with yeast 3′-end processing factors contributing to the removal of Pol II stalled at UV-type DNA lesions, a functional interaction that is conserved between homologous factors in yeast and human cells.

1. Introduction

The biological fitness of an organism depends on the quality and accessibility of its genetic information, which is frequently subjected to internal and external damage. On a daily basis, DNA damage from endogenous cell metabolism and exogenous sources like ultraviolet (UV) radiation can total 10^5 lesions/cell [1]. To preserve genomic integrity, eukaryotic cells rely upon a DNA Damage Response (DDR) that coordinates changes in gene expression with recognition and repair of DNA lesions. As evidenced by numerous genetic diseases, an ineffective DDR can result in neurological disorders, developmental impairments, immunodeficiency, progeria, and cancer [2].

Nucleotide excision repair (NER) plays a crucial role in the DDR since it targets lesions that impede transcription and replication [3]. NER targets a wide variety of DNA damage because it can recognize helix-distorting lesions such as UV-induced cyclobutane pyrimidine dimers (CPDs) as well as bulky DNA adducts that arise from cigarette smoke, the UV-mimetic 4-nitroquinoline 1-oxide (4-NQO), and the chemotherapeutic drug cisplatin [1]. NER is divided into two sub-categories that prioritize repair of genomic regions based on their activity. Transcription-coupled repair (TC-NER) quickly targets DNA lesions from transcribed strands of active genes. Global Genome Repair (GG-NER) responds more slowly and targets lesions in non-transcribed DNA and the non-transcribed strand of active genes [4].

The expeditious response of TC-NER in comparison to GG-NER is due to RNA Polymerase II (Pol II) acting as a direct sensor of damage since Pol II stalls at DNA-distorting lesions. Pol II stalling at a CPD impedes expression of essential genes and prevents access of NER to the damage site. TC-NER is proposed to relieve this obstacle by promoting Pol II backtracking, which provides access of repair factors to the lesion

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[5]. TC-NER in yeast requires the parallel activities of Rad26, a DNA-dependent ATPase; Rpb9, a non-essential Pol II subunit; and Sen1, an ATP-dependent 5′–3′ helicase [6]. Mutations in the human Rad26 homolog CSB cause Cockayne Syndrome, a disorder typified by premature aging and photosensitivity. Mutations in the human Sen1 homolog Senataxin cause ataxia oculomotor apraxia type 2 (AOA2), which is characterized by developmental impairments and sensitivity to DNA damaging agents [7].

If TC-NER fails to resolve stalling, Pol II can be ubiquitinated and targeted for proteasomal degradation *via* a "last resort" mechanism, which provides GG-NER factors with access to the lesion [8]. DNA damage-dependent Pol II degradation requires two distinct sequentially-acting ubiquitin ligase complexes that are conserved from yeast through humans [9]. In yeast, monoubiquitination of the Pol II Rpb1 subunit is promoted by Uba1, Ubc4/5, and Rsp5, which act as the E1 activating enzyme, the E2 conjugating enzyme, and the E3 ubiquitin ligase, respectively. Subsequent addition of polyubiquitin chains to Rpb1 is mediated by Def1 and an E3 ligase composed of Elc1, Ela1, Cul3, and Rbx1. Human cells utilize a similar pathway carried out by a Nedd4, Elongin A/B/C-Cul5-Rbx2 complex [9]. Pol II can also be stalled independently of DNA damage if the processivity of transcription elongation is reduced by nutrient deprivation or treatment with 6-azauracil (6-AU), a drug that depletes NTP levels. Pol II stalling in the absence of DNA damage likewise leads to Rpb1 ubiquitination and degradation by a distinct yet overlapping pathway [10].

The "last resort" pathway for DNA repair must be tightly controlled to prevent inappropriate Pol II degradation. While core components of the ubiquitin ligase machinery have been reconstituted *in vitro* [11], it is not known how Pol II is specifically targeted for degradation at the right place and time *in vivo*. Rsp5 preferentially binds the hyperphosphorylated form of the Pol II CTD, but it is not clear how stalled Pol II is differentiated from elongating Pol II [12,9]. Furthermore, the cross-talk between NER, the "last resort" pathway, and other co-transcriptional events has not been clearly defined.

It is likely that co-transcriptional events help to coordinate the DDR and alleviate Pol II stalling at sites of DNA damage. NER and the "last resort" pathway have previously been linked to factors that promote Pol II transcription efficiency and mRNA 3′-end processing. Transcription elongation and nuclear export mutants show defects in TC-NER [13,14], and mRNA 3′-end processing mutants impede the DDR and cell cycle progression [15]. UV damage inhibits mRNA 3′-end processing in mammalian cells, perhaps as a means to prevent premature release of harmful transcripts [16]. Normally the cleavage stimulatory factor CstF-50 interacts with the Pol II CTD to promote RNA 3′-end processing, but UV-induced inhibition is caused by a transient interaction of CstF-50 with the Bard1 tumor suppressor protein [17]. Furthermore, depletion of CstF-64 enhances UV-sensitivity, reduces Pol II degradation and ubiquitination, and causes a delay in TC-NER [18].

Our labs have demonstrated that UV-induced inhibition of RNA processing is a conserved response between yeast and higher eukaryotes, and UV-type damage induces genome-wide variation in yeast polyadenylation sites [19]. In the current study, we sought to identify additional functional links between mRNA 3′-end processing factors and the DDR. We identified mutations in yeast Cleavage Factor IA (CFIA) and Cleavage and Polyadenylation Factor (CPF) that conferred sensitivity to the UV-mimetic drug 4-NQO. The UV-sensitivity and UV-induced genomic mutation rate of RNA processing mutants was exacerbated in the absence of TC-NER, supporting a broader role for both CFIA and CPF in an alternative repair pathway. CFIA and CPF mutants were defective in ubiquitination and Pol II degradation following UV-type damage, but recruitment of Def1 and the ubiquitin ligase Elc1 was not affected. Overall, these data support a role for 3′-end processing factors in the DDR by removing Pol II stalled at UV-type lesions, a functional interaction that is conserved between yeast and mammalian cells.

2. Methods

2.1. Yeast strains and plasmids

The *RNA15*, *rna15-1*, *PFS2*, *pfs2-1, BRR5, brr5-1, PAP1, and pap1-1* yeast strains were kind gifts of the LaCroute, Keller, Guthrie, and Butler labs [20–23]. The *rad14Δ* mutation was introduced by high-efficiency transformation and homologous recombination with a *rad14:KANMX* PCR product. pRS316-RNA15 was generated by amplifying the region −446 to +1306 (relative to +1 ATG) using PCR primers containing Sal1/Not1 restriction enzyme sites. pRS315-RNA15 was generated through sub-cloning and pRS315-*rna15-1* (L214P) was generated by Quick-change mutagenesis (Agilent). pFL36-PFS2 and pFL36-*pfs2-1* were rescued from the yeast strains above, and pFL38-*PFS2* was generated by subcloning. The BY4742 *rna15Δ* pRS316-*RNA15* and BY4742 *pfs2Δ* pFL38-*PFS2* shuffle strains were generated by high-efficiency transformation and homologous recombination with an *rna15:NATMX6* or *pfs2:NATMX6* PCR product [24]. After overnight growth on YPAD, plates were replica-plated to YPAD + nourseothricin plates (100 μg/mL). Additional disruptions for *rad7Δ*, *rad26Δ*, *rpb9Δ*, and *elc1Δ* were generated as described above using *rad7:KANMX, rad26:HIS3MX*, *rpb9:KANMX*, and *elc1:HPH6MX* PCR products and selection on −His plates or YPAD plates +G418 or hygromycin (200 μg/mL). All mutant strains were confirmed by diagnostic PCR and plasmids were confirmed by sequencing analysis. The pRS424-Myc-Def1 plasmid was a kind gift from the Svejstrup lab [25]. The pRS313-Myc-Def1 plasmid was generated by PCR cloning, and the *ELC1* ORF was used to replace *DEF1* using Gibson Cloning Assembly (NEB). The *RNA15 elc1Δ*, *rna15-1 elc1Δ, RNA15 def1Δ,* and *rna15-1 def1Δ* strains were generated by high-efficiency transformation and homologous recombination with *elc1:KANMX6* or *def1:KANMX6* PCR products and transformed with pRS313-Myc-Def1 or pRS313-Myc-Elc1 for ChIP assays.

2.2. Yeast growth, viability, and mutagenesis

Yeast strains were grown overnight in appropriate media and diluted to OD_{600} = 1.0. Additional 10-fold dilutions were prepared in a 96 well plate prior to using a replica pin plater to spot cultures onto agar plates. YPAD + 4NQO, 5-FOA, or synthetic complete plates were prepared a few days before use, and UV treatment was performed in a Stratalinker UV crosslinker box set to the indicated energy mode. Plates were incubated at 30 °C for several days after treatment. UV-treated plates were wrapped in foil and kept in the dark during the incubation. For viability assays, a saturated overnight culture was plated on YPAD + 4-NQO media, and dilutions of the culture were plated on YPAD without drug to determine cell survival based on colony counting. After 3 days of growth at 30 °C, the percent survival was calculated by dividing the colony number from YPAD + 4NQO plates by the total number of colonies on YPAD without drug (after accounting for dilution factor). For genomic mutation experiments, a saturated overnight culture was adjusted to a similar cell density and plated on SC-Arg + canavanine plates (60 μg/mL), and dilutions of the culture were plated on SC plates. After UV treatment and 7 days of growth in the dark at 30 °C (for *elc1Δ*) or 34 °C (to enhance *rna15-1* temperature sensitivity), the mutation frequency was calculated by dividing the colony number from SC-Arg + canavanine plates by the total number of colonies plated on SC plates (after accounting for dilution factor and plating volume).

2.3. Immunoprecipitation and western blot

Yeast strains were grown in YPAD until reaching exponential phase and treated with 4-NQO (8 μg/mL) or only the 100% ethanol solvent for 2 h. Alternatively, yeast strains were washed with 1X PBS and irradiated with 300 J/m^2 in a Stratalinker crosslinker prior to resuspension in pre-warmed YPAD media and recovery in a dark flask with shaking for 2 h at 30 °C (harvesting at 15, 30, 60, and 120 min). Cell pellets

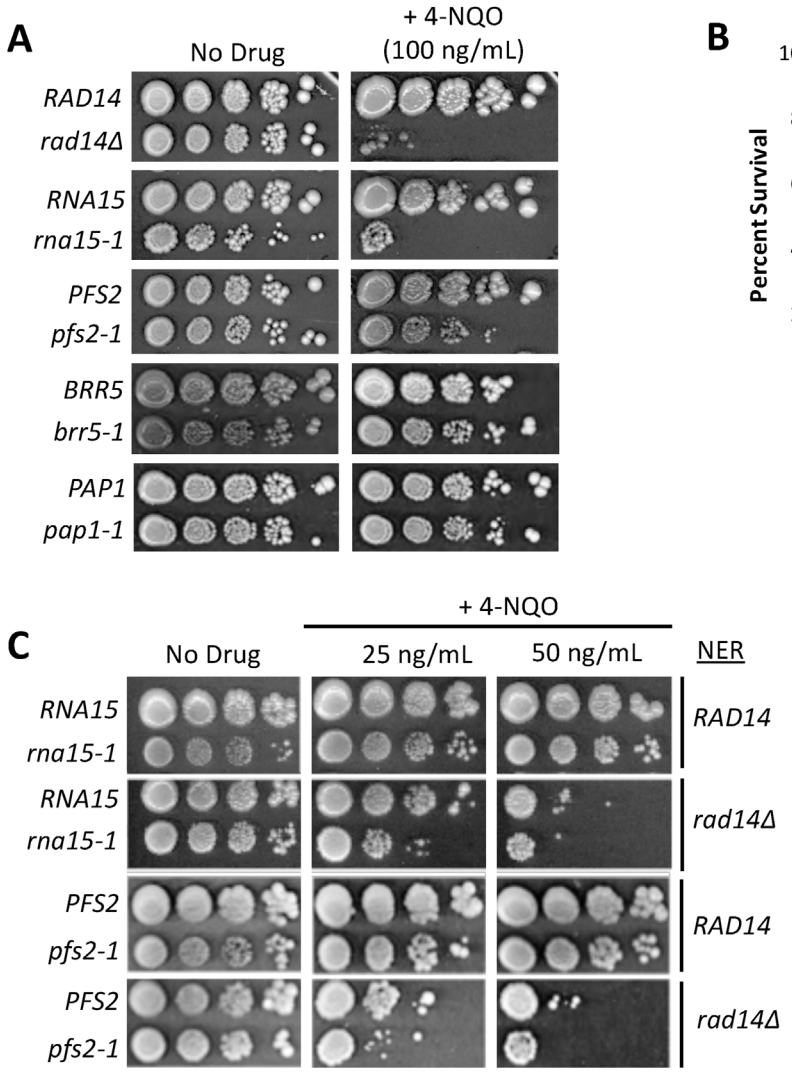

Fig. 1. RNA 3′-end processing factors CFIA (Rna15) and CPF (Pfs2) contribute to the survival of cells exposed to UV-type DNA damage. (A) Serial dilutions of liquid yeast cultures (W303 background) were spotted onto YPAD media plates containing no drug or the UV-mimetic drug 4-NQO (100 ng/mL) and strains were grown at 30 °C for 3 days. The *rad14Δ* mutant inactivates nucleotide excision repair (NER) and serves as a positive control for UV-sensitivity. (B) Saturated yeast liquid cultures were plated on YPAD containing 4-NQO (25 ng/mL for *RAD14/rad14Δ* and *RNA15/rna15-1* or 50 ng/mL for *PFS2/pfs2-1*) and diluted cultures were plated on YPAD without drug. After 3 days of growth at 30 °C the percent survival was calculated by dividing the colony number by the total number of cells plated (as determined from diluted cultures). Error bars show standard deviation of 3 biological replicates. (C) Serial dilutions of liquid yeast cultures were spotted onto YPAD −/+ 4NQO media plates (25 or 50 ng/mL) and strains were grown for 3 days at 30 °C.

were washed with 1X PBS and whole cell protein extracts were prepared using a TCA method [26]. Briefly, cell pellets of 5–10 OD_{600} units were resuspended in 250 µL of 20% TCA. Cells were lysed using an equal volume of glass beads and a vortexer in a 4 °C room (3 × 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 15,000 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. An additional 80 µL of 2X SDS sample buffer was added, and the sample was heated to 95 °C for 5 min, microcentrifuged at top speed for 5 in., and the supernatant was used for Western blot analysis. Western blots were incubated with mouse monoclonal anti-Rpb1 8WG16 antibody (1:5000; SCBT) and anti-actin antibody (1:2000, Abcam) prior to incubation with HRP-conjugated secondary antibody and detection *via* chemiluminescence.

Immunoprecipitation and assessment of Rpb1 ubiquitination was performed as previously described [27]. 50 mL yeast cultures were grown to OD600 = 1.0 and treated with 4-NQO (5 µg/mL) or left untreated (added 100% ethanol solvent) for 30 min in 30 °C shaker. Cultures were harvested and pellets were rinsed with 1X PBS prior to storage at −80 °C. Frozen pellets were resuspended in 500 µL chilled lysis buffer (150 mM NaCl, 50 mM Tris-Cl pH 7.4, 1 mM EDTA pH 8.0, 1 mM EGTA pH 8.0, 0.4 mM Na_4VO_3, 10 mM NaF, 10 mM $Na_4P_2O_7$, 1% Triton-X 100, 0.5% NP-40, 1X protease inhibitors (Roche), 0.2 mM PMSF) and incubated on ice for 5 min. Each sample was further supplemented with 20 µL of 7X protease inhibitors (Roche), 8 µL 10% SDS, 10 uL MG132 (10 mM), 10 uL 0.1 M PMSF, and 10 µL of phosphatase inhibitors 1 M NaF and 0.1 M $Na_4P_2O_7$). Cells were lysed by vortexing in 4 °C (4 × 5 min), with 2 min pauses on ice between runs. After the second run, the lysis buffer was refreshed with protease inhibitors, MG132, PMSF, and phosphatase inhibitors. The supernatant was removed using a gel-loading tip to avoid the glass beads and spun at 15,000 RPM in a microcentrifuge for 10 min at 4 °C. Extracts were quantified by Bradford assay, and 2 mg of total extract was used in an immunoprecipitation reaction refreshed with protease inhibitors, MG132, PMSF, and phosphatase inhibitors as done above. In addition, 5 µL of anti-Rpb1 antibody was added (8WG16; 5 ug/uL; Neoclone),

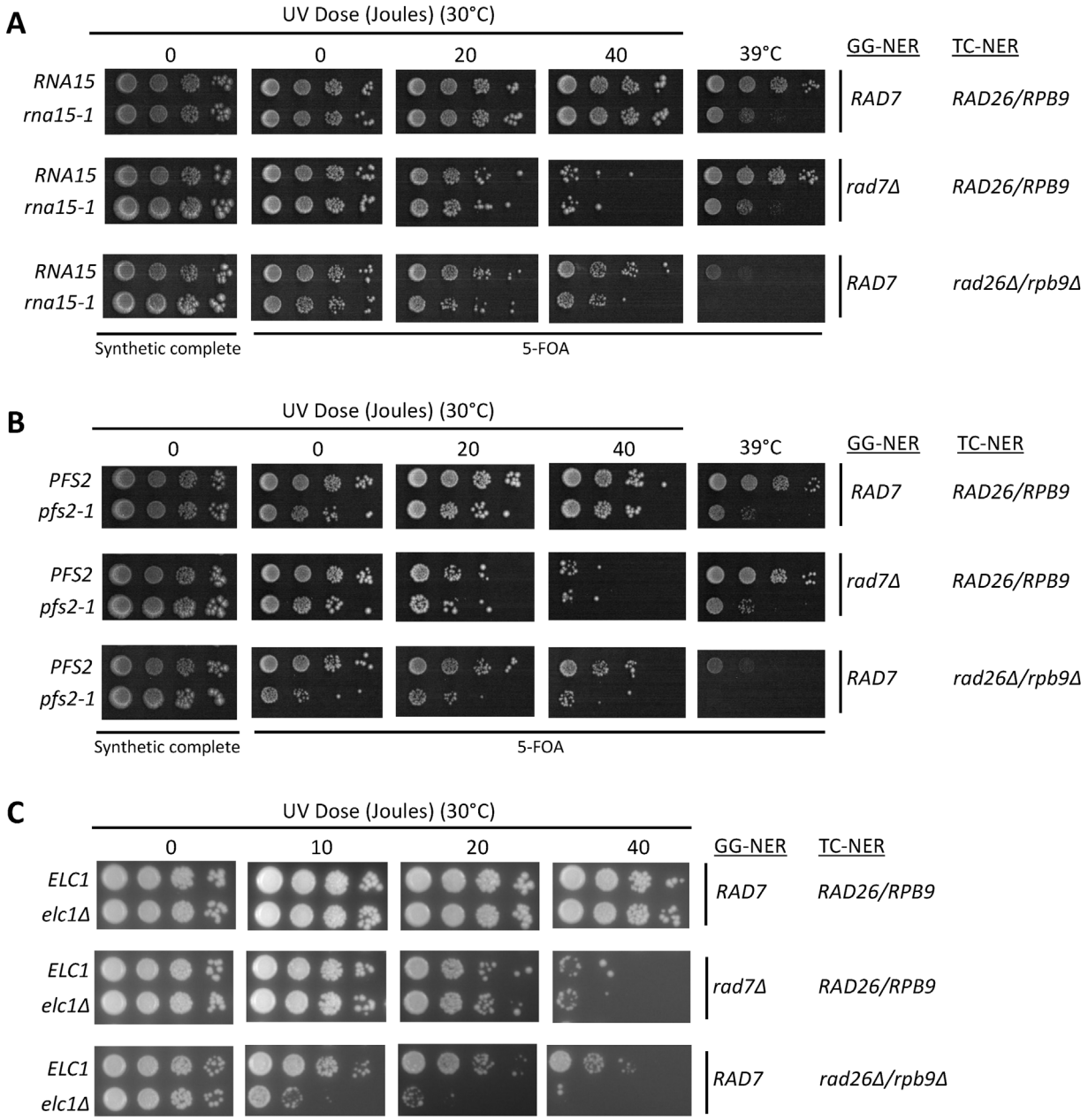

Fig. 2. Mutations in CFIA (*rna15-1*) and CPF (*pfs2-1*) increase UV sensitivity in the absence of transcription-coupled nucleotide excision repair (TC-NER). Serial dilutions of liquid yeast cultures (BY4742 background) were spotted onto synthetic complete and/or 5-FOA media plates, irradiated with varying doses of UV light, and incubated at 30 °C and 39 °C in the dark for 5 days. The *rad7Δ* and *rad26/rpb9Δ* mutants were used to selectively inactivate global genome repair (GG-NER) and transcription-coupled repair (TC-NER), respectively. The 39 °C temperature confirmed the selectivity of the 5-FOA plates and expected temperature sensitivity of *rna15-1* and *pfs2-1* mutants. Elc1 has previously been shown to contribute to GG-NER [30], and the *elc1Δ* mutation was used as a reference control strain.

and the immunoprecipitation was incubated overnight on a rotator at 4 °C. The following day, 60 µL of Protein A-agarose bead suspension was added and incubated for 3 h at 4 °C. Immunoprecipitates were collected by centrifugation at 2500 RPM for 5 min, followed by 2X washes with ice-cold lysis buffer (high stringency with 500 mM NaCl and 0.1% SDS) and 2X washes with ice-cold lysis buffer (low stringency with 0.1% SDS), each time repeating the centrifugation above. After the final wash, the supernatant was discarded and the pellet was re-suspended in 20 µL of 2X SDS sample loading buffer. Samples were heated to 95 °C for 10 min, microcentrifuged at top speed for 5 in., and the supernatant was used for Western blot analysis. Western blots were incubated with mouse monoclonal anti-ubiquitin antibody (1:500; Enzo Life Sciences – ADI-SPA-203), anti-Rpb1 8WG16 antibody (1:5000; SCBT), anti-Rad 53 antibody (1:5000; Durocher lab), and anti-actin antibody (1:2000, Abcam) prior to incubation with HRP-conjugated secondary antibody and detection *via* chemiluminescence.

2.4. Chromatin immunoprecipitation (ChIP) and quantitative PCR (qPCR)

Strains were grown to OD_{600} = 0.8 and either treated with 4-NQO (1.25 µg/mL) or left untreated (added 100% ethanol solvent) for 30 min. Cells were fixed with 1% formaldehyde for 15 min and the remaining procedure was performed as described previously using anti-Rpb1 (4H8; SCBT) and anti-Myc (SCBT) antibodies for immunoprecipitation [28].

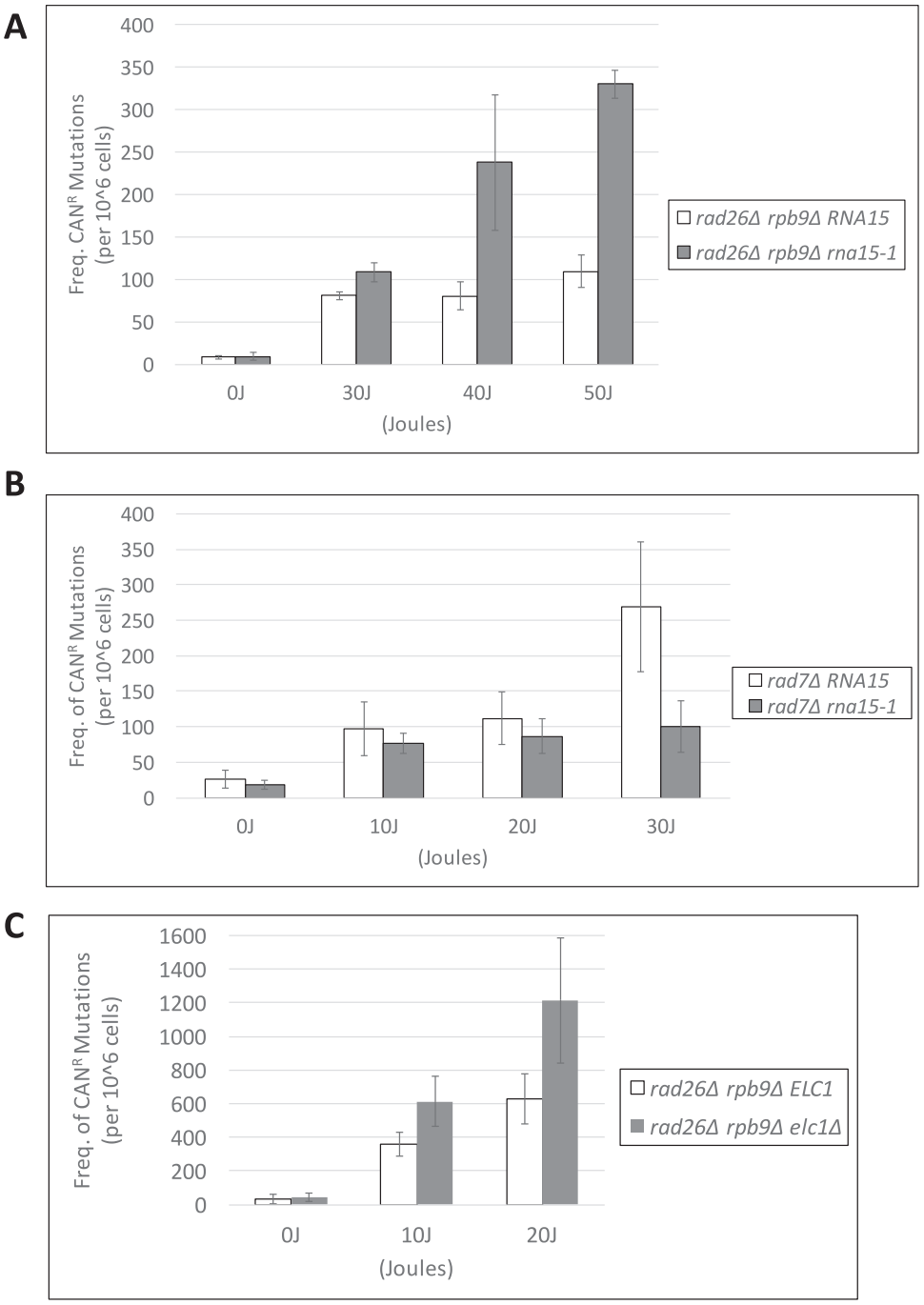

Fig. 3. The *rna15-1* mutation (CFIA) increases the frequency of UV-induced genomic mutation in the absence of TC-NER. The indicated strains (BY4742 background) were grown in liquid culture until saturation and either plated directly on canavanine plates or diluted and plated on synthetic complete plates. Plated cells were irradiated with varying doses of UV light and incubated in the dark for 7 days. The frequency of canavanine-resistance mutations was determined by dividing the number of colonies on canavanine plates by the number of colonies on synthetic complete plates, after normalizing for dilution. Elc1 has previously been shown to contribute to GG-NER, and the *elc1Δ* mutation was used as a reference control strain. Error bars show standard deviation of 3 biological replicates.

3. Results

3.1. RNA 3′-end processing complexes CFIA and CPF contribute to survival of cells exposed to UV-type DNA damage

To identify additional functional links between 3′-end processing factors and the DDR, we tested a variety of CFIA and CPF mutants for sensitivity to the UV-mimetic drug 4-Nitroquinoline 1-oxide (4-NQO). Since most RNA 3′-end processing factors are encoded by essential genes, they have not been reported in previous drug screens of the yeast deletion collection. Thus, we took advantage of temperature-sensitive mutants with known processing defects. We observed that mutations in both CFIA (*rna15-1*) and CPF (*pfs2-1*) reduced growth in the presence of 4-NQO as measured by a serial dilution spot test assay (Fig. 1A). When cell viability was quantified by colony counting, the *rna15-1* and *pfs2-1* mutants exhibited 19- to 6-fold reduced survivorship compared to their wild-type counterpart strains (Fig. 1B). However, not all processing mutants were affected by 4-NQO, suggesting that DNA damage sensitivity is not simply due to reduced 3′-end processing of mRNAs necessary for the DDR. For example, mutants that impair endonucleolytic cleavage (*brr5-1*) or polyA polymerase activity (*pap1-1*) were not 4-NQO sensitive (Fig. 1A). The yeast proteins Rna15 and Pfs2 are homologous to CstF-64 and CstF-50, which have been implicated in mammalian DNA repair [16]. The *rna15-1* and *pfs2-1* yeast mutants were analyzed further to determine the underlying basis of their 4-NQO sensitivity.

Nucleotide excision repair (NER) is the main pathway used by cells to remove bulky lesions, such as those formed by UV light or the UV-mimetic 4-NQO. If NER is not adequately performed, Pol II can become stalled at the lesions, which prevents transcription of essential genes. To

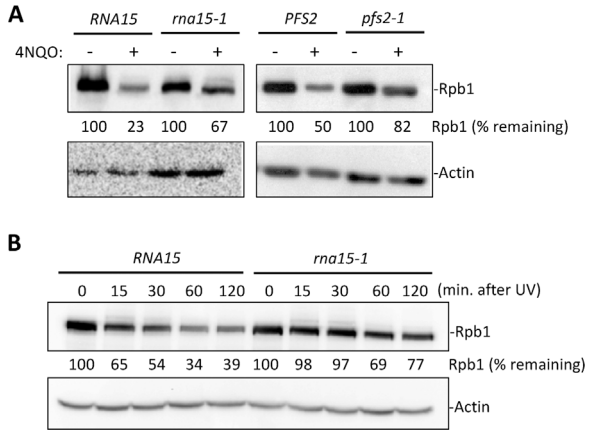

Fig. 4. CFIA and CPF RNA processing factors are required for efficient Pol II degradation following UV-type DNA damage. (A) Immunoblotting was performed using extracts from indicated strains (W303 background) grown at 30 °C and treated with A) −/+ 4 NQO (8 ug/mL) for 2 h or B) + UV (300 J/m^2) and allowed to recover over the indicated time course. Proteins were detected using antibodies against Rpb1 (Pol II) and actin (loading control). Rpb1 level following +UV treatment was normalized to the actin loading control prior to calculating% Rpb1 remaining.

explore the connection of CFIA and CPF mutants with NER, we combined *rna15-1* and *pfs2-1* with a mutation that abolishes the ability of cells to perform NER (*rad14Δ*). The *rna15-1* and *pfs2-1* mutants were not sensitive to the lower drug concentrations used in this assay, and as expected, the *rad14Δ* strains were more 4-NQO sensitive than wild-type *RAD14* strains (Fig. 1C). Interestingly, the *rad14Δ/rna15-1* and *rad14Δ/pfs2-1* double mutants exhibited a synthetic sick phenotype that was more severe than either single mutant alone. This genetic interaction establishes a link between CFIA, CPF, and NER, and it indicates that RNA processing factors have a function in promoting the DDR.

3.2. Mutations in CFIA (rna15-1) and CPF (pfs2-1) increase UV sensitivity in the absence of transcription-coupled nucleotide excision repair (TC-NER)

To further clarify the connection of RNA processing factors to the NER pathway, we combined *rna15-1* and *pfs2-1* with mutations that inactivate one of the two sub-branches of NER, either TC-NER or GG-NER. The *rna15-1* and *pfs2-1* mutations did not obviously alter the UV-sensitivity of the GG-NER deficient strains (*rad7Δ*) (Fig. 2A, B, middle). In contrast, the *rna15-1* and *pfs2-1* mutations exacerbated the UV-sensitivity of the TC-NER deficient strains (*rad26Δrpb9Δ*), particularly at the higher UV dose (Fig. 2A, B, bottom). These experiments use a selection on 5-FOA to remove a covering plasmid containing either *RNA15* or *PFS2*. To confirm the 5-FOA selectivity, we tested growth at 39 °C. As expected, the *rna15-1* and *pfs2-1* strains were temperature sensitive at 39 °C compared to their wild-type counterparts, consistent with the sole presence of mutant alleles in UV-treated strains on 5-FOA (Fig. 2A, B, right).

In contrast to our results, it has previously been reported that CFIA mutations enhance the UV-sensitivity of GG-NER deficient strains [15]. However, this result was obtained using a W303 strain, which commonly contains a *rad5* mutation that increases spontaneous and UV-induced mutations [29]. Our spot test assay and mutagenesis assay (see below) were performed in a non-sensitized BY4742 (*RAD5*) strain background. A similar pattern of genetic interaction was observed for an *elc1Δ* control strain, which exhibited stronger UV-sensitivity in TC-NER vs. GG-NER deficient strains [30] (Fig. 2C). Overall, these genetic interactions support a role for CFIA (Rna15) and CPF (Pfs2) in promoting DNA repair *via* an alternative pathway to TC-NER.

3.3. Mutations in CFIA (rna15-1) increase the frequency of UV-induced genomic mutations in the absence of TC-NER

To further characterize the UV sensitivity of *rna15-1* TCR-deficient strains, we measured the frequency of resistance when yeast were exposed to the toxic drug canavanine. Growth on canavanine can be used as a measure of genomic mutation rate since mutations in the *CAN1* locus are the most common source of drug resistance [40]. In *rad26Δ/rpb9Δ/RNA15* strains, we observed that canavanine-resistance mutations increased in a dose-dependent manner with UV exposure (Fig. 3A). Consistent with a defect in DNA repair, the *rad26/Δrpb9Δ/rna15-1* strains exhibited a ~3-fold increase in the frequency of canavanine resistance mutations at higher UV doses (40–50 J) versus *rad26Δrpb9Δ/RNA15* strains. In the absence of UV, we did not observe a difference in mutation rate. We failed to observe an increased mutation rate in *rna15-1* versus *RNA15* strains in the *rad7Δ* background, which is consistent with the comparable UV-sensitive growth (Fig. 2B). In fact, for an unknown reason, the *rad7Δ/rna15-1* mutant exhibited a reduced mutation rate compared to *rad7Δ/RNA15* at the highest UV dose (Fig. 3B). We compared the UV-induced genomic mutation rate of *rna15-1* with *elc1Δ*, which has previously been shown to reduce the efficiency of GG-NER [30]. The *rad26Δ/rpb9Δ/elc1Δ* strains exhibited a 2-fold increase in the frequency of UV-induced canavanine resistance mutations at the highest treatment dose (Fig. 3C). While the *elc1Δ* control results are consistent with published work, it should be noted that Lejeune et al. (2009) observed a much higher mutation rate in their cananavine resistance assay using a W303 strain background, which was likely sensitized to DNA damage compared to our BY4742 strain (see above) [30]. Taken together, these data indicate that CFIA (Rna15) contributes to the prevention of UV-induced mutagenesis using a pathway outside of TC-NER.

3.4. Mutations in CFIA (rna15-1) and CPF (pfs2-1) reduce the efficiency of Pol II degradation following UV-type DNA damage

An alternative to TC-NER is the last resort pathway, which serves to ubiquitinate and degrade Pol II that has been stalled at UV-induced lesions. To measure the effect of CFIA and CPF mutations on the efficiency of the last resort pathway, we measured protein levels of the Pol II Rpb1 subunit in the absence or presence of UV-type DNA damage. The levels of Rpb1 were reduced to 23% or 50% after 4-NQO treatment of wild-type cells (Fig. 4A), similar to what has previously been reported for UV-type damage [27]. Interestingly, the *rna15-1* and *pfs2-1* mutants stabilized Rpb1, increasing its levels to 67% and 82% of untreated cells, respectively. Similar results were observed in the *rna15-1* mutant after UV treatment as compared to 4-NQO treatment, resulting in a ~2-fold increase in Rpb1 levels at the 30, 60, and 120 min time points after treatment (Fig. 4B). These results indicate that RNA 3′-end processing factors promote the last resort pathway by contributing to proteasomal degradation of Pol II after UV-type damage.

3.5. Mutations in CFIA (rna15-1) and CPF (pfs2-1) reduce the efficiency of Pol II ubiquitination following UV-type DNA damage

Last resort degradation of stalled Pol II requires a series of ordered events, including monoubiquitination, polyubiquitination, and proteasome recruitment. To better determine whether CFIA and CPF RNA processing factors contribute to this process, we immuno-precipitated Rpb1 from extracts following a brief 4-NQO treatment and measured the level of ubiquitination by Western blotting. As expected, 4-NQO treatment resulted in Rpb1 polyubiquitination, which is visible by Western blot as a smear > 230 kDa. The *def1Δ* control extracts were strongly reduced in Rpb1 polyubiquitination, supporting a previously demonstrated role for Def1 in the last resort pathway [31] (Fig. 5A). The CFIA (*rna15-1)* and CPF (*pfs2-1)* mutants likewise reduced Rpb1 polyubiquitination, although to a slightly lesser degree than *def1Δ*

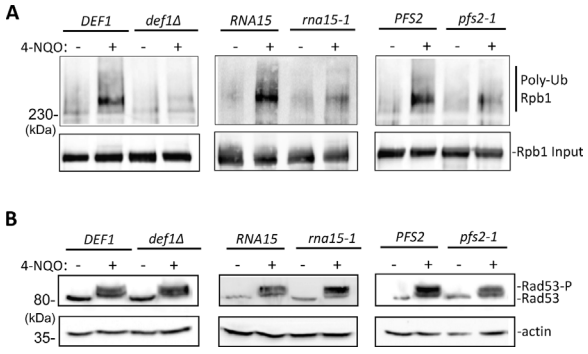

Fig. 5. CFIA and CPF processing factors contribute to efficient Pol II poly ubiquitination following UV-type DNA damage. (A) Immunoblotting was performed using Rpb1 that was immunoprecipitated from indicated strain extracts (W303 background) grown at 30 °C and treated −/+ 4-NQO (5 ug/mL) for 30 min. Approximately equal amounts of immunoprecipitated material was probed with antibodies against Rpb1 and ubiquitin. Def1 is a known member of the Pol II ubiquitination complex and *def1Δ* serves as a positive control. (B) Immunoblotting was performed using input extract from (A) and antibodies against Rad53 and actin (loading control). Phosphorylation of Rad53 (Ra53-P) can be detected due to its slower mobility.

(Fig. 5A). Exposure to 4-NQO was effective, as evident from Rad53 phosphorylation (indicated by appearance of slower mobility bands) and consistent with a DDR (Fig. 5B). Based on these data, we conclude that RNA processing factors CFIA and CPF act upstream of the proteasome and contribute to UV-induced Rpb1 polyubiquitination prior to Pol II degradation.

3.6. UV-induced damage enhances Elc1 and Def1 association with a transcribed gene irrespective of CFIA function

A possible explanation for defective Rpb1 ubiquitination is that CFIA and CPF mutants impair recruitment of the E3 polyubiquitin ligase machinery, which requires Def1 and Elc1. We performed chromatin immunoprecipitation (ChIP) experiments to monitor recruitment of Rpb1, Def1, and Elc1 to the *PMA1* gene (Fig. 6A) before and after 4-NQO treatment in wild-type and CFIA mutant (*rna15-1*) strains. The Pol II (Rpb1) occupancy on *PMA1* matched previously reported ChIP profiles [28], with highest Pol II levels near the 5′-end of the gene and gradual tapering off near the poly(A) site due to transcription termination (Fig. 6B, C, top panel). Interestingly, the *rna15-1* mutant exhibited 2–4 fold reduced occupancy across the gene, perhaps reflecting a previously reported role for CFIA in Pol II initiation or elongation [32,33].

Treatment with 4-NQO reduced Pol II occupancy at *PMA1* in both *RNA15* and *rna15-1* strains, consistent with Pol II stalling and/or depletion from chromatin. Unexpectedly, the ChIP signals for Elc1 and Def1 were higher in the *rna15-1* mutant compared to *RNA15* wild-type strains both in the absence and presence of 4-NQO (Fig. 6B, C, bottom panel). The peak signals for Elc1 and Def1 occupancy were located near the 3′-end of the gene (primer 2616) in 4-NQO treated samples, which may reflect more frequent Pol II stalling in *rna15-1* and support the last resort preference for hyperphosphorylated Rpb1 [12]. Overall these data do not support a role for CFIA (Rna15) in Elc1 or Def1 recruitment to chromatin. If anything, it appears that CFIA mutants lead to a backlog of last resort degradation factors at DNA damage sites.

4. Discussion

The overall goal of this study was to investigate the coordination between mRNA 3′-end processing and the DDR. In addition to yeast CFIA (homolog of mammalian CstF), we have shown that yeast CPF (homolog of mammalian CPSF) is an effector of the DDR. We provided evidence that links UV sensitivity with increased genomic mutation rate in strains deficient for CFIA and TC-NER. We demonstrated that Pol II stabilization in CFIA and CPF mutants following UV-type damage can be explained at least in part by reduced ubiquitination but not reduced recruitment of the Def1 and Elc1 ubiquitination machinery. These data have led us to a model whereby RNA processing factors CFIA and CPF promote the “last resort” pathway of Pol II degradation following UV-type DNA damage (Fig. 7).

4.1. Conserved and contrasting roles for RNA 3′-end processing factors in the DDR

The functional interactions between yeast CFIA and the DDR bear a strong resemblance to mammalian CstF, indicating that eukaryotic cells have utilized RNA processing factors to promote UV resistance over a long evolutionary history. Mirkin et al. (2008) demonstrated that depletion of mammalian CstF-64 in combination with UV treatment diminished cell viability, reduced Pol II ubiquitination and degradation, and caused cell cycle arrest [18]. In addition, CstF-64 was shown to localize to sites of repaired DNA, and depletion of CstF-64 limited the efficiency of TC-NER [18]. Similarly, Gaillard and Aguilera (2014) demonstrated a defect in cell cycle progression in the yeast CFIA mutant *rna14-1* [15]. Consistent with a defect in TC-NER, CFIA mutants were more UV-sensitive in strains deficient for GG-NER. However, unlike mammalian CstF-64 and previously characterized yeast elongation and nuclear export factors, yeast CFIA mutants were not defective for TC-NER or GG-NER in an *RPB2* strand-specific CPD removal assay [13–15]. Together, these data support a conserved general role for RNA 3′-end processing factors in the DDR, but with the mechanism of action partitioned differently between primary and backup repair pathways.

In contrast to CstF, which contributes to both TC-NER and “last resort” Pol II degradation pathways in mammalian cells, our data demonstrate a more focused role for yeast RNA processing machinery in the latter. Similar to what we have shown for CFIA and CPF mutants, *def1*Δ and *elc1*Δ mutants increase the sensitivity of NER-deficient strains, consistent with their role in resolving stalled Pol II complexes [30,31]. In the absence of TC-NER, CFIA and CPF mutants behave like *elc1*Δ, leading to an increased UV-sensitivity and genomic mutation rate. Def1 and Elc1 contribute to Pol II ubiquitination, but Def1 is dispensable for both TC-NER and GG-NER, and Elc1 is only required for GG-NER [30,31]. Interestingly, Def1 is dispensable for Rpb1 ubiquitination and degradation in *rad26Δ* cells, and UV-induced degradation in *rpb9Δ* or *def1Δ* cells is impaired but not completely absent [27]. These observations suggest that an Rpb9- and Def1-independent degradation pathway exists in cells, which may involve CFIA and CPF activity. A role for CFIA and CPF in promoting a sub-pathway of Pol II degradation is further supported by the fact that *rna15-1* and *pfs2-1* mutants increased UV sensitivity in *rad26Δ rpb9Δ* strains, where TC-NER and Pol II degradation are already impaired.

An additional pathway for ubiquitination and degradation has been described in yeast that targets Pol II stalled due to discontinuous transcription (*e.g.* elongation defects) or nutrient deprivation (*e.g.* low NTPs) [10]. This degradation pathway appears evolutionarily conserved since Pol II is ubiquitinated and degraded when transcription is arrested by α-amanitin treatment in higher eukaryotes [34]. The yeast DNA damage-independent pathway uses some components that overlap with DNA damage-dependent degradation, including Def1 and the E2 ubiquitin ligases Ubc4 and Ubc5. In contrast, loss of Rad26, Elc1, and Cul3 did not affect Rpb1 polyubiquitination when Pol II was stalled by a transcription elongation mutation [10]. It is possible that CFIA and CPF contribute to the DNA damage-independent Pol II degradation pathway. We observed a reduction in cell viability in *rad26Δ/rpb9Δ/pfs2-1* cells even in the absence of UV treatment (Fig. 2B). In addition, we observed reduced Pol II occupancy across the *PMA1* gene in the *rna15-1* mutant, consistent with a possible elongation defect (Fig. 6B, C). However, we observed a clear UV-dependent enhancement of growth sensitivity and genomic mutation rate in RNA 3′-end processing mutants. While previous reports have documented Pol II elongation

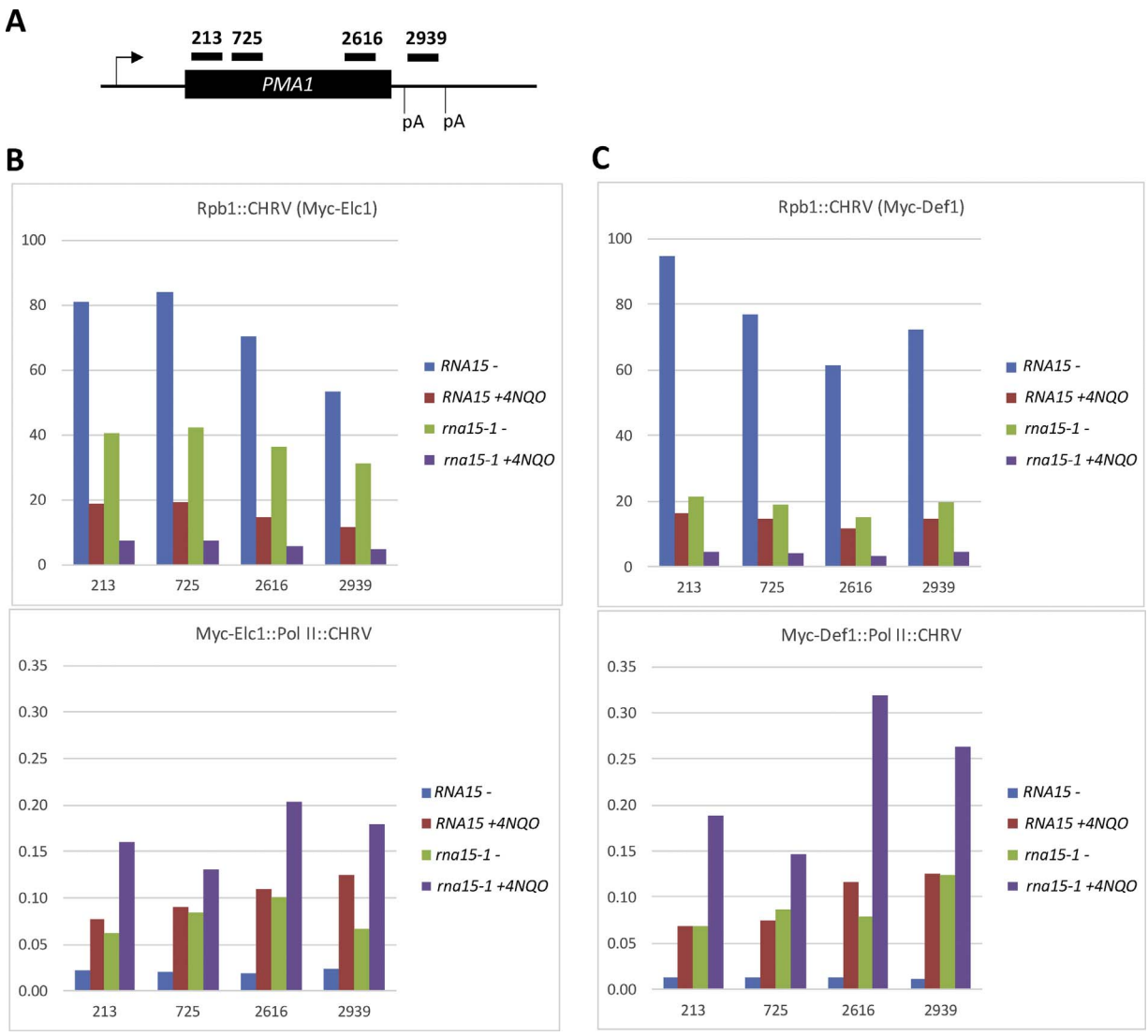

Fig. 6. Elc1 and Def1 recruitment to transcribed regions is increased following DNA damage and undiminished in a CFIA mutant *(rna15-1)*. *RNA15* and *rna15-1* strains (W303 background) were grown −/+ 4NQO (1.25 ug/mL) for 30 min prior to formaldehyde crosslinking and chromatin preparation. (A) ChIP primers are displayed based on their relative position within the *PMA1* gene. (B) ChIP signals are shown for Pol II (Rpb1) occupancy (top) and Myc-Elc1 (bottom) normalized to Rpb1. (C) ChIP signals are shown for Pol II (Rpb1) occupancy (top) and Myc-Def1 (bottom) normalized to Rpb1. The signal at the CHRV intergenic region was taken as background signal, and each qPCR experiment was performed in technical duplicate. The qPCR Ct values for input samples were comparable between cells −/+ 4-NQO treatment, suggesting that reduction in Rpb1 occupancy was not simply due to DNA lesions preventing the qPCR reaction.

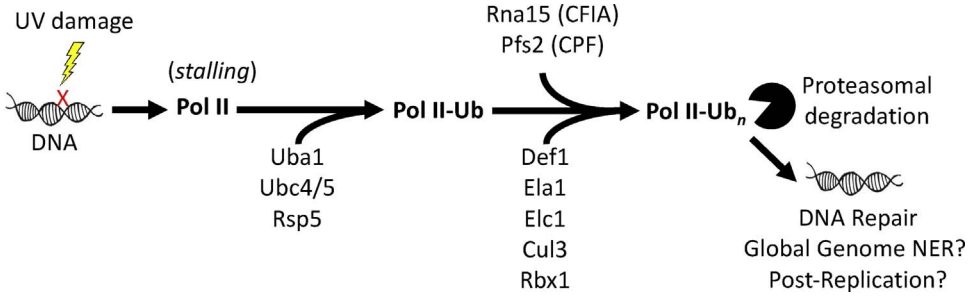

Fig. 7. Model: RNA 3′-end Processing factors CFIA and CPF contribute to DNA repair following UV-induced damage by promoting the last-resort Pol II degradation pathway. Ultraviolet light (UV) creates lesions in DNA (X) that stall the elongating RNA Polymerase II (Pol II) transcription complex. During prolonged stalling, the last resort pathway leads to Pol II mono-ubiquitination (Pol II-Ub) and poly-ubiquitination (Pol II-Ub$_n$), resulting in Pol II degradation and providing DNA repair factors with access to the lesion. RNA processing factors (CFIA and CPF) co-transcriptionally associate with Pol II prior to recognition of RNA sequences at the p(A) 3′-end processing site. Additionally, we propose that CFIA and CPF promote ubiquitination and degradation of stalled Pol II, followed by DNA repair *via* a TC-NER independent pathway.

defects for CFIA mutants, the assay depended on transcription through long GC-rich regions, which may not be generally applicable in yeast [35]. In addition, we observed no evidence of increased Rpb1 poly-ubiquitination in CFIA or CPF mutants in the absence of DNA damage (Fig. 5A). Taken together, these data argue against a significant indirect effect of RNA processing mutants on Pol II elongation or the DNA damage-independent degradation pathway.

4.2. Molecular role of RNA 3′-end processing factors in promoting Rpb1 ubiquitination

In this study, we have expanded our understanding of the RNA

processing machinery involved in the DDR and delineated their role in the "last resort" Pol II degradation pathway. However, the molecular mechanism through which RNA processing factors promote Rpb1 ubiquitination remains to be determined. Since RNA processing factors co-transcriptionally associate with Pol II, we tested whether CFIA recruits polyubiquitination factors after DNA damage. We observed that Elc1 and Def1 occupancy increased within transcribed regions following UV-type stress (Fig. 6B, C). These data are consistent with Def1 activation, nuclear accumulation, and Pol II binding during transcription stress [25]. It was previously reported that Elc1 co-immunoprecipitation with Pol II was unchanged −/+ UV [11], but our ChIP data suggest that like Def1, Elc1 association with Pol II increases after DNA damage. A CFIA mutant did not diminish this association with a transcribed gene, which argues against a role for RNA processing factors in recruitment of Def1 and Elc1.

There are several other explanations that may explain the observed Pol II stabilization in CFIA and CPF mutants. We observed that Elc1 and Def1 occupancy was higher in mutant CFIA versus wild-type strains, which suggests a build-up of degradation factors due to failed activation or rearrangement, preventing access to Rpb1 ubiquitination sites. Alternatively, the RNA processing mutants could prevent recruitment of monoubiquitin ligase proteins like Rsp5, limit assembly of poly-ubiquitin ligase components, or enhance recruitment of antagonistic deubiquitinases like Ubp3 [36]. We also cannot exclude the possibility that the reduction in Pol II on transcribed genes in the *rna15-1* mutant lowers the targetable pool of Pol II, since the elongation complex is the primary target of ubiquitination/degradation. However, studies of another RNA processing mutant (*pcf11-2*) revealed comparable Pol II stabilization as *rna15-1* [15], despite no observed drop in Pol II occupancy on the *ADH1* gene [37].

A significant goal of future studies will be to clarify the precise molecular role of RNA processing factors in "last resort" Pol II degradation and the repair pathways promoted by this process. Our data suggest that the role of mRNA processing factors in the DNA damage response is not limited to TC-NER or NER in general. It will be of particular interest to determine if alternate repair pathways are enhanced by Pol II degradation since Def1 activity has also been shown to contribute to base excision repair (BER) and post-replication repair (PRR) [38,39]. It will be advantageous to take advantage of yeast *in vitro* ubiquitination assays and *in vivo* chromatin association studies, which are likely to reveal additional biological mechanisms shared with the DDR in higher eukaryotic systems.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

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