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Genome-Wide Analysis of Factors Affecting Transcription Elongation and DNA Repair: A New Role for PAF and Ccr4-Not in Transcription-Coupled Repair

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Abstract

RNA polymerases frequently deal with a number of obstacles during transcription elongation that need to be removed for transcription resumption. One important type of hindrance consists of DNA lesions, which are removed by transcription-coupled repair (TC-NER), a specific sub-pathway of nucleotide excision repair. To improve our knowledge of transcription elongation and its coupling to TC-NER, we used the yeast library of non-essential knock-out mutations to screen for genes conferring resistance to the transcription-elongation inhibitor mycophenolic acid and the DNA-damaging agent 4-nitroquinoline-N-oxide. Our data provide evidence that subunits of the SAGA and Ccr4-Not complexes, Mediator, Bre1, Bur2, and Fun12 affect transcription elongation to different extents. Given the dependency of TC-NER on RNA Polymerase II transcription and the fact that the few proteins known to be involved in TC-NER are related to transcription, we performed an in-depth TC-NER analysis of a selection of mutants. We found that mutants of the PAF and Ccr4-Not complexes are impaired in TC-NER. This study provides evidence that PAF and Ccr4-Not are required for efficient TC-NER in yeast, unraveling a novel function for these transcription complexes and opening new perspectives for the understanding of TC-NER and its functional interconnection with transcription elongation.

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Introduction

Synthesis of an RNA transcript by RNA polymerase II (RNAPII) requires the successful completion of at least four steps in the transcription cycle: promoter binding and initiation, promoter clearance, elongation, and termination. While many studies have focused on the regulation of initiation, more recent studies have demonstrated that transcription elongation is a dynamic and highly regulated stage of the transcription cycle capable of coordinating downstream events. Numerous factors have been identified that specifically target elongation [1]. Importantly, multiple steps in mRNA maturation, including pre-mRNA capping, splicing, 3′-end processing, surveillance, and export, are modulated through interactions with the RNAPII machinery [2,3]. It also appears that distinct factors act in specific transcriptional contexts; the requirements of these factors are largely unknown and highlight the need to improve our understanding of elongation *in vivo*. Several lines of evidence indicate that transcript elongation by RNAPII involves frequent pausing and stalling, and an important role of the many accessory factors may be to minimize the negative impact of such events on transcription [4].

Nucleotide excision repair (NER) is an evolutionarily conserved DNA repair pathway that deals with severely distorting DNA lesions including intrastrand crosslinks such as UV-induced pyrimidine dimers (CPDs) and DNA bulky adducts such as those generated by the model carcinogen 4-nitroquinoline-N-oxide (4-NQO). Within NER two damage-sensing pathways are recognized: one for the entire genome, global genome repair (GG-NER), and the other for transcribed strand of active genes, transcription coupled repair (TC-NER). As ongoing transcription is required for TC-NER, damage recognition is likely triggered by the elongating RNAP itself, whose progression gets obstructed at the site of damage. RNAP arrests at injuries in the template strand initiating, likely via additional specific factors, the recruitment of the DNA repair machinery [5,6].

In eukaryotes, the precise mechanism of TC-NER remains poorly understood. Mutations in proteins required for NER lead to severe disorders known as Xeroderma pigmentosum and Cockayne's syndrome. One of these proteins, Cockayne syndrome B protein (CSB), and its yeast orthologue Rad26, share conserved functions [7,8] and represent putative eukaryotic transcription-repair coupling factor (TRCF) candidates. The putative function of CSB as a TRCF has been substantiated by *in vitro* reconstitution

Author Summary

Dealing with DNA lesions is one of the most important tasks of both prokaryotic and eukaryotic cells. This is particularly relevant for damage occurring inside genes, in the DNA strands that are actively transcribed, because transcription cannot proceed through a damaged site and the persisting lesion can cause either genome instability or cell death. Cells have evolved specific mechanisms to repair these DNA lesions, the malfunction of which leads to severe genetic syndromes in humans. Despite many years of intensive research, the mechanisms underlying transcription-coupled repair is still poorly understood. To gain insight into this phenomenon, we undertook a genome-wide screening in the model eukaryotic organism *Saccharomyces cerevisiae* for genes that affect this type of repair that is coupled to transcription. Our study has permitted us to identify and demonstrate new roles in DNA repair for factors with a previously known function in transcription, opening new perspectives for the understanding of DNA repair and its functional interconnection with transcription.

of the TC-NER initiation steps, in which an elongating RNAPII arrested at a DNA lesion was shown to mediate an ATP-dependent incision of the damaged DNA only in the presence of CSB [9]. XPG, one of the structure-specific DNA endonuclease responsible for the removal of an oligonucleotide containing the DNA lesion in NER, is another protein involved in TC-NER. Recent results imply a coordinated recognition of stalled RNAPII by XPG and CSB in TC-NER initiation in mammalian cells and suggest that TFIIH-dependent remodeling of stalled RNAPII without release may be sufficient to allow repair [10]. In yeast, the Rpb4 and Rpb9 subunits of RNAPII have also been shown to contribute to TC-NER [11,12]. Recently, proteins of the THO, Sub2-Yra1 and Thp1-Sac3 complexes, which are involved in mRNP biogenesis and export [13], have also been shown to be required for efficient TC-NER [14].

In addition, RNAPII is subject to ubiquitylation and proteasome-mediated degradation in response to UV damage [15]. It has been proposed that degradation of damage-stalled RNAPII complexes might assist TC-NER [16]. Indeed, recent studies in yeast have shown that arrested RNAPII elongation complexes are the preferred substrate for ubiquitylation, which is mediated by Def1, Rpb9, and the C-terminal repeat domain (CTD) of RNAPII [17–19].

To improve our knowledge of gene products that function at the interface between transcription and DNA repair, we used a yeast mutant library covering the yeast non-essential genes for a genome-wide analysis of the genes conferring resistance to the inosine monophosphate dehydrogenase inhibitor mycophenolic acid (MPA), and the DNA-damaging agent 4-NQO. This approach allowed us to identify new putative candidates for genes involved in transcription and TC-NER. Our study unravels a new function for known transcription complexes in TC-NER and open new perspectives for the understanding of TC-NER and its functional interconnection with transcription elongation.

Results

Genome-Wide Analysis of mRNA Accumulation and 4-NQO- and MPA-Sensitivity

We were interested in analyzing and comparing the genes conferring resistance to 4-NQO and MPA. To explore the functional consequences of the treatments with 4-NQO and MPA,

we first determined the expression levels of the whole genome after treating wild-type cells with either 75 ng/ml 4-NQO or 50 µg/ml MPA during 30 min each. Expression of a total of 2374 genes was evaluated by microarray analysis (data available at www.ncbi.nlm.nih.gov/geo/ under the access number GSE11561). Among these genes, mRNA levels that were at least 2-fold above or below mock treated cells were found for 376 genes in cells treated with 4-NQO and for 295 genes in cells treated with MPA (Table S1). Of the genes affected by either treatment, very few were coincident, which is consistent with the fact that 4-NQO and MPA affect different cellular processes (Figure 1A). Ontology analysis of the 644 genes showing significant variations in expression levels indicated that there is not a relevant class of genes specifically affected by any of the compounds used (data not shown).

For the analysis of genes required for resistance to 4-NQO and MPA, a collection of 4894 yeast haploid knock-out mutants, covering 85% of all yeast genes and virtually all (99,4%) of yeast non-essential genes, were grown in SC medium supplemented either with 150 ng/ml 4-NQO or 25 µg/ml MPA. Cells were incubated at 30°C and growth monitored after 48, 72, and 120 for 4-NQO and 72 hours for MPA. Analysis of the strains showing at least 80% growth inhibition by 4-NQO at the indicated time led to a classification of sensitive strains into three groups (Table S2): group A contains 189 strains whose sensitivity to 4-NQO is observed from early on (48 h) and is maintained over time, B contains 308 strains whose sensitivity was observed only at early time points (48 h), and C contains 100 slow-growing strains whose sensitivity is observed later on (from 72 h). Direct comparison of cell sensitivity to different DNA-damaging agents and stress conditions (4-NQO, methyl-methanesulfonate [MMS], menadione [Mnd], UV, and 37°C) in a selection of 4-NQO-sensitive mutants validated our high-throughput results and confirmed that 4-NQO in addition to being a 'UV-mimetic' agent, causes oxidative damage (Table S3). In the same line, comparison of the mutants found sensitive to 4-NQO with the mutants found sensitive to at least one of five oxidants [20] revealed that 35% of the 4-NQO sensitive strains are sensitive to oxidative damage as well. As compared to a genome-wide study in which deletion strains were pooled and grown competitively in the presence of 4-NQO [21], our set of 4-NQO sensitive strains contains the 10 strains found as top sensitive and 31 out of the 37 most sensitive strains.

Correspondingly, 85 MPA-sensitive deletions showing at least 50% growth inhibition were found (Table S4), of which 40 had been reported in a previous analysis for MPA sensitivity of the yeast disruptome [22] and 45 had not been described as MPA-sensitive to date. We focused on 289 4-NQO-sensitive (groups A and C) and 68 MPA-sensitive deletions showing at least 60% growth inhibition. Ontology analysis of the genes identified for each drug is consistent with the fact that 4-NQO affects DNA repair whereas MPA affects transcription (Figure 1B). Only 25 mutations led to sensitivity to both compounds (Figure 1C). From these mutations, 13 identified known genes involved in aspects of transcription and mRNP biogenesis, and 12 identified genes involved in protein modification, intracellular trafficking, and primary and secondary metabolism. Direct comparison of the genes showing significant variations in their patterns of expression, as determined by microarray analyses, with those showing sensitivity to either MPA or 4-NQO reveals no obvious correlation (data not shown). Therefore, a higher expression of a gene in the presence of 4-NQO or MPA does not imply requirement for resistance, but rather it is the result of an adaptation of the cell to the new conditions of growth.
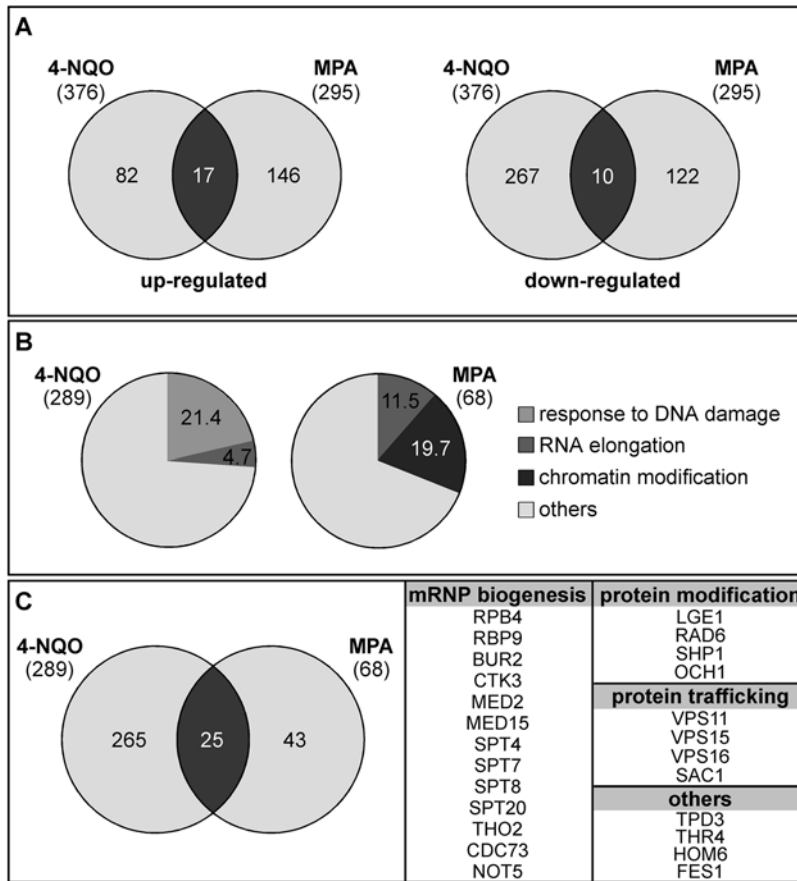
Figure 1. Analysis of genes similarly affected by 4-NQO and MPA. (A) Venn diagram representing the overlap between genes whose expression is changed after treatment with 4-nitroquinoline-N-oxide (4-NQO) and mycophenolic acid (MPA). (B) Fraction of mutant strains sensitive to 4-NQO (left) and MPA (right), classified by GO annotation. The MPA-sensitive set is significantly enriched in genes with the label "RNA elongation" and "chromatin modification" ($p<3.38E-03$ and $p<7.96E-05$, respectively), and the 4-NQO-sensitive set in "response to DNA damage" ($p<6.3E-19$). Enrichment analyses were carried out with the GO Term Finder tool of the SGD. (C) Venn diagram representing the overlap between genes conferring resistance to 4-NQO and MPA. The 25 genes common to both analyses are listed to the right.
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Effect of SAGA, CTK, Mediator, Ccr4-Not, Bre1-Rad6, and Fun12 in Transcription Elongation

We have previously shown that mutants impaired in transcription elongation display lower efficiency in the transcription of long *vs.* short transcription units [23]. To gain insight into the putative defects in RNAPII transcription of 45 MPA-sensitive mutants, the ratios of acid phosphatase activity for a long (*PHO5-lacZ* or *PHO5-LAC4*) *vs.* a short transcription unit (*PHO5*), which is taken as an approximate measurement of *G*ene *L*ength *A*ccumulation of *m*RNA (GLAM) were determined (Table S4). GLAM-ratios were previously used as an indirect estimation of RNAPII elongation [24]. Similar GLAM-ratios were obtained with the two long transcription units (Figure S1). Out of the assayed mutants, 24 showed values below 0.5, which were taken as criteria for candidates with defects in RNAPII elongation. 6 mutants carried deletions of genes encoding subunits of protein complexes that were previously related to RNAPII elongation, including RNAPII (*rpb4Δ* and *rpb9Δ*), THO (*tho2Δ*), Spt4-Spt5 (*spt4Δ*), and PAF (*rtf1Δ* and *cdc73Δ*). 7 mutants carried deletions of genes encoding proteins affecting transcription, for which an implication in transcription elongation has been proposed in the past. These included subunits of SAGA (*spt3Δ* and *spt20Δ*) [25], CDK (*bur2Δ*) [26], and Ccr4-Not (*ccr4Δ*) [27] complexes as well as proteins involved in H2B ubiquitylation (*bre1Δ*, *rad6Δ*, and *lge1Δ*) [28].

Another 4 mutants carried deletions of genes encoding proteins affecting transcription, but that had not been related to RNAPII elongation. These included subunits of Mediator (*med2Δ* and *med15Δ*) and Ccr4-Not (*not5Δ*) complexes as well as the Uvs1 putative transcription factor (*YPL230wΔ*). The remaining 7 mutants affected proteins whose function has not been previously linked to transcription (*tpd3Δ*, *fun12Δ*, *shp1Δ*, *lip2Δ*, *est2Δ*, and *ubp15Δ*) or is unknown (*YJR018wΔ*). To evaluate the significance of these putative new links with RNAPII elongation, and because some mutants of the SAGA (*spt7Δ* and *spt8Δ*), CDK (*ctk1Δ* and *ctk3Δ*), and Mediator (*med12Δ*) complexes did not exhibit expression deficiencies, we extended our analysis to deletions of other functionally related genes.

Gene expression was analyzed in all viable SAGA deletions (Figure 2). We found that, in addition to *spt20Δ* and *spt3Δ*, the absence of three other subunits (Hfi1, Sgf73, and Sgf29) showed a clear gene expression defect (GLAM<0.5) while the remaining 5 viable deletions (*gcn5Δ*, *ada2Δ*, *ubp8Δ*, *ngg1Δ*, and *sgf11Δ*) were only poorly or not affected, as observed for *spt7Δ* and *spt8Δ*.

Since the H2B-ubiquitylases Rad6, Bre1, and Lge1, two PAF subunits (Cdc73 and Rtf1) as well as Bur2 belonged to the MPA-sensitive mutants exhibiting low GLAM-ratios, we extended the analysis to mutants of COMPASS, the complex responsible for H3-K4 methylation. Low GLAM values were observed for some
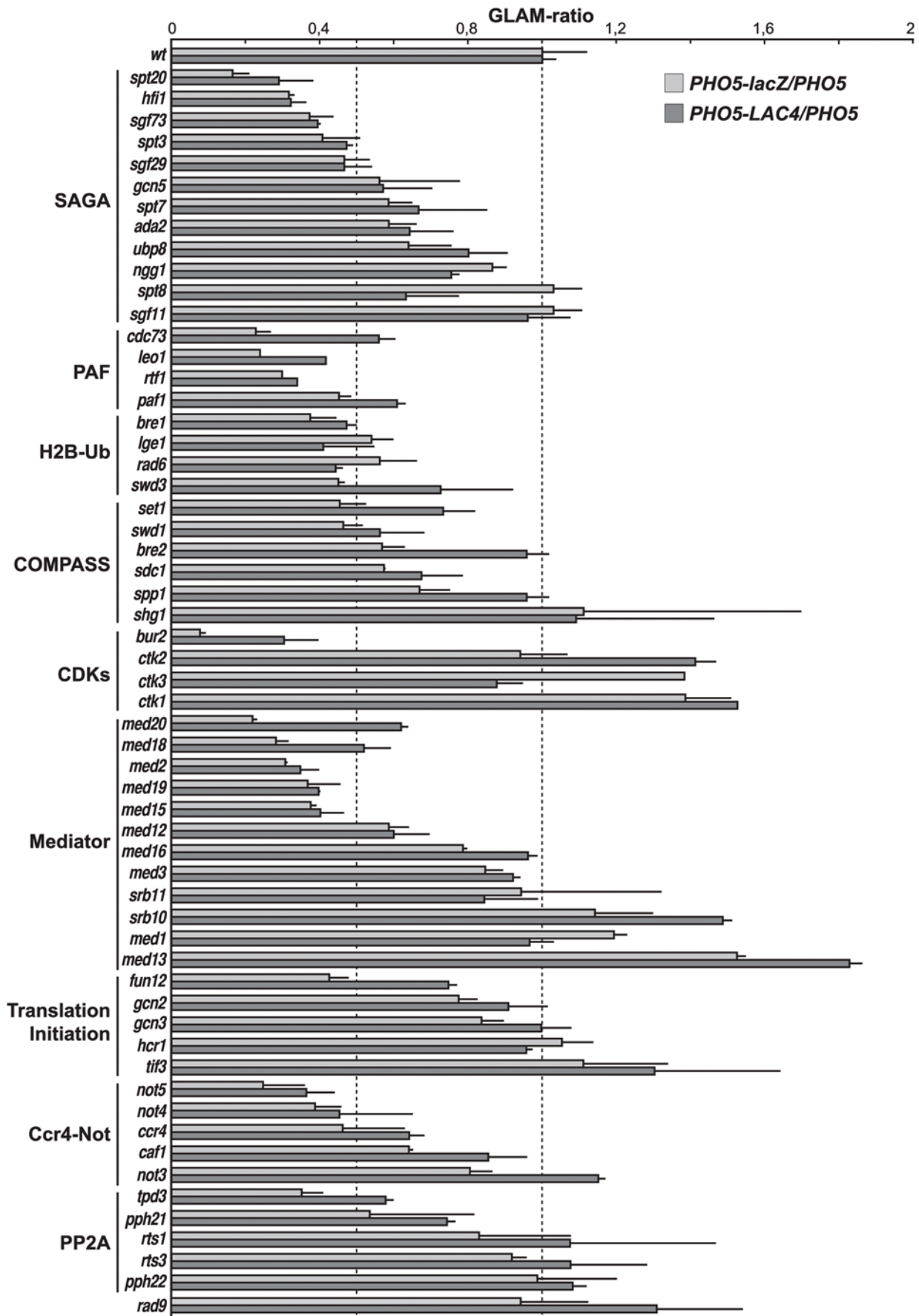
Figure 2. Gene expression analyses of selected MPA-sensitive and functionally related mutants. *PHO5-lacZ/PHO5* and *PHO5-LAC4/PHO5* ratios were calculated after assaying acid phosphatase activity of mutants lacking the indicated gene. Averages of at least three independent assays are shown. Error bars indicate standard deviation.
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of the COMPASS and H2B ubiquitylation mutants (Figure 2), suggesting that both H2B ubiquitylation and H3-K4 methylation may be important for transcription elongation. Interestingly, analysis of Ctk1-Ctk2-Ctk3 (CTDK-I)—the other cyclin-dependent protein kinase involved in transcription elongation [29]—suggests that, despite *ctk1Δ* and *ctk3Δ* being MPA-sensitive, CTDK-I might be dispensable for the expression of long genes.

Most viable deletions lacking Mediator subunits and the viable deletions lacking other subunits of the Ccr4-Not complex were also assayed (Figure 2). The results indicated that in addition to Med2 and Med15, the Med18, Med19 and Med20 subunits might affect transcription elongation, whereas the remaining viable subunits may be dispensable. For the Ccr4-Not complex, *not4Δ* showed low expression of long genes, as observed for *not5Δ* and *ccr4Δ*, whereas this was not the case for *caf1Δ* and *not3Δ*.

Finally, since the only known function of Fun12 is related to translation initiation, we assayed other viable deletions of translational machinery elements playing a role during initiation (Gcn2, Gcn3, Hcr1 and Tif3). To understand the low GLAM-ratios of *tpd3Δ*, all other deletions lacking subunits of the PP2A complex were assayed. Figure 2 shows that neither the translation initiation machinery nor the PP2A complex influence gene expression in a gene length-dependent way.

Since the analysis of GLAM-ratios relies on measurement of enzymatic activities, we decided to assess directly the efficiency of transcription of representative mutants using an *in vitro* elongation assay. This assay is based on a plasmid (pGCYG1-402) in which a hybrid *GAL4-CYC1* promoter containing a Gal4 binding site is fused to a 1.88-kb DNA fragment coding for two G-less cassettes. The first cassette is right downstream of the promoter and is 84-nt-long. The second is located 1.48-kb from the promoter and is 376-nt-long. The efficiency of elongation is determined in whole cell extracts (WCEs) by the values of the ratio of accumulation of the 376- versus the 84-nt-long G-less RNA fragments after RNase T1 digestion [30].

WCEs from representative mutants of Mediator (*med15Δ*), CTK (*bur2Δ*), Bre1-Rad6 (*bre1Δ*), SAGA (*spt20Δ*), Ccr4-Not (*not5Δ*), and PP2A (*tpd3Δ*) complexes as well as the translation initiation mutant *fun12Δ* were analyzed. As can be seen in Figure 3, *bre1Δ*, *spt20Δ*, and *not5Δ* WCEs transcribed the 376-nt G-less cassette with efficiencies around or below 60% of the wild-type levels. These results indicate severe defects in transcription elongation in those subunits of the Bre1-Rad6, SAGA, and Ccr4-Not complexes. Strikingly, *fun12Δ* cell extracts also led to a clear transcription elongation phenotype in our assay (62%). WCEs extracts of *bur2* and *med15* mutants were moderately affected in transcription
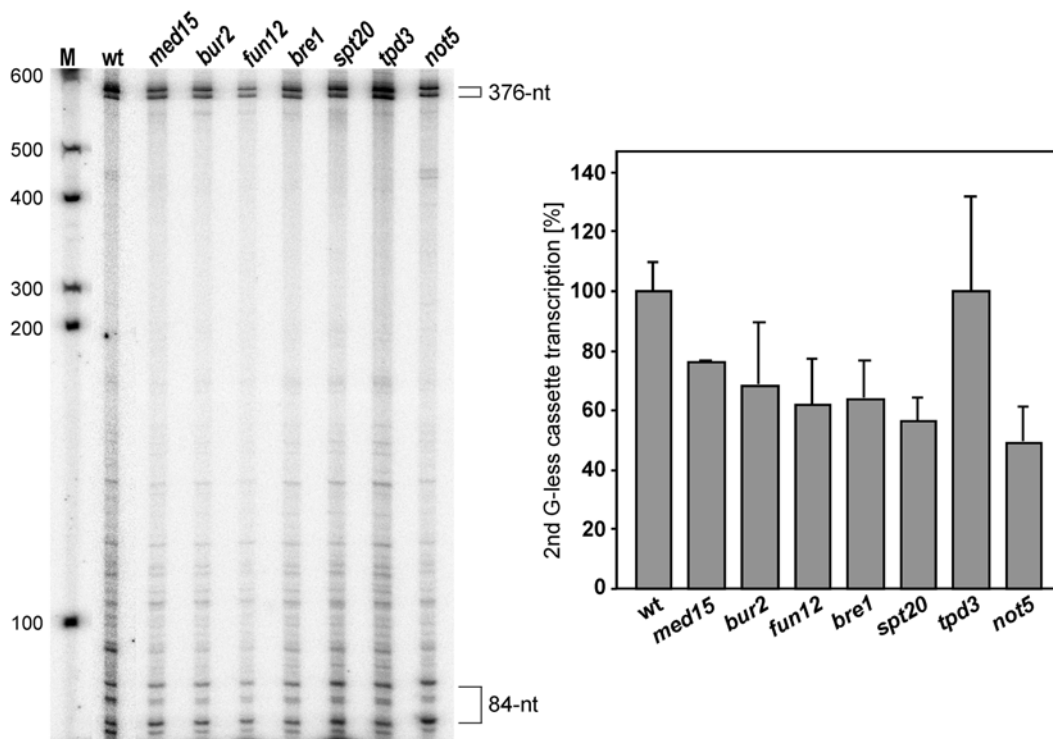

Figure 3. *In vitro* transcription elongation. *In vitro* transcription assay of WCEs from BY4741 (wt), *med15*, *bur2*, *fun12*, *bre1*, *spt20*, *tpd3*, and *not5* strains. Each reaction was stopped after 30 min, treated with RNaseT1 and run in a 6% PAGE. Two bands from each G-less cassette were obtained, probably due to incomplete action of RNaseT1. Efficiency of transcription elongation was determined as the percentage of transcripts that reach the 376-nt cassette in respect to those that cover the 84-nt cassette. Radioactivity incorporated into the cassettes was quantified in a Fuji FLA3000 and normalized with respect to the C content of each cassette. The mean value of the wt (84%) was normalized to 100%. Mean value and standard deviation of three independent experiments are shown.
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elongation, with efficiencies ranging from 68 to 77% of wild-type levels. Transcription elongation efficiencies of *tpd3Δ* WCEs reached wild-type levels, indicating that this mutant was fully transcription elongation-proficient in this assay.

In addition, we aimed at testing whether the candidate mutations changed the distribution of RNAPII along a transcribed unit, as an alternative method to measure elongation [31]. Therefore, RNAPII occupancy was analyzed by ChIP for the representative mutants selected for the *in vitro* assay in the LAUR expression system [32], which contains a 4.15 kb *lacZ-URA3* translational fusion under the control of the *Tet* promoter. The presence of RNAPII was determined at a 5′-end and a 3′-end region of the *lacZ* sequence, as well as within the fused *URA3* gene (Figure 4). The *spt20Δ, not5Δ* mutants and, to a lesser extent, *bur2Δ* were impaired in elongation in this assay, as less RNAPII was found toward the 3′-end than at the 5′-end of the transcription unit. Strikingly, RNAPII appeared to accumulate toward the 3′-end of the gene in *med15Δ* cells. No significant changes in RNAPII distribution were observed in the *bre1Δ, fun12Δ,* and *tpd3Δ* mutants, the latter of which is consistent with the *in vitro* result.

Therefore, our results indicate that subunits of SAGA, Ccr4-Not, Mediator, and, to a lesser extent, CDK affect transcription elongation, as seen with the three different assays tested while the effect of Bre1-Rad6 and the translation factor Fun12 on transcription elongation depends on the assay used.

Genetic Analysis of UV Sensitivity in the Absence of Global Genome Repair

Given the strong dependency of TC-NER on RNAPII transcription and the fact that the few proteins known to be involved in TC-NER are related to transcription, we made use of the results of our MPA-sensitivity screen to select 18 mutants encoding for transcription factors, protein de-ubiquitylase, H2B-ubiquitylase, subunits of the CDK, SWI/SNF, SAGA, Mediator, PAF, Ccr4-Not complexes, and RNAPII and look for those possibly involved in TC-NER. For this purpose, we abolished GG-NER by deleting the *RAD7* gene in the chosen mutants. In the

absence of GG-NER, deficiencies in TC-NER lead to increased UV-sensitivity, a phenotype that we screened for by drop assay (Figure 5). Growth of each double mutant was compared to the growth of *rad7Δ*, giving rise to the classification of 5 mutants as not more sensitive than *rad7Δ* (*dst1Δ, ubp15Δ, ctk3Δ, bur2Δ,* and *spt7Δ*) and 4 mutants as slightly more sensitive to UV than *rad7Δ* (*snf6Δ, spt4Δ, spt8Δ,* and *med12Δ*), this effect being more obvious when higher UV doses were used (data not shown). The remaining 9 strains were much more sensitive to UV than *rad7Δ* (*rad6Δ, rpb9Δ, med2Δ, med15Δ, spt20Δ, spt3Δ, rtf1Δ, cdc73Δ,* and *not5Δ*). Rad6 and Rpb9 are known to be involved in post-replication repair of UV-damaged DNA and TC-NER, respectively [11,33]. However, the 7 other mutants have no known connection to any UV-damaged DNA repair pathway. These mutants include subunits of the mediator (*med2Δ* and *med15Δ*), SAGA (*spt3Δ* and *spt20Δ*), PAF (*rtf1Δ* and *cdc73Δ*), and Ccr4-Not (*not5Δ*) complexes. Given the fact that other subunits of the Mediator and SAGA complexes were represented in the moderately UV-sensitive strains (*med12Δ, spt7Δ,* and *spt8Δ*), we focused on the PAF and Ccr4-Not complexes for a more detailed analysis.

TC-NER Is Impaired in Cells Defective in the PAF and Ccr4-Not Complexes

To refine the UV sensitivity analysis of PAF and Ccr4-Not mutants in the absence of GG-NER, UV survival curves were performed for all viable PAF and Ccr4-Not mutants (*rtf1Δ, cdc73Δ, paf1Δ, leo1Δ, not5Δ, not4Δ, not3Δ, caf1Δ, ccr4Δ*) alone or in combination with the *rad7Δ* mutation (Figure 6). The *rtf1Δ, cdc73Δ, leo1Δ, not3Δ, caf1Δ* and *ccr4Δ* single mutants show no increased UV sensitivity as compared with wild-type cells. However, upon UV irradiation viability of the corresponding double mutants dropped below the levels of the *rad7Δ* single mutant. The *paf1Δ, not5Δ* and *not4Δ* single mutants showed a moderate UV sensitivity, reaching levels very close to that of *rad7Δ* in the case of *paf1Δ* and *not4Δ*. Nevertheless, the viability of the *paf1Δ rad7Δ, not5Δ rad7Δ,* and *not4Δ rad7Δ* double mutants dropped far below the levels of the corresponding single mutants upon UV irradiation.
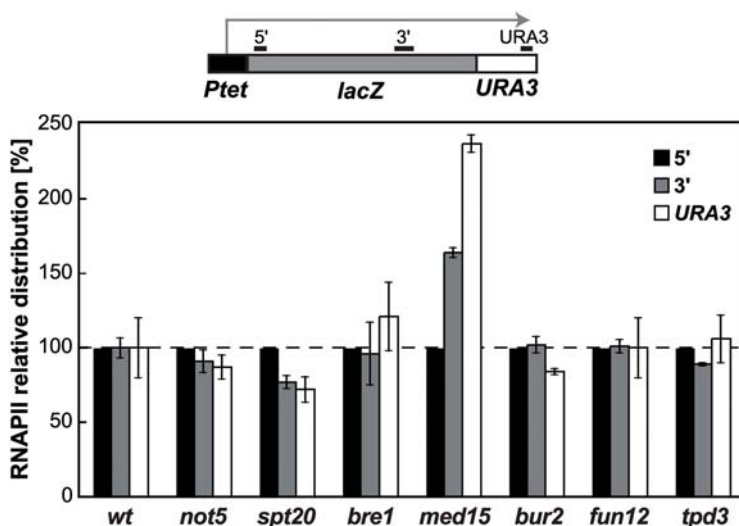
Figure 4. RNAPII occupancy analysis. ChIP analyses in BY4741 (wt), *not5, spt20, bre1, med15, bur2, fun12,* and *tpd3* strains in the LAUR expression system. The scheme of the gene and the PCR-amplified fragments are shown. The DNA ratios in region 5′, 3′ and *URA3* were calculated from their signal relative to the signal of the intergenic region. The recruitment data shown are referred to the value of the 5′ region taken as 100%. ChIPs were performed from 3 independent cultures, and quantitative PCRs were repeated three times for each culture. Error bars, SDs.
doi:10.1371/journal.pgen.1000364.g004

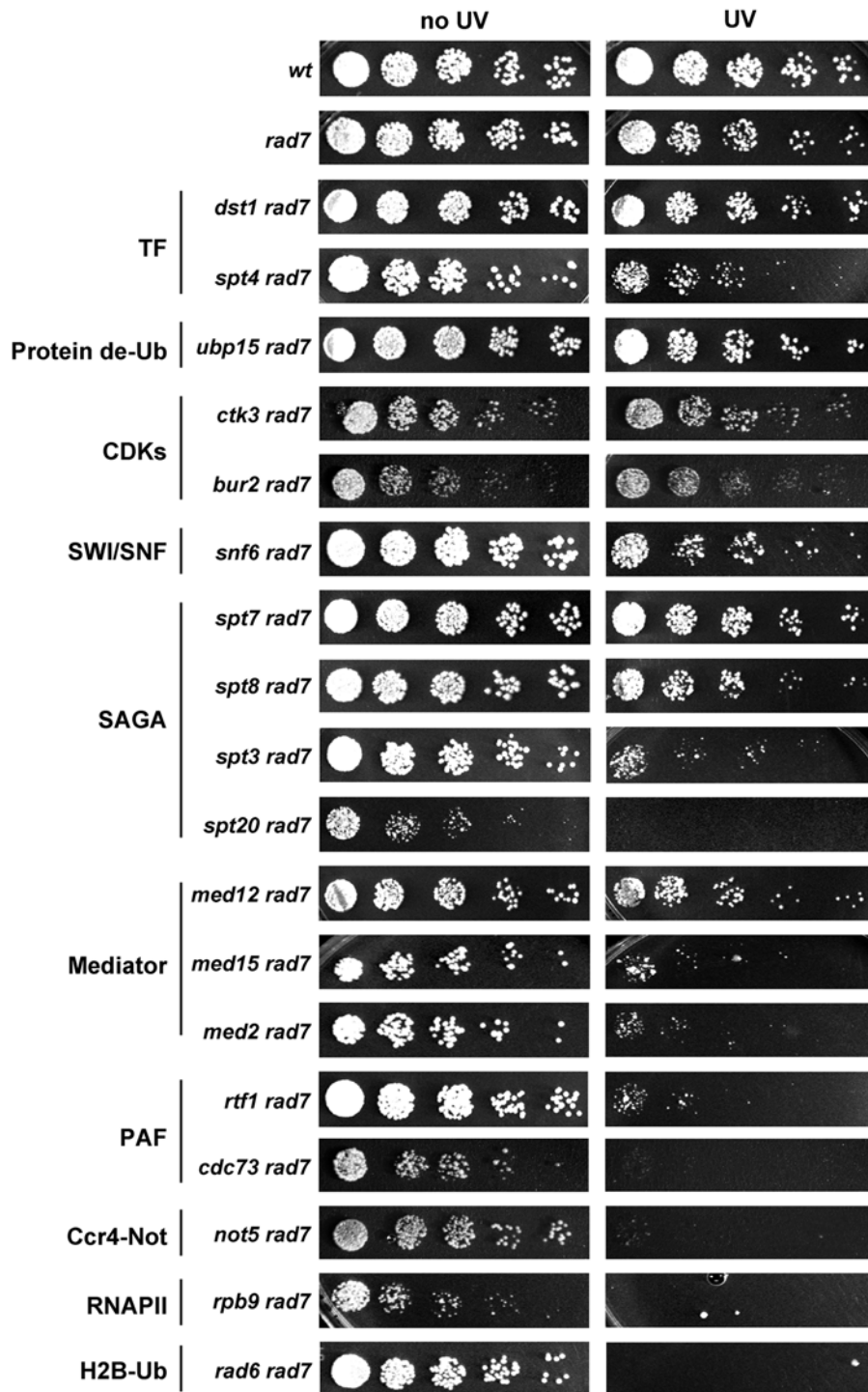**Figure 5. UV sensitivity in the absence of global genome repair in selected MPA-sensitive mutants.** UV sensitivity analysis of 18 mutant strains in which the *RAD7* gene has been disrupted. *rad7* and wild-type strains were used as control. Cell dilutions were dropped on YPDA plates, UV irradiated with 15 J/m^2 and grown at 30°C in the dark for 3 days.
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Both the PAF and the Ccr4-Not complexes have been previously linked to the DNA damage checkpoint pathway [34–37]. Therefore, we wondered whether the observed UV sensitivity might rely on checkpoint activation defects. Firstly, we checked the GLAM-ratios of cells lacking the DNA damage checkpoint protein Rad9 (Figure 2). No transcription defects were observed in this assay. Secondly, we performed UV survival curves of the DNA damage checkpoint *rad9Δ* and the *bre1Δ* mutants alone or in combination with the *rad7Δ* mutation (Figure 7A). The *rad9Δ* single mutant was sensitive to UV irradiation, as previously shown [38]. Deletion of the GG-NER factor Rad7 increased the UV sensitivity of *rad9Δ* cells. Together, these data indicate that a functional DNA damage checkpoint response is important for viability upon UV irradiation both in repair proficient and in GG-
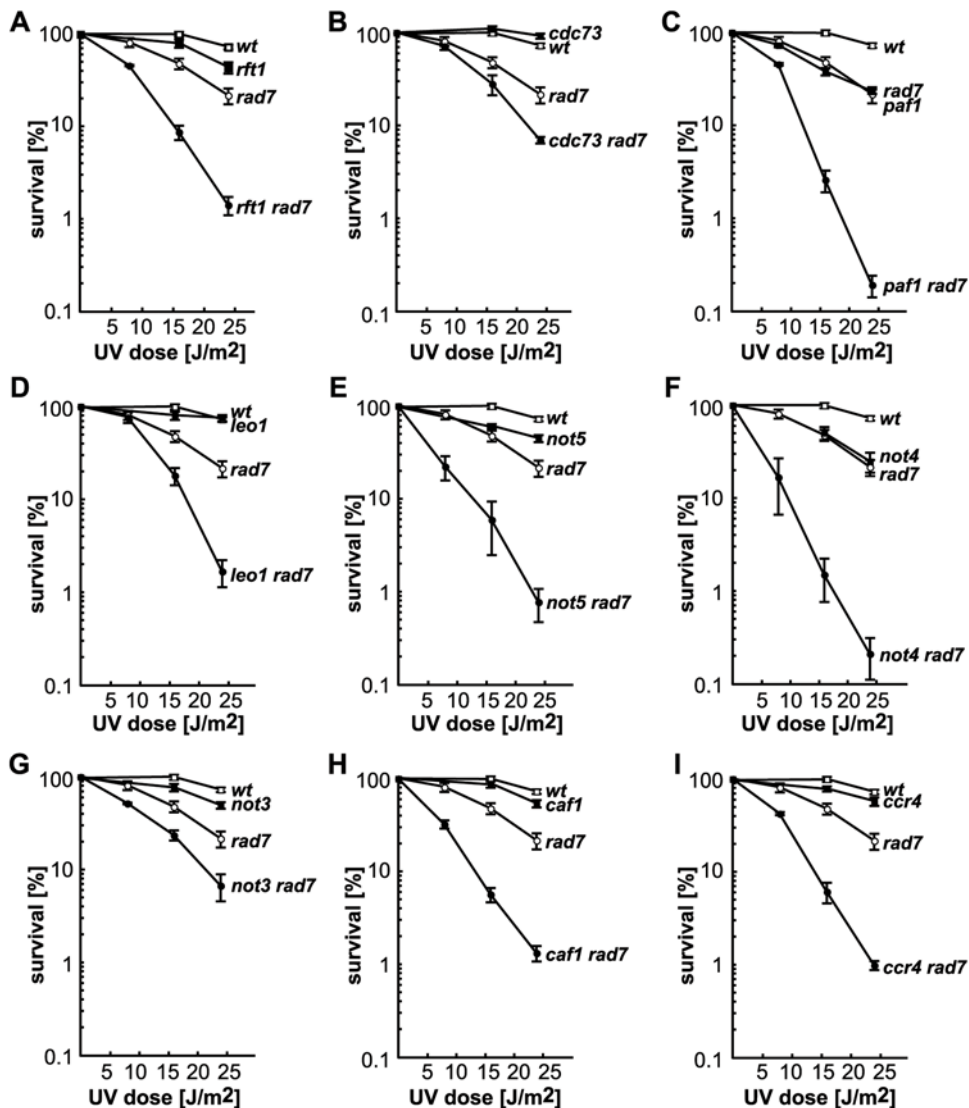
Figure 6. PAF and Ccr4-Not mutants are sensitive to UV in the absence of global genome repair. UV sensitivity curves of strains carrying single and double combinations of the (A) rtf1, (B) cdc73, (C) paf1, (D) leo1, (E) not5, (F) not4, (G) not3, (H) caf1, (I) ccr4, and rad7 mutations. Mutants shown in (A–D) are subunits of the PAF and mutants shown in (E–I) of the Ccr4-Not complex. Average values and standard deviations from 3 independent experiments are shown.
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NER deficient cells. Surprisingly, the *bre1Δ* mutant behaved differently, as the single mutant was not UV-sensitive while the *bre1Δ rad7Δ* double mutant was not more sensitive to UV irradiation than the *rad7Δ* single mutant. Finally, we analyzed the impact of the *rad9Δ* mutation on the UV survival of the *rtf1Δ*, *rtf1Δ rad7Δ*, *not5Δ*, and *not5Δ rad7Δ* strains. A similar set of UV survival curves were performed with the TC-NER mutant *rpb9Δ* as a control. As shown in Figure 7B, a synergistic effect was observed in the absence of GG-NER in mutants of the PAF and Ccr4-Not complexes, since the *rtf1Δ rad9Δ* and *not5Δ rad9Δ* mutants were as sensitive to UV irradiation as the *rad9Δ* mutant alone, while both the *rtf1Δ rad7Δ rad9Δ* and the *not5Δ rad7Δ rad9Δ* strains were significantly more sensitive to UV than the corresponding double mutants. In the TC-NER deficient *rpb9Δ* strains, a synergistic effect with *rad9Δ* was observed independently of the *rad7Δ* mutation. Consequently, the enhanced UV sensitivity of mutants of the PAF and Ccr4-Not complexes in the absence of GG-NER is not due to Rad9-dependent checkpoint activation failure.

Thus, because UV sensitivity in the absence of GG-NER is a phenotype mostly associated with TC-NER deficiencies, we tested whether PAF and Ccr4-Not are required for proficient TC-NER by monitoring the repair rates on the transcribed (TS) and non-transcribed (NTS) strands of the constitutively expressed *RPB2* gene. Molecular analysis of strand-specific removal of UV photoproducts was performed in *rtf1Δ* and *not5Δ* cells. Wild-type and TC-NER-deficient *tho2Δ* [14] strains were used as controls. Repair at various time points after UV irradiation was determined in a 4.4-kb *RPB2* restriction fragment by T4 endonuclease V (T4 endoV) digestion -resulting in ssDNA cleavage at CPD sites-followed by alkaline electrophoresis and indirect end-labeling with strand-specific probes (Figure 8). Non-irradiated and DNA not treated with T4 endoV show the intact restriction fragment. Repair of CPDs is visualized by a time-dependent increase of the T4 endoV-resistant fraction of restriction fragments. In *rtf1Δ* and *not5Δ* cells, repair of the TS was significantly reduced compared to wild-type level. As observed by UV sensitivity assays in the absence
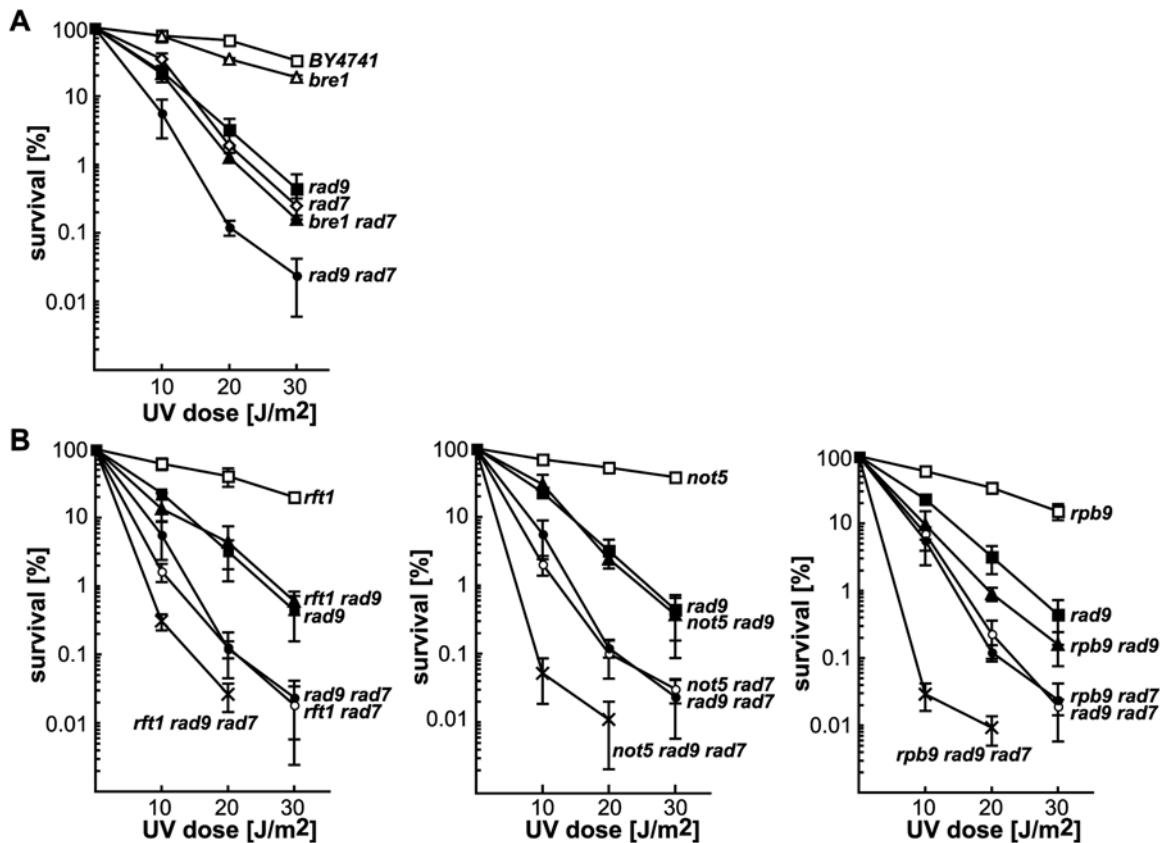
Figure 7. The increased UV sensitivity of PAF and Ccr4-Not mutants in the absence of global genome repair is not due to checkpoint activation failure. (A) UV sensitivity curves of strains carrying single and double combinations of the *rad9* or *bre1*, and *rad7* mutations. (B) UV sensitivity curves of strains carrying single, double, and triple combinations of the *rtf1*, *rad9*, and *rad7* mutations (left), the *not5*, *rad9*, and *rad7* mutations (middle), or the *rpb9*, *rad9*, and *rad7* mutations (right). Average values and standard deviations from at least 3 independent experiments are shown.
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of GG-NER (Figure 6), *not5Δ* cells were more strongly affected in TS repair than *rtf1Δ* cells. The repair deficiencies of these PAF and Ccr4-Not mutants were comparable to those of the TC-NER-deficient *tho2Δ* and *rad26Δ* strains. In the NTS, in contrast to the GG-NER-deficient *rad7Δ* strain, the repair levels of *rtf1Δ* and *not5Δ* were similar to wild-type and *tho2Δ* cells, indicating that GG-NER is not significantly affected in *rtf1Δ* and *not5Δ* cells.

A number of factors have been implicated in the repair of DNA lesions encountered by the RNAPII in eukaryotes, but our knowledge on the mechanisms of TC-NER is scarce. Since proteasome-mediated degradation of UV damage-stalled RNAPII complexes is believed to be alternatively required for DNA repair, we tested whether the effect of PAF and Ccr4-Not effect on TC-NER was dependent on RNAPII degradation. For this, we performed an epistatic analysis of the PAF mutant *rtf1Δ* with both the *def1Δ* and the *rpb9Δ* mutants, which are deficient in RNAPII degradation in response to UV in yeast [16,19]. As can be seen in Figure 9, a synergistic enhancement of the UV sensitivity was observed in both cases, the *rtf1Δ def1Δ* and the *rtf1Δ rpb9Δ* mutants being more sensitive to UV than the corresponding single mutants. Similarly, the *rtf1Δ def1Δ rad7Δ* and the *rtf1Δ rpb9Δ rad7Δ* triple mutants were more sensitive to UV irradiation than the corresponding double mutants. These results suggest that the TC-NER phenotype of PAF mutants is not due to an alteration of the Def1- or Rpb9-mediated degradation of UV damage-stalled RNAPII.

Taken together, our results place the PAF and the Ccr4-Not5 complexes as new factors needed for efficient TC-NER.

Discussion

Genome-wide analyses of the genes conferring resistance to MPA and 4-NQO allowed us to identify new putative candidates of genes involved in transcription elongation and TC-NER. Deletion of some subunits of the SAGA and Ccr4-Not complexes as well as Bur2 show defects in transcription elongation in all the tested assays, while deletion of the Bre1 and Fun12 proteins as well as some subunits of the Mediator complex show defects in transcription elongation as measured by MPA sensitivity, GLAM-ratios and *in vitro* transcription assay, but not by RNAPII occupancy. Notably, we provide genetic and molecular evidence that PAF and Ccr4-Not are required for efficient TC-NER in yeast. Sensitivity analysis of viable deletion mutants showed that the 4-NQO sensitive mutants were enriched in DNA repair genes, while the MPA sensitive mutants were enriched in both DNA repair and transcription genes, in agreement with the known effect of MPA on transcription elongation and 4-NQO on DNA repair. Out of the 25 mutations conferring sensitivity to both compounds, 13 correspond to genes involved in RNAPII transcription and mRNP biogenesis (see Figure 1). Interestingly, the large majority (10) of these mutants shows either TC-NER defects or increased UV sensitivity in the absence of GG-NER (this study; [11,14]).
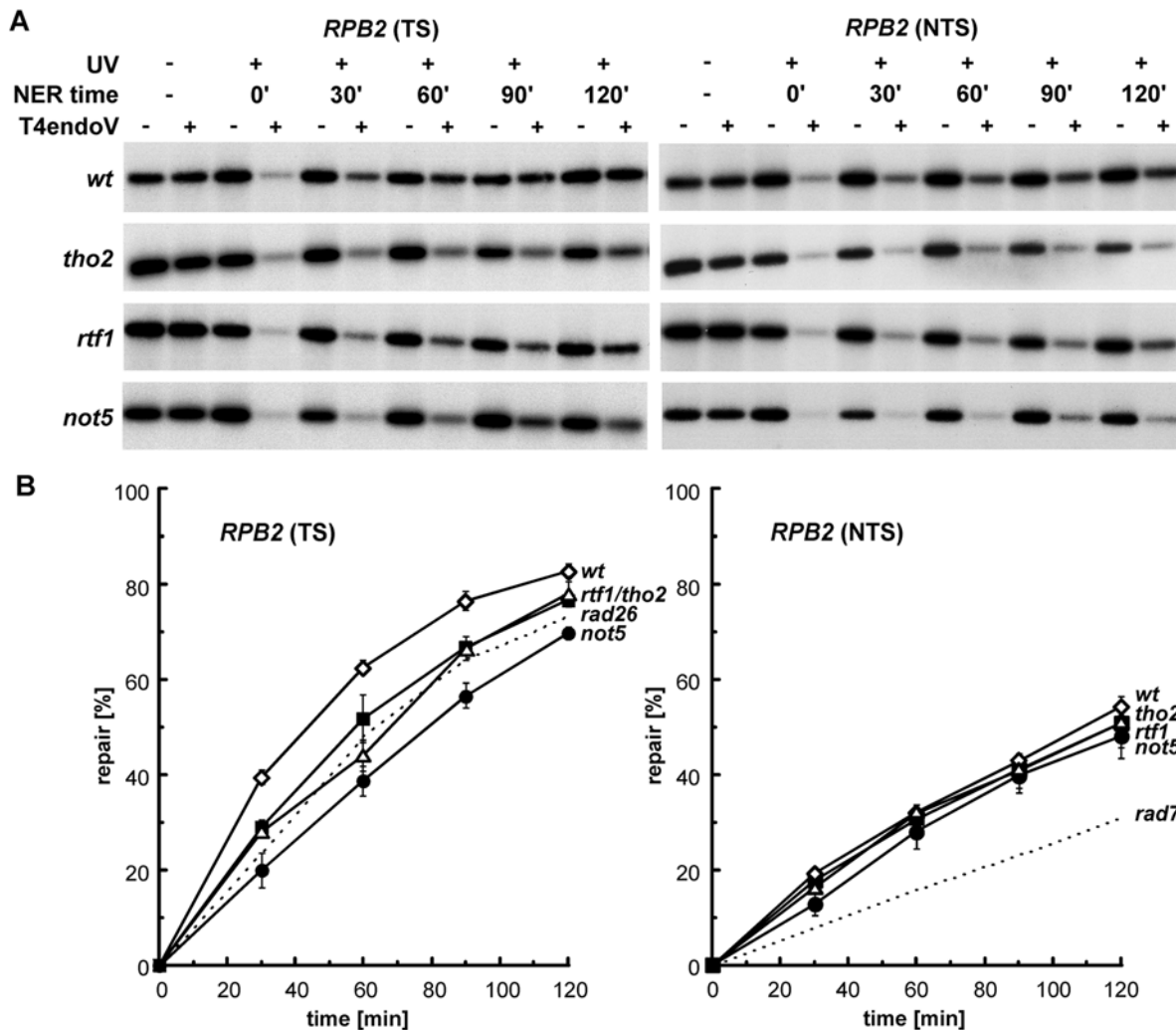
Figure 8. Transcription coupled repair is impaired in PAF and Ccr4-Not deficient cells. (A) Southern blot analysis showing repair of a 4.4 kb (*NsiI/PvuI*) *RPB2* fragment in *tho2*, *rft1*, *not5*, and wt cells. Initial damage was on the average 0.31±0.07 CPD/Kb in the transcribed strand (TS, left) and 0.26±0.06 CPD/Kb in the non-transcribed strand (NTS, right). The remaining intact restriction fragment after treatment of damaged DNA with T4endoV (+UV, +T4endoV) corresponds to the fraction of undamaged DNA. Non-irradiated DNA (−UV) and DNA not treated with T4endoV (−T4endoV) were used as controls. (B) Graphical representation of the repair analysis. The CPD content was calculated using the Poisson expression, −ln (RF$_a$/RF$_b$), where RF$_a$ and RF$_b$ represent the intact restriction fragment signal intensities of the T4endoV- and mock-treated DNA, respectively. Repair curves were calculated as the fraction of CPDs removed *vs.* repair time. Average values derived from two independent experiments are plotted. Repair curves of *rad26* and *rad7* (data taken from [14]) are depicted for the TS and the NTS, respectively (dash lines).
doi:10.1371/journal.pgen.1000364.g008

Previously Characterized Transcription-Initiation Factors with an Effect in Transcription Elongation

After identifying a number of mutants with impairment in the expression of long genes (see Figure 2), we showed that WCEs depleted of representative subunits of the SAGA, Mediator and Ccr4-Not complexes, as well as of the Bre1, Bur2, and Fun12 proteins, were impaired in transcription elongation (see Figure 3). In addition, RNAPII elongation defects were observed by ChIP analysis for mutants of the SAGA, Ccr4-Not, Bur2, and, in a different manner, Med15 (see Figure 4). Therefore, these factors seem to have a putative role in transcription elongation in addition to their previously reported roles in either transcription initiation or other gene-expression processes. Although further molecular and biochemical analyses are required to understand how these mutations affect transcription elongation, our knowledge of the function of these proteins, as discussed below, may provide insight into explaining some of these effects.

SAGA is a 1.8 MDa complex identified as a factor promoting transcription activation through histones H3 and H2B acetylation by its HAT domain [25,39]. One of SAGA's functions consists of the deubiquitylation of histone H2B by its Upb8/Sgf11 module [40]. Interestingly, Gcn5, which belongs to the HAT catalytic core, is recruited to actively transcribed genes in a RNAPII CTD-phosphorylation-dependent manner and promote transcription of a long ORF [41], indicating that SAGA's role is not confined to transcription initiation. Our *in vivo* gene expression analyses of different SAGA-subunit mutants are consistent with a putative defect in transcription elongation. Importantly, the *in vitro* transcription-elongation assay showed that *spt20Δ* extracts, in which the SAGA complex is disrupted, leads to significant elongation defects. Furthermore, RNAPII elongation defects were observed in *spt20Δ* cells by ChIP analysis of RNAPII. However, neither the *upb8* nor the *sgf11* mutants were significantly affected in our assays, suggesting that the H2B de-ubiquitylation module of
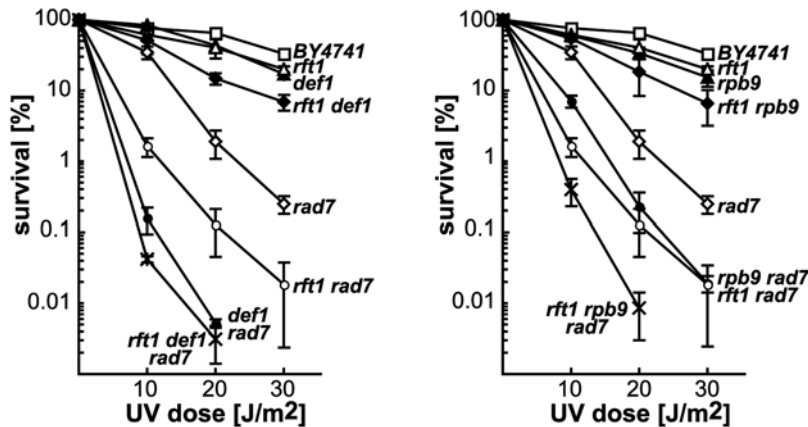
Figure 9. Synergistic increase of UV sensitivity phenotypes in *rft1 def1* and *rft1 rpb9* double mutants. UV sensitivity curves of strains carrying single, double, and triple combinations of the *rft1*, *def1*, and *rad7* mutations (left) or the *rtf1*, *rpb9*, and *rad7* mutations (right). Average values and standard deviations from at least 3 independent experiments are shown.
doi:10.1371/journal.pgen.1000364.g009

SAGA might be dispensable for transcription elongation. Our result portends that SAGA's effect in transcription elongation might not only rely on histone modifications, since the template used in the *in vitro* assay is devoid of histones, although the use of crude extracts does not exclude that some histone deposition occurs spontaneously on the template during the reaction.

Among the analyzed Mediator mutants, some were affected in expression of long genes while others were not. Notably, the affected mutants correspond to subunits of the tail (Med2 and Med15) or head (Med18, Med19, and Med20) domains [42]. The fact that the tested *med15Δ* mutant is impaired in transcription elongation *in vitro* and the observation that RNAPII accumulates toward the 3′-end of the analyzed gene in *med15Δ* cells supports the idea that Mediator influences transcription elongation in a different manner to other factors previously reported. A possible explanation might be that its effect on elongation arises from a stalling of RNAPIIs at the 3′-end of the gene. The putative involvement of Mediator in elongation is further substantiated by the recent findings that it binds to chromatin throughout the ORF of active genes [43]. Interestingly, while other Mediator null mutants showed no effect in our *in vivo* gene expression assay, deletion of the Srb11 subunit of the associated cyclin-dependent Ser/Thr protein kinase complex CDK8 or Med13, to which this domain is attached, appeared to increase the efficiency of expression, in agreement with the involvement of CDK8 in the negative regulation of gene expression [42,44].

Although transcription regulation by Ccr4-Not mostly occurs during the initiation stage, it seems likely that this complex control gene expression at several levels, including regulation of transcription initiation, mRNA deadenylation and degradation, and protein turnover [45–47]. Genetic interactions suggested that Ccr4-Not could also be involved in transcriptional elongation [27]. Our data support an involvement of Ccr4-Not in transcription elongation, as suggested by the deficiency of long gene expression conferred by most viable deletions (the exception is *not3*). Importantly, we show that *not5* cells are impaired in transcription elongation *in vitro* and show RNAPII elongation defects *in vivo*, as determined by ChIP analysis.

Ubiquitylation of histone H2B by Rad6-Bre1 is associated with active transcription [48] and is required for subsequent COM-PASS-mediated methylation of histone H3 [49]. Both chromatin modifications are controlled by PAF and the Bur1-Bur2 cyclin-dependent protein kinase, which associate with the elongating

RNAPII [50,51]. Interestingly, H2B ubiquitylation has been recently shown to be required for efficient reassembly of nucleosomes during transcription elongation [52]. Mutants of the Rad6-Bre1 ubiquitylation complex show a significant reduction in the expression of long ORFs *in vivo* and deletion of *BRE1* leads to elongation defects *in vitro*, suggesting that this complex has a role in transcription elongation. However, no significant alterations of RNAPII occupancy on a transcribed gene were observed by ChIP. Similar findings were obtained in *bur2* mutants, although in this case ChIP analyses also showed an RNAPII distribution consistent with an *in vivo* transcription elongation defect, alluding that the Bur1-Bur2 complex might also be involved in transcription elongation. These findings are in agreement with the previous report that Bur1 and Bur2 are both recruited to transcription elongation complexes [26].

Finally, the Fun12 translation initiation factor also affects transcription elongation in all assays but RNAPII occupancy measurement, although we show that this is not a general feature of translation initiation factors. Further studies would be required to better understand the function of Fun12 and how this might influence transcription elongation. In this sense, it is worth noticing that mutants showing elongation deficiencies in the *in vitro* transcription elongation assay but not in RNAPII ChIP assays have been reported previously, as it is the case of PAF complex mutants [31,53]. It is likely that the interconnection of transcription with other nuclear processes like RNA processing and export may be responsible for a different behavior of particular mutations *in vitro* and *in vivo*. Further development of novel transcription elongation assays may be required to solve such cases. In any case, the putative implication of a number of known transcription-initiation factors and other factors in transcription elongation opens new perspectives about the function of these proteins in transcription that demand further studies.

Novel Role for the PAF and Ccr4-Not Complexes in TC-NER

Among all factors analyzed, a striking observation of this study is that two known transcription factors, PAF and Ccr4-Not, have a novel role in TC-NER. Although evidence for a direct involvement of these complexes in TC-NER, as any other previously reported, would require the development of a TC-NER *in vitro* assay, the fact that PAF and Ccr4-Not work during transcription elongation makes it plausible that both complexes

affect TC-NER directly. The PAF complex is present at promoters and coding regions of all active genes tested and has been involved in transcription initiation and elongation, as well as in the 3′-end formation of polyadenylated and nonpolyadenylated RNAPII transcripts [49,54–56]. It is required for association of the H2B monoubiquitylating enzyme Bre1-Rad6 with hyperphosphorylated RNAPII and therefore for H2B monoubiquitylation [48,57], as well as for Set1 and Set2 recruitment and therewith for correct H3-K4 and H3-K36 methylation [50]. PAF also functions in the regulation of histone acetylation [58]. An involvement of histone modifications in the repair of UV damages by NER was found only for monoubiquitylation at H3-K79 [59] and for H3 and H4 histone acetylation [60,61]. Interestingly, residues in both the N- and C-terminal tail of H2A have been recently found important for UV survival [62]. Since PAF's role in transcription elongation is not confined to the regulation of histone modifications [53,63], and that *bre1Δ* cells were not sensitive to UV irradiation in the absence of GG-NER (see Figure 7), it remains to be determined whether the TC-NER phenotype of PAF mutants relies on some alteration of histone modifications.

In addition to genetic interactions (synthetic lethality) between the PAF and Ccr4-Not complexes [64], a functional interplay was inferred from the observation that both PAF and Ccr4-Not mutants give rise to HU-sensitivity and a deregulation of *RNR* gene transcription [36,65,66]. In addition, both the PAF and the Ccr4-Not complexes have been previously linked to the DNA damage checkpoint pathway. The PAF complex is responsible for the recruitment of the Bre1-Rad6 ubiquitylation complex to transcribed genes [48] and H2B ubiquitylation by the Bre1-Rad6 complex has been shown to be necessary for the activation of the DNA damage checkpoint [34]. The Ccr4-Not complex has been shown to promote cell cycle transition from G1 to S phase after ionizing radiation [35] and its deadenylase subunits Ccr4 and Caf1 were shown to influence Crt1 abundance *via* mRNA poly(A) tail length regulation, which in turn regulates the expression of a number of DNA-damage inducible genes. Furthermore, Ccr4 and Caf1 show complex genetic interactions with a number of DNA damage checkpoint genes in response to HU and MMS [37]. More recently, Ccr4 was reported to modulate the timing of gene expression of G1-phase cyclins [67], which are key regulators of the G1-S checkpoint.

Thus, the TC-NER deficiencies of mutants of the PAF and Ccr4-Not complexes could rely on some failure in the activation of the DNA damage checkpoint response. UV survival analysis of *rad9Δ* cells revealed that a functional DNA damage checkpoint response is important for viability upon UV irradiation both in repair proficient and in GG-NER deficient cells (see Figure 7). Our epistatic analysis of UV survival in double and triple combinations of *rad9Δ* with the *rtf1Δ*, *not5Δ*, or *rpb9Δ* mutations indicate that removal of the Rpb9 TC-NER factor as well as Rtf1 and Not5 increase the sensitivity of *rad9Δ rad7Δ* double mutants, suggesting that the enhanced UV sensitivity of mutants of the PAF and Ccr4-Not complexes in the absence of GG-NER is not due to Rad9-dependent checkpoint activation failure. These findings are in agreement with the observation that PAF and Ccr4-Not mutants are impaired in the repair of UV lesions exclusively on the transcribed strand of an active gene (see Figure 8), while *rad9Δ* has been previously reported to alter the repair efficiency of both the transcribed and the non-transcribed strands [68].

Interestingly, despite Bre1-Rad6 being required for Rad9-dependent DNA damage checkpoint activation [34] and *rad9* cells being sensitive to UV independently of GG-NER, *bre1* mutants were not sensitive to UV, not even in the absence of GG-NER. Thus, the Rad9 protein has functions in DNA damage checkpoint that are not dependent on the Bre1-Rad6 complex.

Recently, subunits of the Ccr4-Not complex were shown to be required for tri-methylation of H3K4 and PAF recruitment in a Bur1/Bur2-independent manner [69]. Notably, Ccr4 and Caf1, the two major yeast deadenylases [70], did not share this phenotype, suggesting a functional distinction between the cytoplasmic deadenylase activity of the Ccr4-Caf1 module and the nuclear function of the Not proteins of the Ccr4-Not complex. However, *CCR4* shows similar genetic interactions (synthetic lethality) with *BUR1* and *BUR2* as well as 6-AU sensitivity as *NOT2* and *NOT4* mutants do [27,69]. Our analysis of UV survival in the absence of GG-NER in viable Ccr4-Not mutants indicates that *caf1* and *ccr4* share the TC-NER phenotype of *not* mutants (see Figure 6). Furthermore, deletion of *BUR2* did not lead to increased UV sensitivity in the absence of GG-NER (see Figure 5), suggesting that the TC-NER deficiencies of Ccr4-Not mutants are not a consequence of misrecruitment of the PAF complex.

Is There a Link between Transcription Elongation Efficiency and TC-NER?

The finding that both the PAF and Ccr4-Not complexes lead to impaired TC-NER could suggest that transcription might be altered in a similar way in these mutants, this alteration being the cause for the inefficient TC-NER. Strikingly, all factors known to be required for TC-NER are also somehow involved in transcription elongation. Although original studies suggested that *RAD26*, the *CSB* yeast homolog, might be a transcription-repair coupling factor by analogy to the bacterial TRCF, this idea has not been validated. Instead, several studies have opened the possibility that Rad26/CSB might have a role in transcription elongation in the absence of DNA damage [71,72]. Mutants of the Rpb9 and Rpb4 RNAPII subunits, which both have an effect on transcription elongation, confer TC-NER phenotypes [11,12]. Mutants of the THO/TREX and Thp1-Sac3 complexes, which show impairment in transcription elongation, also lead to TC-NER deficiencies [14].

In light of these observations, it is conceivable that proficient transcription elongation might be a pre-requisite for efficient TC-NER. However, transcription-elongation mutants like *spt4* do not show defects in TC-NER [73]. On the contrary, *spt4* suppresses the TC-NER defects of *rad26*, indicating that reduced RNAPII elongation can even act positively on TC-NER. Another example arising from our study is *bur2*, which shows poor expression of long genes *in vivo* and has a general role in transcription elongation [49] but no increase in UV sensitivity in the absence of GG-NER. Thus, it appears that the TC-NER phenotype is not directly linked to elongation efficiency. Furthermore, analysis of TC-NER in a TFIIE mutant in which transcription is significantly reduced demonstrated that TC-NER occurs even at low levels of RNAPII transcription [74].

Another possibility is that the signaling of damage-stalled RNAPII to the repair factors might occur via post-translational modification of the RNAPII, such as ubiquitylation or CTD phosphorylation; and that this signaling might be affected in mutants leading to TC-NER phenotypes. The assumption that CTD phosphorylation might be important for TC-NER is supported by the finding that *KIN28* mutants –the TFIIH subunit with CTD kinase activity- are affected in TC-NER [75]. Along this line, we have proposed the existence of feedback mechanism acting on the RNAPII holoenzyme in response to mRNP biogenesis and export deficiencies associated with THO/TREX and Thp1-Sac3 mutations [14]. As a result of this feedback mechanism, the elongating RNAPII would no longer be proficient for TC-NER. Noteworthy, both the PAF and Ccr4-Not complexes have functions in mRNA 3′-end processing [54–56,76,77], which

might lead to a feed-back mechanism on the elongating RNAPII as proposed for THO and Thp1-Sac3 mutants.

Interestingly, bacterial TRCF has the ability to promote elongation of backtracked polymerases, resulting in polymerase release in case elongation cannot take place, as is the case at sites of UV lesions [78]. By analogy, it is conceivable that PAF and Ccr4-Not, and perhaps THO and Thp1-Sac3 complexes, might also be capable of such anti-backtracking activity. Finally, RNAPII is subject to ubiquitylation and proteasome-mediated degradation in response to UV-generated DNA damage [4]. Degradation of damage-stalled RNAPII complexes is believed to be alternatively required for DNA repair and depends on the Def1 and the Rpb9 proteins in yeast [16,19]. Our epistatic analysis of the PAF mutant *rft1* and the *def1* and *rpb9* mutants (see Figure 9) suggest that PAF is not acting in the degradation of stalled RNAPII upon UV irradiation. Further studies will be required to understand how PAF and Ccr4-Not controls TC-NER.

In conclusion, our study open new perspectives to understand TC-NER by providing evidence for TC-NER being a process intimately linked to transcription elongation, a number of specific transcription factors having a dual role in transcription and TC-NER.

Materials and Methods

Strains and Plasmids

All strains used were purchased from Euroscarf and are isogenic to BY4741. The *rad7Δ::URA3* strains were obtained by direct replacement of the *RAD7* gene. The *rtf1Δ::KAN rad9Δ::KAN* and *rft1Δ::KAN rpb9Δ::KAN* strains were obtained by genetic crosses. The *not5Δ::KAN rad9Δ::HYGR* strains were obtained by direct replacement of the *RAD9* gene. The *rpb9Δ::HIS3 rad9Δ::KAN* strains were obtained by direct replacement of the *RPB9* gene. The *def1Δ::HYGR* strains were obtained by replacement of the *DEF1* gene in *rft1Δ::KAN rad7Δ::URA3* diploids and subsequent tetrad dissection. Plasmids pCYC1-402 [30] and pCM184-LAUR [32] were described previously.

Expression Profiling Experiments

Yeast cells were grown at 28°C in 10 ml YPD medium to an OD_{660} of 0.6. The appropriate compounds were added to reach a final concentration of 75 ng/ml (4-NQO) or 50 μg/ml (MPA). Cell harvesting, purification of total RNA and transcriptional profiling were performed using DNA microarrays containing PCR-amplified fragments of *S. cerevisiae* ORFs as previously described [79]. For each condition assayed three independent experiments were performed and dye-swapping was carried out in each case. Data were combined and the mean calculated yielding 2374 genes significant for statistical analysis. Induction or repression was considered significant when the mean of the ratios was at least 2-fold above or below mock treated cells.

High-Throughput Screen for MPA and 4-NQO Sensitivity

The screenings of the complete collection of yeast haploid mutant strains were performed as described [80], using MPA (25 μg/ml), or 4-NQO (150 ng/ml).

In Vivo Gene Expression Analysis

An indirect measurement of *G*ene *L*ength *A*ccumulation of *m*RNA, GLAM ratios were determined by measuring acid phosphatase of plasmid gene expression constructs sharing the same "short" transcription unit (GAL1pr::PHO5), but differing in the 3′UTR of the "long" one (GAL1pr::PHO5-lacZ and GAL1pr:: PHO5-LAC4) as described [24].

In Vitro Transcription Elongation Assay

Yeast cells were grown in rich YEPD medium at 30°C to an OD_{600} of 1 and WCEs were prepared as described [30]. Each *in vitro* transcription reaction was performed with 100 mg WCEs and 100 ng of purified Gal4-VP16, using as template the pCYC1-402 plasmid containing the two G-less cassette used to assay transcription elongation.

ChIP Assay

Yeast cells harboring pCM184-LAUR were grown in synthetic complete medium (SC) medium at 30°C to an OD_{600} of 0.5. Samples were taken and ChIP assay were performed as described previously [81]. Primer sequences are available upon request.

UV Survival Curves and Drop Assays

UV survival curves were performed as described [14]. UV irradiation was performed using germicidal lamps (Philips TUV 15 W) and a UVX radiometer (UVP) for the curves shown in Figure 6, and in a BS03 UV irradiation chamber and UV-Mat dosimeter (Dr. Gröbel UV-Elektronik GmbH) for the curves shown in Figures 7 and 9. For the drop assays, yeast cells were grown in YPD-rich medium to an OD_{600} of 0.7. Serial dilutions (100-, 500-, 1000-, 5000-, and 10000-fold) were dropped on YPAD plates, irradiated with 15 J/m^2 UV light, and incubated in the dark at 30°C for 3 days.

Gene- and Strand-Specific Repair Assays

Irradiation and repair at the *RPB2* gene, mapping of CPDs, and analysis and quantification of membranes were carried out as described [14].

Supporting Information

Figure S1 Correlation between PHO5-lacZ/PHO5 and PHO5-LAC4/PHO5 gene expression ratios (Pearson coefficient of 0.75). Values plotted are those shown in Table S4.
Found at: doi:10.1371/journal.pgen.1000364.s001 (0.45 MB EPS)

Table S1 Analysis of genes whose expression is affected by 4-NQO and MPA. Expression of a total of 2374 genes was determined by microarray analysis after treating wild-type cells with either 75 ng/ml of 4-NQO or 50 μg/ml of MPA for 30 min each. The genes showing mRNA levels that were at least 2-fold above or below mock treated cells for each treatment are listed. The complete microarray data are available at http://www.ncbi.nlm.nih.gov/geo/ under the access number GSE11561.
Found at: doi:10.1371/journal.pgen.1000364.s002 (0.06 MB PDF)

Table S2 Analysis of mutant strains leading to 4-NQO sensitivity. Growth was monitored after 48, 72, and 120 hours in 4-NQO-containing media (150 ng/ml) and the sensitive strains classified into three groups. Group A contains 189 strains whose sensitivity to 4-NQO is observed from early on (48 h) and is maintained over the course of the experiment (up to 120 h for group A1, and up to 72 h for group A2), group B contains 315 strains whose sensitivity was observed only at early time points (48 h), and group C contains 100 slow-growing strains which showed 4-NQO sensitivity only at later time points (from 72 h on and up to 120 h). The strains of group B were not considered as significantly inhibited by 4-NQO and excluded from further analyses. Values between 60% and 80% and above 80% inhibition are highlighted (orange and red, respectively).
Found at: doi:10.1371/journal.pgen.1000364.s003 (0.07 MB PDF)

Table S3 Genetic analysis of sensitivity to DNA-damaging agents. Thirty 4-NQO-sensitive strains of group A that encompassed mutations of the SAGA, PAF, CDKs, and Mediator complexes as well as proteins involved in RNAPII transcription, mRNA processing and degradation, chromatin remodeling, DNA-damage response, and translation initiation were analyzed for drug sensitivity. The indicated strains were spotted as 10-fold serial dilutions on complete minimal medium (SC) and minimal medium containing 0.1 μg/ml 4-nitroquinoline-N-oxide (4-NQO), 0,015% methyl methane sulfonate (MMS), or 0.1 nM menadione (Mnd). UV sensitivity (UV) was assessed following irradiation with 70 J/m². Plates were grown for 3 days at 30°C or 37°C and cell viability for each condition was scored as + for growth as wild-type, as +/− for moderate growth defects, and as - for severe growth defects. Most of the UV sensitive strains were also sensitive to 4-NQO, consistent with the fact that the bulky adduct produced by 4-NQO get repaired by NER (exceptions are *ctk2*, *spt5*, *bdf1*, *lsm1*, *npl6*, and *fps1*). However, 9 of the 4-NQO sensitive strains were not sensitive to UV (*spt20*, *bur2*, *med2*, *med16*, *med20*, *hpr1*, *spt4*, *rpb9*, and *swi6*), indicating that 4-NQO is more than a 'UV-mimetic' agent. Those strains appeared mainly sensitive to Mnd, reflecting their deficiencies in the presence of oxidative damage. Comparison of strain sensitivity to 4-NQO, MMS, Mnd, and heat stress did not lead to significant clustering of cross-resistance, indicating that each drug leads to its own response, as observed previously for 15 DNA-damaging agents including MMS and 4-NQO [21,82].
Found at: doi:10.1371/journal.pgen.1000364.s004 (0.09 MB DOC)

Table S4 Analysis of mutant strains leading to MPA sensitivity. Growth was monitored after 72 hours in MPA-containing media (25 μg/ml). Strains showing at least 50% growth inhibition are listed together with their GLAM-ratios. Values above 60% inhibition and ratios below 0.5 are highlighted in red. n.d., not determined.
Found at: doi:10.1371/journal.pgen.1000364.s005 (0.03 MB PDF)

Acknowledgments

Author Contributions

Conceived and designed the experiments: HG JB LV ARG JA JLR SC AA. Performed the experiments: HG CT JB CGA MJQ LV MLGR ARG. Analyzed the data: HG AM JA JLR SC AA. Contributed reagents/materials/analysis tools: AM. Wrote the paper: HG SC AA.

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