selected parasites that lost expression of the integrated thymidine kinase marker. We subcloned ganciclovir-resistant and pyrimethamine-sensitive parasites from this selection to produce T. gondii strains, cp1-1 and cp2-1. Uracil auxotrophs cp1-1 and cp2-1 have two tandem copies of the targeting plasmid integrated into the CPSII locus. Only the CPSII locus was disrupted, and integration was achieved by homologous recombination in CPSII sequences on the 5’ side of the BamHI sites of the 6.6-kb CPSII HindIII fragment (data not shown). We maintained the uracil auxotrophs in culture in medium supplemented with 0.2 mM uracil.

**Murine virulence assay**

We obtained tachyzoites by allowing infected HFF monolayers to lyse completely. Tachyzoites were purified by filtration through sterile 3-μm nucleopore membranes, washed in PBS and collected by centrifugation. We resuspended tachyzoites pellets in PBS and counted them under the microscope. Tachyzoites were injected intraperitoneally (i.p.) in 0.2 ml into mice 6–8 weeks. The actual p.f.u. in the inoculum was determined by plaque assay. In all of the mouse injection experiments and for all of the parasite strains tested, the p.f.u./parasite ratio was between 0.4 and 0.6. We used four mice per parasite strain.

**Competing interests statement**

The authors declare that they have no competing financial interests.

Correspondence and requests for materials should be addressed to D.J.B. (e-mail: David.J.Bizik/Dartmouth.edu). The sequence of the 6.6-kb CPSII HindIII fragment has been deposited with GenBank (accession code AY059630).

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**A Rad26–Def1 complex coordinates repair and RNA pol II proteolysis in response to DNA damage**


Eukaryotic cells use multiple, highly conserved mechanisms to contend with ultraviolet-light-induced DNA damage. One important response mechanism is transcription-coupled repair (TCR), during which DNA lesions in the transcribed strand of an active gene are repaired much faster than in the genome overall. In mammalian cells, defective TCR gives rise to the severe human disorder Cockayne’s syndrome (CS). The best-studied CS gene, CSB, codes for a Swi/Snf-like DNA-dependent ATPase, whose yeast homologue is called Rad26 (ref. 4). Here we identify a yeast protein, termed Def1, which forms a complex with Rad26 in chromatin. The phenotypes of cells lacking DEF1 are consistent with a role for this factor in the DNA damage response, but Def1 is not required for TCR. Rather, def1 cells are compromised for transcription elongation, and are unable to degrade RNA polymerase II (RNAPII) in response to DNA damage. Our data suggest that RNAPII stalled at a DNA lesion triggers a coordinated rescue mechanism that requires the Rad26–Def1 complex, and that Def1 enables ubiquitination and proteolysis of RNAPII when the lesion cannot be rapidly removed by Rad26-promoted DNA repair.

In order to purify Rad26, yeast whole-cell extract derived from a strain expressing Myc-decahistidine tagged Rad26 (MRad26) was fractionated into a DNA-free ‘soluble’ fraction and a salt-stable chromatin fraction as previously described. MRad26 partitioned approximately equally between these fractions and was purified to homogeneity by a combination of conventional and affinity chromatography. As can be seen in Fig. 1a, there was a noticeable difference between the polypeptide composition of Rad26 isolated from the soluble fraction and that isolated from chromatin after high salt extraction. Silver staining of MRad26 isolated from the soluble fraction as previously described. As can be seen in Fig. 1a, there was a noticeable difference between the polypeptide composition of Rad26 isolated from the soluble fraction and that isolated from chromatin after high salt extraction. Silver staining of MRad26 isolated from the soluble fraction as previously described.

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**Supplementary Information**

accompanies the paper on Nature’s website (http://www.nature.com).
acrylamide gel electrophoresis (PAGE) and silver staining revealed that Def1HH from the soluble fraction was a single polypeptide (Fig. 1b). In contrast, essentially all the Def1HH protein extracted and isolated from chromatin was associated with MHRad26 (Fig. 1c). We note that the chromatographic behaviour on MonoQ of Rad26–Def1 purified from chromatin (upper panel, elution peak in fraction 14) was clearly distinct from that of free Rad26 purified from the soluble fraction (lower panel, peak in fraction 11), confirming the association of the proteins in a true complex.

The amino-acid sequence of Def1 predicts a protein with unusually extensive regions of low complexity, such as a large region with homology to coiled-coil domains and a very high glutamine content over almost the entire protein. The high glutamine content probably explains the aberrant gel-electrophoretic properties of the protein, and the extensive regions of low complexity means that a convincing metazoan homologue of the protein has not yet been identified by database searching.

To explore the in vivo function of Def1, the DEF1 gene was deleted in different genetic backgrounds and the resulting strains were subjected to phenotypic analysis. def1 mutants were viable, but slow growing. Because Def1 was isolated as a Rad26-associated protein, we first investigated whether its absence conferred a rad26-like phenotype (Fig. 2). RAD26 deletion does not confer ultraviolet light (UV)-sensitivity on its own, but significantly increases the UV-sensitivity of a rad16 (or rad7) strain2. We found that def1 strains, as well as rad26 def1 double-mutant strains were only slightly UV-sensitive, while def1 mutation dramatically increased the UV-sensitivity of a rad16 strain (Fig. 2a). We note that the rad16 def1 double mutant was even more UV-sensitive than a rad14 strain, which is completely defective in nucleotide excision repair (NER)1. In addition, def1 mutation increased the UV-sensitivity of a rad14 strain (Fig. 2b), but (like rad26) failed to increase the UV-sensitivity of strains defective in other repair pathways, such as recombination repair (rad52) and the damage tolerance/post-replication repair (rad6/rad18) pathway (Fig. 2c, and data not shown). These genetic interactions establish a connection between DEF1 and NER, and indicate that Def1 has a function during DNA damage.

We next investigated whether def1 cells have defects in preferential repair of the transcribed strand of an active gene, and whether def1
mutation might affect transcript elongation (Fig. 3). Transcription-coupled repair of the RPB2 gene has previously been shown to be severely reduced in rad26 strains\(^7\). By contrast, def1 cells exhibited more or less normal TCR of RPB2 because they preferentially repaired the transcribed strand of an active gene with initial rates of repair almost indistinguishable from those of the wild type (Fig. 3a, b). In contrast to the rad26 rad16 double mutant, which has a TCR level at RPB2 that is even lower than that of rad26 cells\(^7\), def1 rad16 double mutants and def1 rad16 rad26 triple mutants had initial TCR rates which were indistinguishable from those of the respective parental strains containing DEF1 (data not shown). These data indicate that def1 mutation does not significantly affect the rate of TCR.

Several observations indicated a role for Def1 during transcript elongation (Fig. 3c–e). First, def1 cells were as sensitive to the transcript elongation inhibitor 6-azauracil (6AU) as strains lacking the prototype elongation factor, TFIIIS\(^8\) (coded for DST1). Second, def1 dst1 double mutants were extremely 6AU sensitive (Fig. 3c). Moreover, whereas def1 and dst1 single mutants grew at both 30\(^\circ\)C and 37\(^\circ\)C, the double mutant grew very slowly at 30\(^\circ\)C and was unable to grow at the elevated temperature (Fig. 3d). Several alleles of the RPO21 (RPB1) gene, coding for the largest subunit of RNAPII, that are compromised for transcript elongation were previously isolated\(^9,10\). Combination of def1 mutation with four of these alleles, rpo21-7, -17, -18, and -23 was lethal or gave rise to a new phenotype (Fig. 3e, and data not shown), in further support of the notion that DEF1 influences transcript elongation by RNAPII.

One model that could explain the results described so far would be that cells degrade irreversibly stalled RNAPII as a last resort when a transcription block, such as a DNA lesion, cannot be repaired or bypassed. We therefore investigated the possibility that Def1 is required for proteolysis of RNAPII in response to DNA damage (Fig. 4a). As expected from previous studies\(^11,12\), RNAPII was degraded in response to UV-irradiation in wild-type cells. Surprisingly, UV-induced RNAPII degradation occurred more rapidly and to a greater extent in rad26 cells than in wild-type cells. Given the greatly reduced rate of TCR in rad26 cells, this result indicates that RNAPII degradation in itself is not sufficient for TCR, as has been suggested\(^13\). In contrast, cells lacking DEF1 did not degrade RNAPII at all, leading to apparent accumulation of the protein in response to DNA damage. Damage-induced RNAPII degradation did not require functional NER or damage tolerance pathways, as it still took place in rad14 and rad18 cells, respectively (data not shown). Significantly, the deletion of Rad26 re-activated RNAPII degradation in def1 cells (Fig. 4a). Together, these results demonstrate that Def1 is absolutely required for damage-induced degradation of RNAPII in the presence of Rad26, and, conversely, that Rad26 protects RNAPII from degradation during DNA damage.

We finally investigated the mechanism of Def1-dependent RNAPII degradation. In yeast, the ubiquitin ligase Rsp1 has previously been shown to be required for UV-induced RNAPII degradation through the ubiquitin-mediated degradation pathway\(^12\). We detected monoubiquitinated Rpb1 protein in both wild-type and def1 cells in the absence and presence of UV-irradiation (Fig. 4b). In contrast, a polyubiquitinated Rpb1 intermediate was only detected in wild-type cells in response to a damage-inducing dose of UV-

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**Figure 3** The effect of DEF1 deletion on TCR and transcript elongation. **a**, Repair of DNA lesions in the transcribed (TS) and non-transcribed strand (NTS) of an active gene. Damage-dependent T4 endonuclease V restriction gives a measure of remaining lesions. **b**, The result from **a** and those from two other independent experiments were quantified. The graphs represent averages of these three experiments. The stippled line shows the slower repair of the transcribed strand in rad26 cells for reference. This line represents the average of more than eight independent experiments\(^7\) and is shown without error bars for clarity. **c**, Strains of the indicated genotype were grown in the absence of uracil (control), or in the absence of uracil and presence of 6-azauracil (6AU). A 6AU concentration (50 mM) to which the single mutants were only moderately sensitive was used to demonstrate the hyper-sensitive phenotype of the def1 dst1 double mutant. **d**, Strains of the indicated genotype were grown at either 30 or 37\(^\circ\)C. **e**, The effect of combining DEF1 deletion with rpo21 alleles\(^5,10\) was tested in def1 rpo21 cells carrying RPO21 on a URA3-marked plasmid and the indicated rpo21 allele on a TRP-marked plasmid. Survival in the absence of the wild-type RPO21 allele was tested on 5-FOA-containing synthetic media. Besides the synthetic phenotypes shown here, def1 rpo21-7 (ref. 9) was also inviable, and def1 rpo21-23 (ref. 9) failed to grow on rich medium containing 1 M NaCl (data not shown).
irradiation (Fig. 4b, compare lane 1 and 2). Significantly, UV-irradiation failed to give rise to polyubiquitinylated Rpb1 in def1 cells (lane 4), indicating that Def1 is required for targeting RNAPII for degradation via the ubiquitin-mediated proteolysis pathway.

Taken together, our results suggest that cells have a dual response to DNA damage-stalled RNAPII. According to this model, stalling of RNAPII leads to the recruitment of not only transcript elongation factors14, such as TFIIH, but also the transcription-coupling repair factor Rad26, and Def1. Rad26 and Def1 might play an important role when the stall is persistent, such as at DNA lesions. Our data support the notion that stalled RNAPII and/or DNA damage leads to the establishment of a stable, DNA-associated Rad26–Def1 complex. The chromatin-specific association of Rad26 and Def1 is reminiscent of the Elongator–RNAPII interaction, whose stability is dependent on a hyper-phosphorylated state of the RNAPII carboxy-terminal domain5, but the precise mechanism of Rad26–Def1 complex formation and maintenance requires further investigation.

The activity of the Swi/Snf-like Rad26 protein might enable an alteration of the structure of the RNAPII–DNA damage interface, allowing TCR and resumption of transcript elongation. However, when this is not possible and RNAPII is left permanently trapped, a more drastic action might be required. Here, Def1 would be needed for removal of RNAPII by ubiquitin-mediated degradation. This would provide an attractive explanation for the UV-sensitivity data, such as the extraordinary sensitivity of def1 rad6 and def1 rad4 double mutants: in the absence of both NER and Def1-mediated RNAPII degradation, induction of even a few DNA lesions becomes lethal.

We noted that cells lacking Rad26 efficiently degrade RNAPII in response to UV-damage, whereas cells lacking Def1 still preferentially repair lesions in the transcribed strand of an active gene, indicating that even though Rad26 and Def1 form a complex they do not absolutely require each other for function. However, rad26 cells, as well as rad14 cells (data not shown), degrade RNAPII faster than the wild type in response to UV-damage, and Def1 is no longer absolutely required for RNAPII degradation in the absence of Rad26. This indicates that DEF1-dependent RNAPII degradation is not merely a byproduct of another DEF1 function, and also suggests that an important function of Rad26 might be to protect RNAPII from degradation to allow time for repair and that Def1 is absolutely required to overcome this inhibition. When Def1 is absent, there is nothing to counteract the degradation-inhibitory effect of Rad26, and no RNAPII degradation occurs. By contrast, in the absence of Rad26, Def1 can mediate very rapid degradation of RNAPII. Finally, when both Rad26 and Def1 are absent, this regulatory component of the damage response is gone, and Def1-independent degradation is observed. On the basis of the biochemical evidence it seems reasonable to assume that Rad26 and Def1 regulate their respective activities by forming a functional complex.

An involvement of DEF1 in the overall cellular damage response has recently been indicated by the finding that it is one of some 200 yeast genes that are upregulated under 26 cell-damaging conditions, a surprisingly large fraction of which code for protein degradation factors15. Besides shedding light on the molecular role of Def1 in the overall cellular DNA-damage response, our data also indicate a role for the factor in RNAPII transcript elongation. It is thus important to note that even though Def1 might not be an elongation factor in the classical sense, cells lacking DEF1 have several phenotypes that indicate elongation defects even in the absence of UV-induced DNA damage. Def1 also may play a role in other cellular processes, because only a minor fraction of the protein is associated with Rad26, whereas approximately half of cellular Rad26 was found in a complex with Def1. The phenotypes of def1 and def1 dst1, as well as the lethal consequence of deleting DEF1 in RNAPII elongation mutants suggest a requirement for either TFIIH-mediated backtracking or Def1-mediated RNAPII ubiquitination/removal for overall efficient transcription. We also note that ubiquitinated proteins are recruited to the 26S proteasome via components of the 19S regulatory particle16, and that the 19S complex has recently been shown to affect transcript elongation as well as DNA repair, independent of proteolysis17–19. It is an important future task to determine whether the role of Def1 in elongation occurs via degradation of stalled polymerases, or whether it might affect elongation via the intriguing degradation-independent function of the 19S regulatory complex.

Given that UV-induced RNAPII ubiquitination and degradation has been reported in both yeast and mammalian cells11,12,20–22, it is likely that Def1 function has also been conserved in evolution. In this connection, we note that while yeast cells lacking Rad26 have a phenotype that cannot easily be distinguished from that of wild-type cells, mutation of the CSB counterpart results in a severe syndrome in humans5. Cultured cells from CS patients are UV-sensitive23, completely unable to perform TCR24, and exhibit a severe deficiency in recovery of RNA synthesis after DNA damage25. These cellular phenotypes are either absent or less pronounced in yeast rad26 mutants. One possible explanation for the noticeable differences between the phenotypes observed in yeast and human cells could be that the function of CSB and the presumed Def1 homologue are both compromised by CSB mutation, whereas Rad26 and Def1 can perform their functions more or less independently in yeast. In support of this explanation, gel-filtration experiments have shown that in contrast to yeast Rad26, human CSB normally exists in a large protein complex8, which might contain human Def1. It is thus likely to be significant that RNA polymerases remain stalled at a site of damage for longer than usual in CS cells27,28, rather than being quickly degraded as appears to happen in yeast. Indeed, CSB cells have defects in RNAPII ubiquitination20, and are compromised21, although not fully defective21, for RNAPII degradation. On the basis of the phenotypes of yeast def1 cells reported here, we suggest that this compromised ability to remove RNAPII by degradation, as an alternative to TCR, is an important contributing factor to the severe consequences for genome integrity observed in cells from patients suffering from Cockayne’s syndrome27,28.

Figure 4 DEF1 is required for UV-induced ubiquitination and degradation of Rpb1. a. Degradation of RNAPII in response to UV irradiation. Western blots probed with the antibodies indicated on the right are shown. Similar results were obtained whether Rpb1 was detected with antibody directed against hyper-phosphorylated or hypo-phosphorylated C-terminus Rpb1. Tubulin acts as a loading control. Coomassie-staining of total protein extracts further demonstrated that protein degradation was not general, but specific for RNAPII, as previously reported22. b. Ubiquitination of RNAPII immunoprecipitated from UV-irradiated cells. Western blots probed with the antibodies indicated on the right are shown. Arrows on left indicate slower-migrating polyubiquitinated Rpb1 forms.

Methods

Yeast manipulation

Yeast manipulation was done as previously described. Strains expressing amino-terminal tagged Rad26 (Myc-decachistidine-TEV-Rad26, MHRad26), and/or carboxyl-terminal
His-HA tagged Def1 (Def1-decachistidine-HA, Def1H1H) had wild-type phenotypes. For purification purposes, tagging was done in the protease-deficient PV36 strain. Details of affinity tagging are available on request.

To construct def1 p202 strains, DEF1 was replaced with the LEU2 marker in YF2277 (ref. 9) (containing the wild-type RPO21 gene on a URA3 CEN plasmid). These cells and control YF2277 cells were transformed with TRP1 CEN plasmids expressing p202 alleles. ura3 cells were selected on 5-FOA-containing media.

**Protein purification and identification**

During purification, Def1H1H was detected by 12CA5 (anti-HA) antibody, whereas MHRad26 was detected either by 9E10 (anti-Myc) antibody or by a polyclonal Rad26 antibody. Yeast extract preparations and chromatography of proteins on resins such as Bio-Rex-70 (BioRad), nickel-agarose (Qiagen), DEAE Sepharose (Pharmacia), and M MonoQ H5/5 (Pharmacia) was done as previously described for other factors3. Approximately 1 kg of yeast paste from the strains indicated above was processed. Rad26 from the soluble (DNA-free) fraction was eluted in the 600 mM step from Bio-Rex-70. After nickel-agarose chromatography, eluted proteins were bound to 9E10-adsorbed protein A-Sepharose (Pharmacia), washed with A-500 (ref. 5), and eluted with either 150 mM NaCl, 100 mM glycine pH 2.5; or by tobacco etch virus (TEV) protease digestion according to the manufacturers’ instructions (Gibco Life Technologies). Def1H1H from the soluble fraction was recovered in BioRex-70 flow-through and was loaded directly onto a DEAE Sepharose resin. After washing with B-150 (ref. 5), proteins were eluted with B-1000 (lacking DTT and EDTA) containing 200 mM imidazole, the eluate was recovered in BioRex-70 flow-through and was loaded directly onto a DEAE Sepharose resin. After washing with B-150 (ref. 5), proteins were eluted with B-1000 (lacking DTT and EDTA) and incubated with nickel-agarose overnight. After elution with B-500 (lacking DTT and EDTA) containing 200 mM imidazole, the eluate was adsorbed to 12CA5-adsorbed protein A-Sepharose, washed with A-300, and eluted as described4. Purification from the chromatins fractions was done as follows: release of proteins from soluble (500 mM KCl) soluble chromatins fractions by treatment with I-ammonium sulphate (and in some cases also DNAse and RNAase treatment) was done as described4. Purification on nickel-agarose and antibody-affinity resin was done as described above. Proteins eluted from 12CA5-affinity resin (Def1H1H purification) were further fractionated by loading onto MonoQ H5/5, which was resolved by a ten-column volume gradient from 100 to 1,000 mM KCl in buffer B (ref. 5).

**Other techniques**

Protein identification was done after trypsin digestion of gel-fractionated proteins as described6. A peptide representing the C-terminal 20 amino acids of Rad26 was cross-linked to keyhole limpet haemocyanin (Calbiochem) and used to immunize rabbits (Murex) for antibody production. TCR assays were performed as described7. Methods for investigating RNAPII degradation were as described8, except that UV-irradiation adsorbed to 12CA5-adsorbed protein A-Sepharose, washed with A-300, and eluted as described.

Purification from the chromatins fractions was done as follows: release of proteins from soluble (500 mM KCl) soluble chromatins fractions by treatment with I-ammonium sulphate (and in some cases also DNAse and RNAase treatment) was done as described4. Purification on nickel-agarose and antibody-affinity resin was done as described above. Proteins eluted from 12CA5-affinity resin (Def1H1H purification) were further fractionated by loading onto MonoQ H5/5, which was resolved by a ten-column volume gradient from 100 to 1,000 mM KCl in buffer B (ref. 5).

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**Structural basis for acidic-cluster-dileucine sorting-signal recognition by VHS domains**

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Specific sorting signals direct transmembrane proteins to the compartments of the endosomal–lysosomal system. Acidic-cluster-dileucine signals present within the cytoplasmic tails of sorting receptors, such as the cation-independent and cation-dependent mannose-6-phosphate receptors, are recognized by the GGA (Golgi-localized, γ-ear-containing, ADP-ribosylation-factor-binding) proteins3–5. The VHS (Vps27p, Hrs and STAM) domains3 of the GGA proteins are responsible for the highly specific recognition of these acidic-cluster-dileucine signals5–10. Here we report the structures of the VHS domain of human GGA3