selected parasites that lost expression of the integrated thymidine kinase marker[18]. We subcloned ganciclovir-resistant and pyrimethamine-sensitive parasites from this selection to produce *T. gondii* strains, *cps1-1* and *cps2-1*. Uracil auxotrophs *cps1-1* and *cps2-1* 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 *Bam*HI sites of the 6.6-kb *CPSII Hin*dIII 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 nuclepore 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 aged 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. to parasite ratio was between 0.4 and 0.6. We used four mice per parasite dose with each strain. Experiments with groups of mice were repeated twice. Mice were monitored for 40 d, and in some experiments for more than 1 year. We cared for mice according to NIH guidelines.

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Competing interests statement

The authors declare that they have no competing financial interests.

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

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Eukaryotic cells use multiple, highly conserved mechanisms to contend with ultraviolet-light-induced DNA damage[1]. 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[2]. In mammalian cells, defective TCR gives rise to the severe human disorder Cockayne's syndrome (CS)[3]. 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 transcript 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 (MHRad26) was fractionated into a DNA-free 'soluble' fraction and a salt-stable chromatin fraction as previously described[5]. MHRad26 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 MHRad26 isolated from the soluble fraction showed a single protein band (MHRad26) (Fig. 1a, lane 1), whereas MHRad26 extracted and purified from the salt-stable chromatin fraction appeared to be a doublet (lane 3). Both fractions were analysed by western blot analysis, and the proteins from the doublet were identified by a combination of peptide mass fingerprinting using matrix-assisted laser desorption/ionization-time-of-flight (MALDI-TOF) mass spectrometry (MS), and mass spectrometric sequencing using NanoES triple-quadrupole MS/MS (ref. 6). We found that the slower-migrating protein purified from chromatin was Rad26, whereas the faster-migrating protein was the product of the YKL054C ORF on budding yeast chromosome XI (predicted M_r 83,900). We named this gene *DEF1* (RNAPII degradation factor 1).

In order to investigate the biochemical behaviour of Def1 and to confirm its association with Rad26, decahistidine-haemagglutinin (HA) tagged Def1 (Def1HH) was immuno-purified from extracts derived from *MHRAD26 DEF1HH* cells. The majority of Def1 was found in the soluble (DNA-free) fraction, but only very small amounts of Rad26 could be immunoprecipitated with it from this fraction (data not shown). As in the case of Rad26, SDS–poly-

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[4], but significantly increases the UV-sensitivity of *rad16* (or *rad7*) strains[7]. 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*

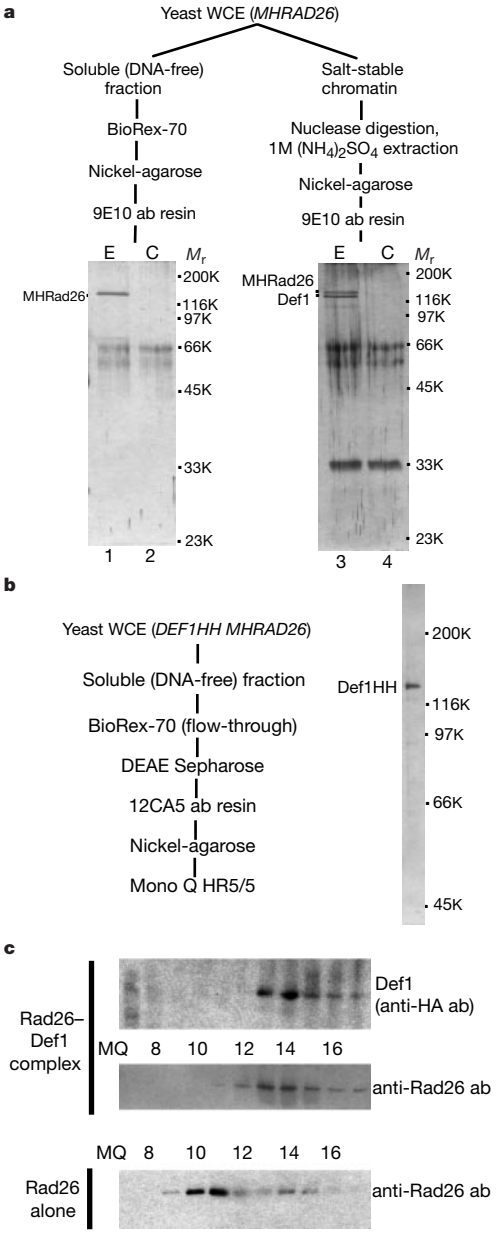

Figure 1 Affinity-purification of Rad26 and Def1. **a**, Purification of Rad26. E, eluate from affinity-resin. C, control eluate from antibody-resin not incubated with protein to show background antibody bands. **b**, Purification of Def1 from the soluble (DNA-free) fraction using antibody-affinity chromatography. Purified proteins are designated on the left side of the silver-stained SDS–PAGE gels, and size markers are indicated on the right. **c**, Purification of Def1 from salt-stable chromatin. Western blots on MonoQ fractions of Def1–Rad26 complex (upper panels) and Rad26 alone (lower panel) were performed with the antibodies indicated on the right.

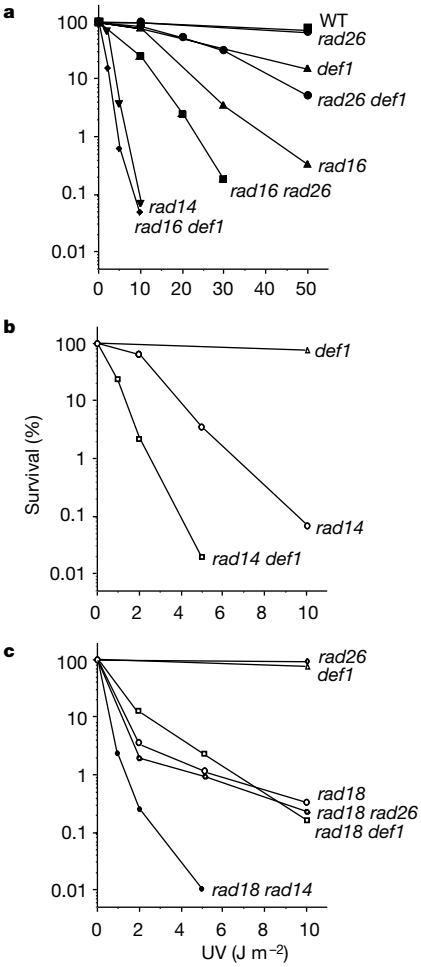

Figure 2 UV-sensitivity of *def1* strains. **a**, Comparison of strains lacking various genes involved in global genome repair and TCR. **b**, Genetic interaction of *def1* and *rad14*. **c**, Lack of genetic interaction of *DEF1* with the damage tolerance/post-replication repair pathway. We note that the UV doses used in **b** and **c** were lower than in **a**. All experiments were done at least in triplicate. Error bars are omitted for clarity. WT, wild type.

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[4,7]. 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, TFIIS[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 °C and 37 °C, the double mutant grew very slowly at 30 °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 compromized 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-

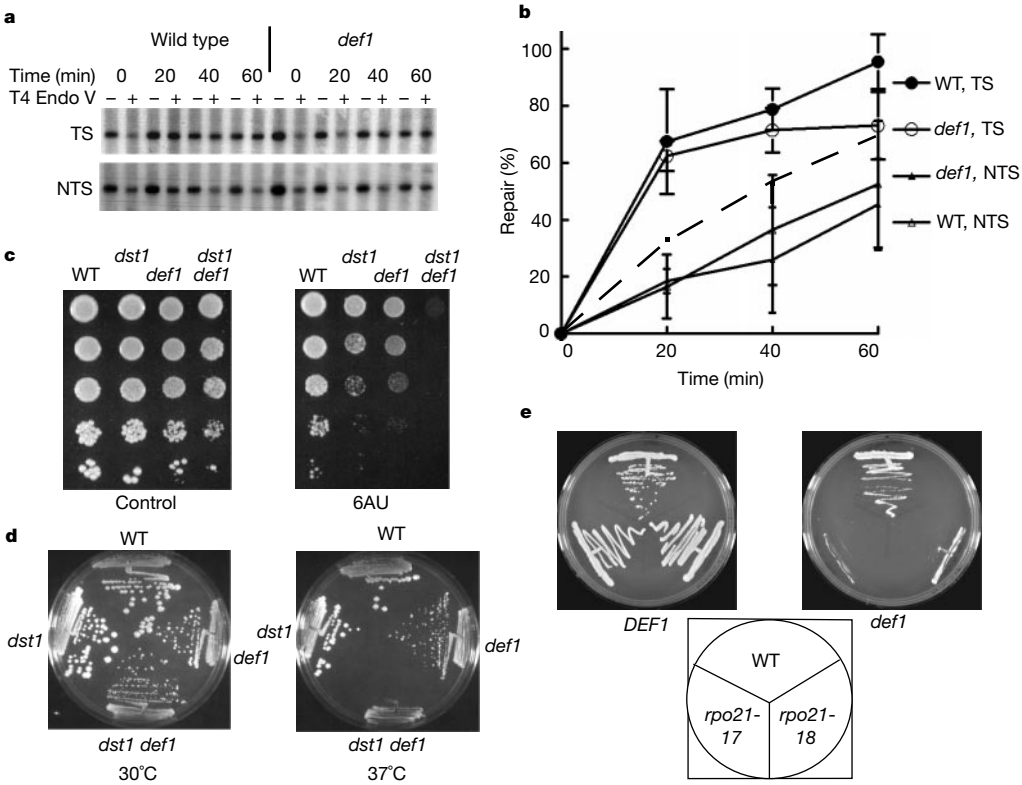

Figure 3 The effect of *DEF1* deletion on TCR and transcript elongation. **a**, Repair of DNA lesions in the transcribed (TS) and non-transcribed strand (NTS) of an active gene. Damage-dependent T4 endonuclease V restriction gives a measure of remaining lesions. **b**, The result from **a** and those 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[4,7] 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 °C. **e**, The effect of combining *DEF1* deletion with rpo21 alleles[9,10] 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 polyubiquitinated 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 factors[14], such as TFIIS, 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 domain[5], 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 inter-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.

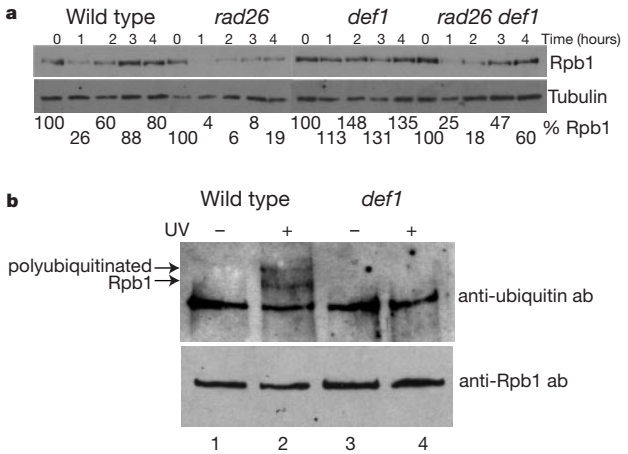

Figure 4 *DEF1* is required for UV-induced ubiquitination and degradation of Rpb1. **a**, Degradation of RNAPII in response to UV irradiation. Western blots probed with the antibodies indicated on the right are shown. Similar results were obtained whether Rpb1 was detected with antibody directed against hyper-phosphorylated or hypo-phosphorylated C-terminal domain of 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 reported[12]. **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.

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 factors[15]. 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 may also 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 TFIIS-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 particle[16], and that the 19S complex has recently been shown to affect transcript elongation as well as DNA repair, independent of proteolysis[17–19]. 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 cells[11,12,20–22], 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 humans[3]. Cultured cells from CS patients are UV-sensitive[23], completely unable to perform TCR[24], and exhibit a severe deficiency in recovery of RNA synthesis after DNA damage[25]. 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 compromized 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 complex[26], 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 cells[27,28], rather than being quickly degraded as appears to happen in yeast. Indeed, CSB cells have defects in RNAPII ubiquitination[20], and are compromized[21], although not fully defective[22], for RNAPII degradation. On the basis of the phenotypes of yeast *def1* cells reported here, we suggest that this compromized 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 syndrome[27,28]. □

Methods

Yeast manipulation

Yeast manipulation was done as previously described[5]. Strains expressing amino-terminal tagged Rad26 (Myc-decahistidine-TEV-Rad26, MHRad26), and/or carboxyl-terminal

His-HA tagged Def1 (Def1-decahistidine-HA, Def1HH) had wild-type phenotypes. For purification purposes, tagging was done in the protease-deficient PY26 strain. Details of affinity tagging are available on request.

To construct *def1 rpo21* 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 *rpo21* alleles[9]. *ura3*$^-$ cells were selected on 5-FOA-containing media.

Protein purification and identification

During purification, Def1HH 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 BioRex-70 (Bio-Rad), nickel-agarose (Qiagen), DEAE Sepharose (Pharmacia), and MonoQ HR5/5 (Pharmacia) was done as previously described for other factors[5]. 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 BioRex-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). Def1HH 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 dithiothreitol (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 described[6].

Purification from the chromatin fractions was done as follows: release of proteins from salt-stable (500 mM KOAc) soluble chromatin fragments by treatment with 1 M ammonium sulphate (and in some cases also RNAse and DNAse treatment) was done as described[5]. Purification on nickel-agarose and antibody-affinity resins was done as described above. Proteins eluted from 12CA5-affinity resin (Def1HH purification) were further fractionated by loading onto MonoQ HR5/5, which was resolved by a ten-column volume gradient from 100 to 1,000 mM KOAc in buffer B (ref. 5).

Other techniques

Protein identification was done after trypsin digestion of gel-fractionated proteins as described[6]. 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 described[29]. Methods for investigating RNAPII degradation were as described[12], except that UV-irradiation (30 J m^{-2}) was done on cells re-suspended in saline. Detection of ubiquitinated RNAPII by western blotting was done after immunoprecipitation of polymerase using 8WG16 (ref. 30) antibodies.

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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[1]. 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) proteins[2–5]. The VHS (Vps27p, Hrs and STAM) domains[6] of the GGA proteins are responsible for the highly specific recognition of these acidic-cluster-dileucine signals[7–10]. Here we report the structures of the VHS domain of human GGA3