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Rad10-YFP focus induction in response to UV depends on *RAD14* in yeast

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Abstract

Rad14 is a DNA damage recognition protein in yeast Nucleotide Excision Repair (NER) and believed to function early in the cascade of events. The function of Rad14 presumably precedes that of the Rad1-Rad10 endonuclease complex, which functions in a downstream step incising DNA 5' to the site of DNA damage. We investigated whether recruitment of Rad10 to UV-induced DNA damage sites in live cells is dependent on Rad14 using fluorescence microscopy. Experiments were carried out using *Saccharomyces cerevisiae* strains in which the gene for Rad14 was fused to Cyan Fluorescent Protein (Rad14-CFP) and that of Rad10 was fused to Yellow Fluorescent Protein (Rad10-YFP). Rad14-CFP forms nuclear localized CFP fluorescent foci in response to UV-irradiation with the peak induction occurring 15 minutes post-irradiation. In contrast, Rad10-YFP foci form in response to UV with the peak induction occurring 2 hours post-irradiation. Recruitment of Rad14-CFP is not dependent on the *RAD10* gene but Rad10-YFP is recruited to UV-induced YFP foci in a *RAD14*-dependent fashion. Time-lapse experiments indicate that Rad14-CFP foci are transient, typically persisting less than 6 minutes. Together these data support the model that yeast NER protein assembly is step-wise whereas Rad14 required to recruit Rad10 and Rad14 involvement is transient.

Keywords

Rad14; Rad1; Rad10; Nucleotide Excision Repair; Yeast

Introduction

Nucleotide Excision Repair (NER) in eukaryotes repairs DNA via a cascade of biochemical interactions requiring more than 30 proteins. NER repairs a variety of DNA lesions including those induced by UV light such as cyclobutane pyrimidine dimers (CPDs) and 6,4-photoproducts (6,4-PPs). Genetic defects in NER in humans lead to several cancer-prone conditions including *Xeroderma pigmentosum*. NER can function in two modes with differing recognition steps: global genome repair (GGR) and transcription-coupled repair (TCR). In GGR, the DNA lesion is first recognized by the Rad4-Rad23 complex, whereas in TCR it is recognized by the transcriptional machinery. Damage recognition is followed by

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the unwinding of DNA surrounding the damage site by helicases Rad3 and Ssl2. Helicase unwinding is followed by 5' and 3' incision of the damaged strand by the Rad1-Rad10 complex and Rad2 respectively (Friedberg et al., 2005). Rad14 binds the lesion site as part of a damage recognition/ verification step at a point in time, which lies somewhere between the initial damage recognition event and the 5' DNA strand incision step, however the precise timing has not been shown directly. Following incision, the ~25 base pair damaged DNA oligonucleotide is removed from the site and the gapped DNA is filled in a template-directed fashion by DNA polymerase and the nick sealed by DNA ligase (Friedberg et al., 2005).

The timing and molecular basis for each successive recruitment event during NER is not fully understood. Specifically, the timing of the arrival of Rad14 in relation to the other early-acting factors is not known. It is also not understood whether the arrival of Rad14 recruits factors acting downstream of Rad14, such as the Rad1-Rad10 complex, and whether the role of Rad14 is short or prolonged. Knowledge of such biochemical details would increase our understanding of the molecular triggers driving the progression and regulation of NER with respect to other DNA repair pathways and may aid in understanding the molecular pathogenesis of human cancers, especially skin cancer.

Direct physical interactions between Rad14 and the Rad1-Rad10 complex have previously been recognized (Guzder et al., 2006). Further, in fixed human fibroblasts, it has been shown that the various protein participants in NER do not all arrive at DNA damage sites simultaneously and that the arrival of some downstream factors, notably ERCC1, the human homolog of *S. cerevisiae* Rad10, cannot be recruited to UV damaged DNA in a cell line deficient in the *XPA* gene, the human homolog of yeast *RAD14* (Volker et al., 2001). Hence, recruitment of the Rad1-Rad10 complex to NER sites may analogously be dependent on Rad14, but this has not been shown experimentally. Moreover, the timescale of action of Rad14 at an NER site is unknown.

Rad14 is not known to have cellular functions other than its role as a damage recognition/ verification factor in NER. However, the Rad1-Rad10 complex is required for other cellular processes including several modes of DNA double-strand break repair (Fishman-Lobell et al., 1992; Friedberg et al., 2005; Ivanov et al., 1996; Krogh and Symington, 2004; Ma et al., 2003). *RAD1* and *RAD10* function exclusively in complex with each other and both are required for several types of mitotic recombination events, including sister chromatid exchange (SCE) (Friedberg et al., 2005; Kadyk and Hartwell, 1993; Klein, 1988; Schiestl and Prakash, 1988, 1990). Phenotypic differences have been observed when examining spontaneous versus UV-induced SCE in *rad1Δ* mutants, which indicate that Rad1-Rad10 is required for a subset of spontaneous SCE events, but not UV-induced SCE (Kadyk and Hartwell, 1993). Therefore investigations into the recruitment patterns of the Rad1-Rad10 complex following UV damage would be expected to reflect Rad1-Rad10 participation in NER, but not necessarily participation in UV-induced SCE.

Recent studies have demonstrated the utility of fluorescence microscopy in monitoring biochemical events in live yeast cells (Barlow et al., 2008; Lisby et al., 2004; Moore et al., 2009). Two key advantages of this experimental approach are especially noteworthy. First, the gene that encodes a given fluorescent protein under study is placed in the same chromosomal location as the native gene and also under the control of the endogenous promoter, thereby avoiding physiologically irrelevant over-expression of the fluorescent genes. Second, this technique avoids fixation of cells prior to staining with fluorescent antibodies, thereby enabling investigation of the real time dynamics of the proteins under study (Barlow et al., 2008; Lisby et al., 2004; Moore et al., 2009). Using this technology, the present study demonstrates that yeast Rad14 tagged with cyan fluorescent protein (Rad14-

CFP) forms foci in response to UV-C irradiation independent of the presence of the *RAD10* gene. In addition, time-lapse experiments with Rad14-CFP show that CFP foci form within a few minutes of UV irradiation and are temporally transient. Finally, experiments with Rad10-YFP show that induction of Rad10-YFP foci in response to UV is dependent on *RAD14* suggesting that Rad10-YFP foci observed in response to UV are NER repair events.

Materials and methods

Preparation of yeast strains Rad14-CFP and rad14Δ

The *S. cerevisiae* *RAD14* gene was genetically fused in frame at the chromosomal locus with the DNA coding region of CFP to prepare the Rad14-CFP strain in the W303-1A genetic background by the adaptamer-mediated PCR method as described previously (Friedberg et al., 2005; Reid et al., 2002). The resulting strain was backcrossed to the WPF006-13C strain to produce the final Rad14-CFP strain (PF031-10D) which was used in microscopy experiments. The presence of the CFP tag was confirmed by PCR and fluorescence microscopy. Dye terminator sequencing confirmed clean splicing of the CFP label with the Rad14 gene and no mutations in the Rad14 coding region.

Expression of the full-length Rad14-CFP polypeptide in the Rad14-CFP strain was confirmed by immunoblotting of yeast whole cell extracts from appropriate strains using an α -Rad14 antibody. 200 μ g total protein was loaded to each lane of the gel as assayed with the Bio-Rad Protein Assay Reagent according to manufacturer's instructions (Bio-Rad, Hercules, CA) based on the method of Bradford (1976). SDS PAGE gels for Western hybridization were NuPAGE® Novex 4–12% Bis-Tris (Invitrogen, Carlsbad, CA) using 2-(N-morpholino)ethanesulfonic acid (MES) running buffer according to manufacturer's recommendations. Bands were sized based on migration of Benchmark™ protein standards (Invitrogen) run in adjacent lanes. The Rad14 antibody was prepared as a rabbit polyclonal to Rad14 protein that had been over-expressed in *E. coli* using standard techniques. The crude rabbit polyclonal antibody was affinity purified with pure recombinant Rad14 protein expressed as a GST-Rad14 fusion and immobilized on an AminoLink Plus Column (Pierce, Rockford, IL) according to manufacturer's instructions. As a loading control, the immunoblot was stripped using standard methods and rehybridized with α - β -actin (Abcam, Cambridge, MA; ab8224). All other Rad14-CFP strains used in this study were prepared by genetic crosses of the appropriate parent strains with PF031-10D and are listed in Table 1. All strains used in this study are haploids.

An isogenic disruption mutant of *RAD14* (PF032-1D) was prepared by PCR amplification of the *rad14Δ::LEU2* deletion cassette from the MGSC139 strain (Verhage et al., 1996) and transformation into the W303-1A background. Transformants were selected on Synthetic Complete (SC) agar lacking leucine and the presence of the deletion cassette confirmed by PCR. The resulting strain was backcrossed to strain WPF006-13C to give strains PF032-1D and PF032-12C.

UV survival studies

UV survival experiments were conducted as previously reported (Moore et al., 2009).

General microscopy

Microscopy was conducted on a Zeiss AxioImager M1 microscope (Zeiss, Thornwood, NY) with a plan-apochromat 100x, 1.4 numerical aperture (NA) objective oil immersion lens as previously reported (Moore et al., 2009). Except for time-lapse experiments, an 800 ms exposure time was used to acquire fluorescent images of Rad14-CFP. Unless otherwise indicated, images were acquired as Z-stacks of 11 images in which the focal plane offset

was 0.3 μm per slice, so as to image the entire thickness of the cell with some margin on each side. Foci were counted by manually inspecting images for regions of bright, punctate fluorescence. Publication quality images were contrast enhanced to make the fluorescent foci visible. Data represent percentages from at least 100 cells per data point unless otherwise stated.

Induction of foci by UV-irradiation

Cultures for UV pulse-chase experiments were started and propagated in SC medium supplemented with 200 $\mu\text{g/mL}$ Adenine (SC + ade) at 23 $^{\circ}\text{C}$. Overnight cultures were freshly diluted (to 0.1 OD_{600}) and incubated (3 h) prior to irradiation with UV. Cells were transferred to sterile 110 $\times$ 15 mm Petri dishes and exposed to UV-C light (20 J / m^2 , lamp model CC12T6L, Atlantic Ultraviolet Corporation Hauppauge, NY), transferred to fresh culture tubes, wrapped in aluminum foil and incubated for the desired amount of time prior to processing for microscopy.

Timelapse UV pulse-chase experiments

In time-lapse experiments, cells were UV irradiated as droplets directly on microscope slides and quickly mounted with mounting medium (2% low-melting agarose in SC + ade medium as previously described (Moore et al., 2009)). Due to the low abundance of the Rad14-CFP chromophore and the associated photobleaching, Z-stacks were recorded which contained only three slices (as opposed to 11 slices in other experiments). Foci were only counted in the second slice image, using images from the first and third slices to verify the presence of the focus in the second slice. Hence, foci counts for these experiments represent only approximately 30% of each cell thickness (assuming an average cell diameter of 3 μm). Additionally, successive time-points were imaged using 800 ms (12 min) 1.2 sec (at 15 min) and 2 sec (at 18 min) instead of each image being only 800 ms as in the other experiments presented.

DAPI stained images

DAPI stained images were prepared as previously reported (Moore et al., 2009).

Results

The *Saccharomyces cerevisiae* *RAD14* gene was tagged on the N-terminus with Cyan Fluorescent Protein (CFP) using previously described methodology (Reid et al., 2002). The resulting yeast strain expresses a Rad14-CFP fusion protein product that migrates at the expected 73 kDa polypeptide during SDS PAGE, whereas a wild-type strain containing the unlabeled Rad14 gene migrates as a 43 kDa band during SDS PAGE (Fig. 1A). A control lane containing an extract from a Rad14 deletion mutation (*rad14 Δ*) exhibits only background signal with the α -Rad14 antibody. As expected, Rad14-CFP protein is expressed at comparable levels to wild-type Rad14. The immunoblot was also probed with β -actin, which indicates consistent loading among the lanes (Fig. 1A). The Rad14-CFP strain exhibits nuclear localized cyan fluorescence indicating the presence of nuclear Rad14-CFP protein, as expected (Fig. 1B). The CFP signal colocalizes with 4',6-diamidino-2-phenylindole (DAPI) stained DNA in the nucleus (Fig. 1B).

To ensure that the CFP label does not compromise Rad14 protein function in NER, the UV sensitivity of the Rad14-CFP strain was tested. The Rad14-CFP strain exhibits wild-type levels of survival to UV-C irradiation (hereafter "UV"), indicating that the presence of the fluorescent protein domain does not negatively impact Rad14 protein function in NER (Fig. 1C).

Next, we investigated the ability of the Rad14-CFP strain to act as a reporter of NER activity following UV. The Rad14-CFP strain exhibits nuclear localized CFP foci that are induced following UV (Fig. 2A). The Rad14-CFP strain was monitored as a function of time to determine the peak time for UV-induced CFP focus formation. Peak Rad14-CFP focus induction occurs at approximately 15 minutes following UV (Fig. 2B). In contrast, a strain containing Rad10-YFP (described previously (Moore et al., 2009)) exhibits peak Rad10-YFP focus induction approximately 2 hours following UV (data not shown). During UV pulse-chase experiments, cultures are growing in log phase during the course of the experiment which may explain the increasing background levels of spontaneous foci observed in non-irradiated control samples (white bars in Fig. 2B).

To determine the persistence of UV-induced Rad14-CFP foci, short time-lapse experiments were conducted in which Rad14-CFP cells were imaged at 12, 15 and 18 min following UV exposure (Fig. 3). Foci were observed appearing and disappearing over the course of the time-lapse (Fig. 3A). In 118 cells imaged in this fashion, 20 (~17%) contained Rad14-CFP foci present at 15 minutes. Of these 20 foci, 17 (representing ~14.4% of cells) were present only at the 15 minute time-point (Fig. 3B). The remaining 3 foci present at 15 minutes (representing ~2.5% of cells) were also present at 18 minutes following UV (Fig. 3B). At 18 minutes following UV, 17 foci were observed that were not present at the 15 minute time-point (representing ~14.4 % of cells). Additionally, foci were observed in all phases of cell cycle and in approximately the same percentages (17.5% of S phase cells, 21.4% of G2/M phase cells and 14.9% of G1 phase cells). Hence, 15 minutes following UV irradiation (when Rad14-CFP foci are present in the greatest numbers [Fig.2B]), approximately 17% of the cells contain Rad14-CFP foci and 17 of 20 of these foci persist for fewer than 6 minutes. The time-lapse images were acquired by imaging only 3 focal planes per time-point and foci only counted in the center image explaining why percentages of Rad14-CFP foci observed are lower than in Fig. 2B. Owing to the low abundance of the Rad14-CFP protein, photobleaching made it difficult to acquire more than approximately 3–4 images per field of cells and also hampered attempts at colocalization experiments with a strain containing Rad14-CFP and Rad10-YFP (data not shown).

Given that prior literature has revealed a direct protein-protein interaction between Rad1 and Rad14 (Guzder et al., 2006), we investigated whether there was a genetic dependence of recruitment of the Rad1-Rad10 complex (the presumed later-acting complex) on Rad14 (the presumed earlier-acting factor). The genetic requirements for Rad14-CFP and Rad10-YFP focus induction were examined by monitoring focus formation of either Rad14-CFP or Rad10-YFP in the deletion mutant background of the other gene. Both Rad14-CFP/*RAD10* and Rad14-CFP/*rad10Δ* strains showed approximately 2-fold induction of Rad14-CFP foci 15 minutes following UV (Fig. 4A). Rad14-CFP focus induction peaks at 15 minutes and then begins to fall off with time (Fig. 2B) explaining why no induction was observed at 120 minutes with either the Rad14-CFP/*RAD10* or Rad14-CFP/*rad10Δ* strains. However, in reciprocal experiments conducted with *RAD14*/Rad10-YFP and *rad14Δ* Rad10-YFP strains, Rad10-YFP focus induction at 120 minutes was approximately 1.7-fold in the *RAD14*/Rad10-YFP but abolished in the *rad14Δ* Rad10-YFP (Fig. 4B). This result shows that Rad10-YFP focus induction present at 120 minutes post-irradiation is dependent on *RAD14*. The 120 minute time-point was chosen because the peak of Rad10-YFP induction in response to UV occurs at 120 minutes post-irradiation (data not shown). At 15 minutes post-irradiation, no Rad10-YFP focus induction was observed in either the *RAD14* wild-type or *rad14Δ* strains indicating that Rad10-YFP foci are not formed in significant numbers at early time-points following UV and that this lack of induction is not altered in a *rad14Δ* mutant background. Taken together these data indicate that Rad14 induction occurs earlier than Rad10 and that Rad14 is required for recruitment of the Rad1-Rad10 complex to UV-induced foci.

Discussion

Rad14 is known to be a DNA damage recognition / verification factor that participates earlier in NER, and Rad10 is part of the heterodimeric Rad1-Rad10 complex incising DNA 5' to the DNA lesion as a later step (Friedberg et al., 2005). The precise temporal relationships of the NER factors and the genetic requirements for successive recruitment events have only been investigated more recently (Mocquet et al., 2008; Solimando et al., 2009; Volker et al., 2001). Appropriately, much of this work has focused on studies in human fibroblasts using NER-deficient cell lines deriving from *Xeroderma pigmentosum* patients. Experiments in human fibroblasts, however, require the use of over-expression systems for the fluorescent proteins or require that cells be fixed prior to visualization using fluorescent antibodies. It is therefore important, not only to establish whether the findings in human cells are similar in lower eukaryotic organisms, but also to conduct temporal investigations in live cells expressing the labeled proteins from their endogenous genes and promoters, as afforded in our yeast model system. This study provides real-time data acquired in live cells addressing the relative recruitment patterns of Rad14 and Rad10 in response to UV irradiation.

These experiments show that an N-terminally labeled Rad14-CFP protein is a faithful reporter of Rad14 participation in NER as it is expressed at comparable levels to endogenous wild-type Rad14, is localized in nuclei and gives rise to a yeast strain exhibiting wild-type survival in response to UV irradiation. Importantly, Rad14-CFP fluorescent foci form in response to UV. It is not surprising that Rad14-CFP foci form rapidly following UV irradiation as Rad14 functions as a DNA damage recognition factor (Bankmann et al., 1992; Friedberg et al., 2005) which would be expected to be recruited to UV lesions early during repair. This study shows that in a live cell this is in fact the case and that most Rad14-CFP foci persist for only a few minutes (< 6 min, Figs. 3B and C).

It is noteworthy that high levels of background foci (~50%) are observed for both Rad14-CFP and Rad10-YFP, even in the absence of UV irradiation (Fig. 2B, Fig. 4A and 4B). In the case of Rad14-CFP, this indicates that spontaneous levels of DNA damage are frequently and continuously being repaired by NER in all phases of the cell cycle. In the case of Rad10-YFP, the high levels of spontaneous foci observed may reflect recruitment of Rad10-YFP to both repair and recombination sites. Rad10-YFP has already been shown to be recruited to I-SceI-induced double-strand breaks and IR-induced nuclear foci, which likely reflects roles in synthesis-dependent strand annealing and single-strand annealing, respectively (Moore et al., 2009).

An additional interesting observation is that peak focus induction for Rad14-CFP and Rad10-YFP are highly offset from each other with respect to time (15 min post-irradiation for Rad14-CFP versus 120 min post-irradiation for Rad10-YFP). Minor temporal offset in peaks of induction might be expected since Rad14 may act earlier in NER than Rad10 and therefore be recruited sooner (Fig. 5). However, since Rad14-CFP foci typically only persist for a few minutes (Figs. 3B and C), one might expect a more limited temporal offset for downstream recruitment of Rad10. The more dramatic offset observed suggests several possible explanations. First, the time-scale of action of Rad10-YFP in NER may be slower than that of Rad14-CFP. Second, Rad14 may play a previously unrecognized role in signaling other UV-induced DNA damage responses involving Rad10 that are carried out on a longer time-scale and for which the roles of Rad14 and Rad10 are not temporally overlapping. Third, Rad14 recruitment may be required in advance of Rad10, but there may be another biochemical processing step required between recruitment events (Fig. 5). This last possibility seems less likely owing to evidence that a physical protein-protein complex can form between Rad1 and Rad14 (Guzder et al., 2006). Because previously reported UV

survival studies showed that Rad10-YFP survival is comparable to wild-type cells (Moore et al., 2009), we think it unlikely that the lag in recruitment is a result of the fluorescent tag. Thus far, Rad10-YFP/Rad14-CFP colocalization studies are precluded for technical reasons.

Experiments conducted with strains containing *rad14Δ* Rad10-YFP and Rad14-CFP/*rad10Δ* show that Rad14 is required for downstream recruitment of Rad10, analogously to what has been observed in fixed human fibroblasts in which in an *XPA* deficient fibroblast cell line, ERCC1 cannot be recruited to UV-DNA damage as it can in an *XPA* wild-type cell line (Volker et al., 2001). Our data support the model that Rad14 may directly recruit the Rad1-Rad10 complex via the reported physical interaction between Rad14 and Rad1 (Guzder et al., 2006) (Fig. 5).

It is interesting that we observed a *RAD14*-dependence on Rad10-YFP recruitment to UV-induced fluorescent foci given that Rad10 has additional roles in the cellular responses to DNA damage (Friedberg et al., 2005). As described, Rad10 is also required for several modes of DNA double-strand break repair and mitotic recombination (Daley and Wilson, 2005; Friedberg et al., 2005; Ivanov and Haber, 1995; Ivanov et al., 1996; Kadyk and Hartwell, 1993; Ma et al., 2003; Moore et al., 2009). Intriguingly, differing phenotypes are observed with *rad1Δ* mutants when examining spontaneous versus UV-induced SCE (Dong and Fasullo, 2003; Kadyk and Hartwell, 1993). In the case of spontaneous SCE, *RAD1* deletion mutants (*rad1Δ*) are comparable to wild-type strains, but *rad1Δ rad51Δ* double mutants are synergistically defective, exhibiting a 10-fold reduction in the numbers of SCE's as compared to *rad51Δ* mutants (Dong and Fasullo, 2003). Since *RAD51* is required for a subset of SCEs (those involving homology searching), these data suggest a requirement of the Rad1-Rad10 complex in the subset SCE's that are not mediated by homology searching, perhaps those occurring in repeat sequences. Conversely, with regard to UV-induced SCEs, previous studies have shown that the magnitude of UV-induced SCE is approximately 10-fold higher in *rad1Δ* mutant strains as compared to wild-type strains (Kadyk and Hartwell, 1993). If *RAD1* were required for UV-induced SCE, the frequency of SCE would be expected to be diminished in a *rad1Δ* background. Since the opposite result is observed, it suggests, not only that there is no requirement for *RAD1* in UV-induced SCE, but also that SCE is suppressed by the function of *RAD1* following UV, probably due to efficient repair of UV lesions by NER. In support of this possibility, the same study showed that *rad1Δ rad50Δ* double mutants exhibit UV-induced recombination levels comparable to wild-type cells indicating that the increased reliance on SCE for repair in the absence of *rad1Δ* is connected to DSB repair and not NER. Presumably SCE levels are increased in a *rad1Δ* background because unrepaired UV lesions stall a larger number of replication forks thereby increasing the levels of template switching and gap repair which can give rise to 3' overhanging ends that would need to be excised, a process that could require Rad1-Rad10.

Our results support these prior published observations regarding SCE by showing that nuclear Rad10-YFP foci only accumulate following UV irradiation if the NER damage recognition factor Rad14 is present, suggesting that all the UV-induced Rad10-YFP foci are NER foci and none represent recombination foci. Alternatively, our observation could indicate that Rad10 participation in UV-induced recombination events may be fleeting or may involve too few copies of Rad10 to enable the formation of foci visible under the experimental conditions employed. Our data further support a requirement for *RAD10* in spontaneous SCE since we observed background levels of Rad10-YFP foci present at all stages of cell cycle and did not vary substantially either in the absence of *RAD14* or in the absence of UV. The background foci observed in the absence of UV in both Rad14-CFP and Rad10-YFP cells likely reflect repair of spontaneous DNA damage by NER and HR. These data provide support for analogous investigations in human cells showing the timing of

assembly of NER factors and strengthen our understanding of the genetic conservation of the NER pathways between the yeast *S. cerevisiae* and humans.

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Figure 1.

Characterization of the Rad14-CFP strain. **A**, The Rad14-CFP strain exhibits an increased polypeptide size consistent with Rad14 expressed as a CFP fusion protein. Immunoblot of equal quantities of whole cell extracts (WCE) from the indicated yeast strains probed with an α-Rad14 rabbit polyclonal antibody. Lane 1: MagicMark® protein standard, lane 2: WT (W1588-4C), lane 3: Rad14-CFP (PF031-10D), lane 4 *rad14Δ* PF032-1D). The wild-type strain (lane 2) shows a Rad14 band at the expected 43 kDa while the Rad14-CFP strain (lane 3) shows a band at the expected 73 kDa. A Rad14 deletion mutant (*rad14Δ*, lane 4) shows only background binding. The immunoblot was stripped and reprobed with an antibody to β-actin as a loading control which shows consistent loading among the lanes (lower panel). **B**,

The Rad14-CFP protein is nuclear localized. CFP fluorescence (upper image) colocalizes with the DAPI fluorescence signal (middle image) confirming the nuclear localization of CFP. A differential interference contrast (DIC) image of the same cell is also shown (lower image). C, Function is not compromised with Rad14-CFP in response to UV. UV survival experiments were conducted on the Rad14-CFP strain in parallel with isogenic wild-type and *rad14Δ* deletion mutant strains. The strains shown are the unlabeled wild-type strain (WT), W1588-4C (■), the Rad14-CFP strain, PF031-10D (▲) and *rad14Δ*, PF032-1D (●). Percentage survival is reported as a function of UV-C dosage at 254 nm (J / m^2). Error bars show the standard error of three independent experiments as the percentage of plated cells.

Figure 2.

The Rad14-CFP strain exhibits nuclear localized cyan fluorescent foci that are present in greater numbers following UV-C exposure. An asynchronous culture of Rad14-CFP strain (PF031-10D) was exposed to UV-C (20 J / m²), incubated for the indicated times and imaged. **A**, A bright punctate region of cyan fluorescent signal is visible within the nucleus of a cell 15 minutes post-irradiation as indicated by the orange arrow. **B**, Graph of foci counts following UV-induction of the Rad14-CFP strain. The plotted values represent means (λ 's) from ideal Poisson distributions least squares fit to the observed distributions of foci per cell. The least squares deviations were propagated into the overall errors which are

depicted as the error bars in the figure. Gray bars show data from UV-induced samples; white bars show data from non-induced controls.

Figure 3.

Rad14-CFP foci are transient. Time-lapse experiments were conducted with the Rad14-CFP strain (PF031-10D) in which cells were irradiated with UV-C ($20 \text{ J} / \text{m}^2$) and imaged at 12, 15, 18 and 30 minutes post-irradiation, respectively. **A**, DIC and CFP images from a representative cell. The 15 and 18 minute images exhibit Rad14-CFP foci that are not present at the other time-points (indicated by the gold arrows). **B**, Quantification of time-lapse experiments. The pie chart shows percentages of cells containing Rad14-CFP foci 15 and 18 minutes following UV irradiation. Data are broken down according to cells containing foci present only in the 15 minute image, those cells containing foci at 15 minutes that persisted to 18 minutes and those containing foci at 18 minutes that were not present at 15 minutes. Only one cell (shown in Fig. 3A) contained a focus at 15 minutes that had disappeared by 18 minutes, but in which there was a new focus in a different location visible at 18 minutes. No foci were observed at 12 minutes that persisted into the 15 minute time-point, presumably by chance (data not shown). **C**, Statistical analysis of the key data presented in the pie chart in B. The plotted values represent means (λ 's) from ideal Poisson distributions least squares fit to the observed distributions of foci per cell. The least squares deviations were propagated into the overall errors which are depicted as the error bars in the figure.

Figure 4.

RAD14 is required to recruit Rad10-YFP to YFP foci induced by UV-C, but *RAD10* is not required to recruit Rad14-CFP. Strains were cultured, induced with UV-C ($20 \text{ J} / \text{m}^2$), incubated for the indicated times and imaged as Z-stacks of $11 \times 0.3 \mu\text{M}$ slices, thus imaging the entire cell thickness. Fluorescent foci were counted and data from three independent experiments averaged. Data are shown comparing: A. the Rad14-CFP/*RAD10* strain (PF031-10D, black bars) and Rad14-CFP/*rad10Δ* strain (PF033-5D, gray bars) and, B. the *RAD14*/Rad10-YFP (WPF006-4C, black bars) and *rad14Δ*/Rad10-YFP strains (PF032-12C, white bars). Data are either for UV-C induced cells (+) or non-induced cells (-). Error bars represent the standard error from the three experiments. Student's t-tests were performed on all pair-wise combinations of averaged data. Key comparisons that were significantly different ($p < 0.005$) are indicated with "***" above the data pair with the corresponding p value shown next to the asterisks.

Figure 5.

Model for recruitment of Rad1-Rad10 with respect to Rad14. Following UV-irradiation, DNA lesions form and early-acting NER factors (e.g. Rad4-Rad23) initiate the repair process. Rad14 binds the damage site as a damage recognition/ verification factor which is necessary for recruitment of Rad1-Rad10. Rad1-Rad10 recruitment may occur at approximately the same time as Rad14 or later. Rad14 is transiently recruited to the damage site and, as depicted in the figure, may or may not depart the damage site prior to Rad1-Rad10 recruitment. The timing and order of events between Rad14 recruitment and Rad1-Rad10 recruitment may be rapid and direct or slower and separated by other intermediate biochemical processing.

Table 1

Strains Used in This Study

Name	Genotype	Where published
W303-1A	<i>MATa ade2-1 lys2Δ trp1-1 can1-100 his 3-11,15 leu2-3,112 ura3-1 rad5-535</i>	(Thomas and Rothstein 1989)
W1588-4C	<i>MATa ade2-1 lys2Δ trp1-1 can1-100 his 3-11,15 leu2-3,112 ura3-1</i>	(Zhao et al. 1998)
W3646-11D	<i>MATa lys2Δ can1-100 his 3-11,15 bar1::LEU2 ura3-1</i>	(Thomas and Rothstein 1989)
WPF006-4C	<i>MATa lys2Δ trp1-1 can1-100 his 3-11,15 leu2-3,112 ura3-1 Rad10-YFP</i>	(Moore et al. 2009)
WPF006-13C	<i>MATa lys2Δ trp1-1 can1-100 his 3-11,15 leu2-3,112 ura3-1</i>	(Moore et al. 2009)
PF033-6C	<i>MATa trp1-1 his 3-11,15 leu2-3,112 ura3-1 rad10::KanMX</i>	(Moore et al. 2009)
WPF033-6C	<i>MATa ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3-1 rad10::KanMX</i>	(Moore et al. 2009)
PF031-10D	<i>MATa lys2Δ trp1-1 can1-100 his 3-11,15 leu2-3,112 ura3-1 Rad14-CFP</i>	This manuscript
PF033-5D	<i>MATa trp1-1 can1-100 his 3-11,15 leu2-3,112 ura3-1 rad10::KanMX Rad14-CFP</i>	This manuscript
PF032-1D	<i>MATa lys2Δ trp1-1 can1-100 his 3-11,15 leu2-3,112 ura3-1 rad14::LEU2</i>	This manuscript
PF032-12C	<i>MATa lys2Δ trp1-1 can1-100 his 3-11,15 leu2-3,112 ura3-1 Rad10-YFP rad14::LEU2</i>	This manuscript

All strains in this study are haploid and derivatives of W303-1A and W303-1B (Thomas and Rothstein, 1989). Additionally, all strains are wild-type for the *ADE2* and *RAD5* genes unless otherwise noted.