Tracking DNA double-strand breaks

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DNA DOUBLE-STRAND BREAKS ARE NOT SUFFICIENT TO INITIATE RECRUITMENT OF TRF2

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The human telomere binding factor TRF2 is essential at telomeres, facilitating the formation and stabilization of t-loops and suppressing local ATM-mediated damage response. Bradshaw et al. recently reported that TRF2 accumulates at nuclear sites damaged by high-intensity laser beams, presumptively in response to DNA double-strand breaks (DSBs), and that it arrives before other DNA repair-related proteins, including ATM. To characterize the type of lesion responsible for triggering TRF2 recruitment, we produced a variety of localized nuclear damage and then quantified TRF2 colocalization with appropriate DNA damage markers. Although we found that TRF2 is indeed recruited to sites damaged by a high-intensity multiphoton laser beam, we did not find any evidence for such recruitment after we exposed cells to lower-intensity sources of ultraviolet radiation or to ionizing radiation, indicating that archetypal radiogenic DNA lesions such as DSBs are insufficient to trigger TRF2 recruitment.

In an effort to confirm the original observation that TRF2 is recruited to sites damaged by high-intensity lasers (for example, multiphoton lasers or pulsed laser microbeams), we exposed HeLa cells expressing green fluorescent protein (GFP)-tagged TRF2 to a highly focused beam from an 800-nm pulsed multiphoton laser. With this source, coincident absorption of two photons results in energy deposition equivalent to that produced by a single 400-nm

Figure 1 TRF2 response to photo induced DNA damage. (a) Live-cell confocal images of a nucleus after high-intensity multiphoton laser exposure in the presence of Hoechst 33258 demonstrates recruitment of TRF2 to the exposed region. Representative time points are shown for images captured at 10-s intervals after exposure. (b) Cell nuclei do not show any recruitment of TRF2-GFP (green) to damage sites as marked by DDB2-mCherry (red) after exposure to localized ultraviolet C irradiation (1,000 J/m², t = 5 min after illumination).
photon (Supplementary Methods online). We measured TRF2 recruitment to exposed nuclear regions by live-cell imaging and compared it with the recruitment of Ku80-GFP, a nonhomologous end-joining (NHEJ) protein, or xeroderma pigmentosum C (XPC)-GFP, a critical nucleotide excision repair (NER) protein. When cells were exposed in the presence of the photosensitizing dye Hoechst 33258, we observed, within 10 s, recruitment of both Ku80 and XPC to laser-damaged sites (15% of maximum laser output; Supplementary Fig. 1 online). Recruitment of TRF2 to these damaged sites occurred within 20 s of exposure and persisted for the 3-min duration of the experiment, but only after a 1.6-fold increase in laser power (to 24% of maximum laser output; Fig. 1 and Supplementary Table 1 online). Fluorescence intensity of TRF2 at telomeres was not measurably affected. These results are consistent with those of Bradshaw et al.3 in that we confirmed TRF2 recruitment to damaged nuclear regions within seconds of exposure.

Hoechst 33258 promotes the precise photochemical reaction that produces DSBs after ultraviolet A exposure4. This reaction originally formed the basis for concluding that γH2AX foci were generated specifically in response to ultraviolet A laser–induced DSBs5. However, even in the absence of Hoechst, boosting the output of our multiphoton laser beam to 60% of the maximum resulted in Ku80 and XPC being rapidly recruited to damaged nuclear regions. Interestingly, TRF2 recruitment again required a 1.6-fold increase in power output for visualization (to 95% of the maximum; Supplementary Fig. 2 online). These results highlight the fact that laser energy output and the presence of photosensitizers can greatly influence results. In contrast to multiphoton treatment, we did not observe TRF2 recruitment after exposure to a more conventional, less intense 405-nm laser beam in the presence of Hoechst, whereas both NER and NHEJ proteins were rapidly and abundantly recruited.

Although ionizing radiation produces a multitude of DNA lesions, it is perhaps best known for its ability to produce DSBs6. α-particles deposit their energy along defined tracks that produce dense linear distributions of DSBs that are readily recognizable after detection of γH2AX by immunofluorescence7. In one series of experiments, an average of one to two α-particles from a 241Am source traversed HeLa cell nuclei in a longitudinal trajectory (Supplementary Methods). We observed significant accumulation of γH2AX as well as various DNA damage-response proteins (NBS1, MRE11, MDC1 and 53BP1) as early as 90 s after α-particle exposure (Fig. 2). However, quantitative analysis of fluorescence intensity at damaged sites did not show any significant accumulation of TRF2 (Fig. 2c). Furthermore, TRF2 was not recruited to α-particle–induced damage sites in primary human dermal fibroblasts or in the ALT-positive osteosarcoma cell line U2OS, indicating that failure of TRF2 to accumulate at damage sites
Figure 2 TRF2 fails to colocalize with DNA damage generated by α-particle irradiation. 
(a) Maximum intensity projection from a reconstructed stack of images of a U2OS nucleus 90 s after longitudinal transversal by two α-particles shows well-defined damage tracks (MDC1) with no change in TRF2 staining pattern. (b) Maximum-intensity projection of a HeLa nucleus (outlined in white for clarity) ∼10 min after perpendicular exposure to 200 α-particles delivered to an area ∼5 µm in diameter (yellow) shows robust γH2AX accumulation but no recruitment of TRF2 (0 of 111 cells from three independent experiments). (c) TRF2 fluorescence intensity (mean ± s.d.; n = 10) in HeLa cells 10 min after α-particle exposure does not show any increase in TRF2 at DSBs relative to background fluorescence. The intensity of TRF2 signals at telomeres remains essentially unchanged.

is independent of telomere maintenance mechanisms (Supplementary Fig. 3 online). Also, consistent with previous observations², TRF2 did not colocalize with ionizing radiation–induced foci in response to 5 Gy of ¹³⁷Cs γ-rays (data not shown).
We considered the possibility that damage from one or two $\alpha$-particle tracks might be insufficient to trigger TRF2 recruitment. To resolve this issue, we used a charged-particle microbeam to deliver defined numbers of $\alpha$-particles to specified locations (Supplementary Methods). Delivery of either 200 or 400 $\alpha$-particles (roughly 30 or 60 Gy) to a defined nuclear area of less than 5 µm$^2$ resulted in well-defined damage clusters marked by $\gamma$H2AX and MDC1 (Fig. 2b and Supplementary Fig. 4 online). However, even at these high fluences (producing thousands of DSBs in a limited volume), we never observed TRF2 at exposed nuclear regions (Fig. 2b,c and Supplementary Fig. 4).

Finally, we considered the possibility that TRF2 was, in fact, recruited to sites of high-intensity laser-induced damage not in response to DSBs but instead in response to DNA damage more characteristic of ultraviolet exposure (for example, cyclobutane pyrimidine dimmers and 6-4 photoproducts). We used ultraviolet C light (254 nm) to expose HeLa cells expressing both TRF2-GFP and mCherry-tagged DDB2, a heterodimeric protein involved in NER. We confined ultraviolet damage to discrete nuclear volumes by passing light through a polycarbonate filter (5 µm pore size) as described previously (Supplementary Methods) and performed live-cell imaging to monitor recruitment of the fluorescently tagged proteins to damaged sites. Exposures of 100 J m$^{-2}$ resulted in rapid accumulation of DDB2. However, even after 1,000 J m$^{-2}$, no aggregation of TRF2-GFP occurred within 15 min of exposure (Fig. 1b).

The lesion spectrum produced by high-intensity laser systems is not well characterized, and compared with other DNA-damaging agents, it seems uniquely capable of eliciting TRF2 recruitment. Although DNA damage characteristic of that produced by ionizing radiation, such as DSBs, is undoubtedly present after such exposure, the precise mechanism of damage formation is unknown, and clearly, unusual features associated with such concentrated energy deposition result in additional lesion types in DNA or other nuclear constituents. It is certainly not our intent to cast aspersions on the use of laser microbeams for the study of biological phenomena. Indeed, we believe that in many cases, laser microbeams (when combined with appropriate controls, discriminating damage markers and suitable endpoints) can be used effectively to study DNA damage responses. However, our results strike a cautionary note regarding the interpretation of resultant data, and they illustrate the importance of combining different sources of DNA damage before drawing conclusions about localization or dynamics of any DNA damage regulator. In conclusion, although the initiating lesion(s) responsible for laser microirradiation–induced TRF2 recruitment remains elusive, it seems unlikely to us that TRF2 has a biologically relevant role in the early response to exogenous DNA damage, particularly DSBs.
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AUTHOR CONTRIBUTIONS

E.S.W. and J.S. participated in all aspects of the study including assistance with and/or performance of radiation exposures, immunostaining, image capturing and analysis, and manuscript preparation. J.E. performed and/or assisted with all laser exposure experiments. B.P. assisted with Columbia microbeam exposures. M.S.L. generated TRF-GFP and DDB2-mCherry cell line and carried out UVC exposures. P.M.K. designed and performed fluorescence intensity analysis. R.L.U. assisted with overall study design. J.A.A. and S.M.B. shared senior author responsibilities.

REFERENCE LIST

**SUPPLEMENTARY MATERIALS**

**Supplementary Methods**

**Cell culture.** HeLa cells and U2OS cells were cultured as monolayers in Dulbecco’s Minimal Essential Medium (Gibco BRL, Invitrogen Corporation, Carlsbad, CA, USA), supplemented with 10% fetal calf serum. Cells were incubated in a 37°C incubator in an atmosphere of 10% CO₂ in air. Primary Human Dermal Fibroblasts (HDF) were cultured as monolayers in MEM alpha medium (Gibco), supplemented with 10% fetal calf serum and were incubated in a 37°C incubator in an atmosphere of 5% CO₂ in air.

**Laser-induction of local DNA damage and live cell imaging.** For induction of multiphoton damage, a Coherent Mira mode-locked Ti:Sapphire laser was used at 800 nm with a pulse length of 200 fs and repetition rate of 76 MHz. In parallel experiments, a 30 mW 405 nm diode laser supplied by Zeiss was used to induce local damage. Exposures were done with and without Hoechst 33258 (Sigma, bisbenzimidazole derivative, supravital minor groove-binding DNA stain with AT selectivity; final concentration 10 µg/ml added 30 minutes prior to treatment). All treated cells were analyzed at the same magnification and zoom factor using low laser power to minimize monitor bleaching during data collection. The region to be damaged was always the same size and shape, while laser treatment was done with calibrated lasers to exclude variations in dose. Confocal images of living HeLa cells expressing GFP-tagged TRF2, Ku80 and XPC (manuscript in preparation) were obtained using a Zeiss LSM 510 microscope equipped with a 25 mW Ar laser at 488 nm and 40X 1.3 N.A. oil immersion lens. Images of single nuclei were taken at a lateral sample interval of 100 nm. GFP fluorescence was detected using a dichroic beamsplitter (HFT488) with an additional 505-530 nm bandpass emission filter placed in front of the photomultiplier tube.

**Ionizing Radiation-induced DNA damage**

**Longitudinal exposure to one or two α-particles (high LET, densely ionizing).** Our procedure is described elsewhere in detail. In short, cells were cultured on ultra-thin Mylar film in custom-made culture dishes and irradiated with α-particles using an Americium (²⁴¹Am) source with an activity of 140 kBq. Cell cultures were irradiated so that α-particles entered at an angle of 30° from the horizontal plane, on average. Cells were fixed immediately after exposure (90 seconds), 5 minutes, 10 minutes, 30 minutes and 60 minutes post-exposure. Preparations were observed using a Leica (Wetzlar, Germany) fluorescence microscope (DM RA HC). Micrographs were recorded using a cooled CCD camera (KX1400, Apogee Instruments, CA, USA). Stacks of 40 images were collected at intervals of 200 nm in the Z direction. The stacks of images were reconstructed and rendered using Huygens software (Scientific Volume Imaging, Hilversum, The Netherlands) and maximum intensity projections were made using Image Pro Plus software (Mediacybernetics, Carlsbad, CA, USA).

**Perpendicular exposure to a high dose of localized α-particles.** Cells were cultured on ultra-thin polypropylene foil in custom-made culture dishes and then irradiated with 7.2
MeV α-particles (range ~60µm, initial stopping power 80 keV/µm). The charged particles are focused with a series of electrostatic lenses to a beam diameter of less than 5µm. A detailed description of ion beam generation, cell-targeting techniques and beam positioning has been described elsewhere. Cells were exposed to 0, 200, or 400 α-particles delivered perpendicularly (approximately 0, 30, and 60 Gy, respectively) and fixed immediately, 30 min, and 60 min after irradiation. Preparations were observed using a Zeiss (Thornwood, NY, USA) fluorescence microscope (Axioplan 2ie MOT). Images were captured using a CCD camera (model CV-M4+CL, JAI PULNiX Inc., San Jose, CA, USA). Stacks of 40 images were collected at intervals of 200nm in the Z direction. The stacks of images were reconstructed and rendered as above.

Irradiation of cells with γ-rays (low LET, sparsely ionizing). Cells were cultured on chamber slides (Nalge Nunc International, Naperville, IL, USA), then exposed to 5 Gy 137Cs γ-rays (Mark I, J.L. Shepard) and fixed immediately, 5 min, 10 min, 30 min, and 60 min following exposure. Preparations were observed using a Zeiss (Thornwood, NY, USA) fluorescence microscope (Axioplan 2ie MOT). Images were captured using a CCD camera (model CV-M4+CL, JAI PULNiX Inc., San Jose, CA, USA). Stacks of 10 images were collected at intervals of 500nm in the Z direction. Each image was analyzed individually for co-localization.

Scoring. Maximal accumulation of TRF2 at damage sites was previously shown to occur approximately 2 minutes following exposure; this was corroborated by our results from multiphoton laser experiments. Accordingly, we primarily investigated time points less than 10 minutes, although later time points were also examined as noted. Cells were scored qualitatively for the presence or absence of TRF2 recruitment to damage sites. Positive recruitment was scored when noticeable changes in the staining pattern of TRF2 were observed, or when there was an increase in the incidence of TRF2 foci at the damage site. A subset of these cells was also quantitatively analyzed as described in “Image Analysis.”

Image analysis. The amount of TRF2 fluorescence co-localizing with DNA damage markers relative to the amount fluorescence in the background and on the telomeres was quantified by image analysis. Image analysis was performed using a custom-made macro created in Matlab (MathWorks, Natick, Massachusetts, USA), using DipImage (Quantitative Imaging Group, Delft University of Technology, Delft, The Netherlands), an image library for Matlab. 3D images of cells co-stained for TRF2, γH2AX (or MDC1) and DNA were used. For analysis of telomere-associated TRF2, the TRF2 channel was thresholded using the isodata algorithm. For the areas obtained by thresholding, the average TRF2 intensity was determined. For analysis of the TRF2 signal in the damage-containing areas, the γH2AX channel was thresholded using the isodata algorithm. Damage-containing areas that also contained telomere-associated TRF2 were excluded from analysis. The intensity of TRF2 was measured in the remaining areas. To measure intensity of TRF2 in the background, the damage-containing areas were expanded by dilation, the damage-containing and telomere-associated areas were subtracted and, in the remaining areas, the average TRF2 intensity was determined.
Immunofluorescence. Cells were fixed onto substrate (Mylar, polypropylene, or glass) in 2% paraformaldehyde in PBS for 6 minutes and then rinsed in PBS. Cells were permeabilized with 0.2% Triton X-100 in PBS for 6 minutes followed by 30 minutes blocking in 5% milk solution. Primary antibodies were mixed in 5% milk solution and incubated for 1 hour. For γH2AX / TRF2 dual staining, Rabbit anti-γH2AX (Trevigen #4411 PC-100) was used in combination with Mouse anti-TRF2 (Imgenex #IM6-124A). Alternatively, dual staining with Mouse anti-γH2AX (Upstate Biotechnology #05-636) and Rabbit anti-TRF2 (Santa Cruz #sc9143 H-300) was used. Following incubation with primary antibodies, cells were rinsed in PBS and secondary antibodies in 5% milk solution were added for 1 hour. Secondary antibodies used were Goat anti-mouse CY3 (Jackson ImmunoResearch Laboratories, West Grove, PA, USA, #115-165-100), Goat anti-rabbit FITC (Jackson, #111-095-144), Goat anti-mouse Alexa488 (Invitrogen #A11029), and Goat anti-rabbit Alexa594 (Invitrogen #A11012). Cells were then rinsed in PBS and mounted onto slides with Vectashield antifade with DAPI (Vector Laboratories, Burlingame, CA, USA) and coverslipped.

UVC-induction of local DNA damage and live cell imaging. HeLa cells expressing TRF2-GFP were transiently transfected with DDB2-mCherry using Lipofectamine transfection reagent (Invitrogen, Breda, The Netherlands) according to manufacturer instructions. After transfection, cells were cultured for an additional 24 h to allow expression of the fusion proteins before experiments were performed. Cells were UVC-irradiated on an Axiovert 200M widefield fluorescence microscope using a UVC source containing four UV lamps (Philips TUV 9W PL-S) above the microscope stage. The UV dose rate was measured to be 3 W/m² at 254 nm. For induction of local UV-damage, cells were UV irradiated through a polycarbonate mask (Millipore Billerica, Massachusetts, USA) with pores of 5μm and subsequently irradiated for 39s (100 J/m²) or 390s (1000 J/m²). The response of TRF2-GFP and DDB2-mCherry upon UV irradiation was measured on a Zeiss Axiovert 200M widefield fluorescence microscope, equipped with a 100x Plan-Apochromat (1.4 N.A.) oil immersion lens (Zeiss, Oberkochen, Germany) and a Cairn Xenon Arc lamp with monochromator (Cairn research, Kent, U.K.). Images were recorded with a cooled CCD camera (Coolsnap HQ, Roper Scientific, USA). A 375-490 excitation filter, 490 dichroic mirror and 525-40 band-pass emission filter was used for EGFP imaging (monochromator: 470 nm ± 20 nm). A 375-580 excitation filter, 585 long-pass dichroic mirror and 620-60 band-pass emission filter was used for mCherry imaging (monochromator: 550 nm ± 20 nm).
## Supplementary Table 1. Summary of protein responses following exposure to various DNA damaging sources.

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<th>Protein Recruitment</th>
<th>General DSB Marker</th>
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<td>Live cell imaging</td>
<td>TRF2: Present (24% laser power)</td>
<td>NHEJ: Ku80 (15%)</td>
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<td>NER: XPC (15%)</td>
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<tr>
<td>800 nm multiphoton laser – Hoechst 33258</td>
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<td>TRF2: Present (95% laser power)</td>
<td>NHEJ: Ku80 (60%)</td>
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<td></td>
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<td>NER: XPC (60%)</td>
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<tr>
<td><strong>UVA</strong></td>
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<td>405 nm laser + Hoechst 33258</td>
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<td>N.D.</td>
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<td>XPC present (live cell)</td>
<td>γH2AX present (IF)</td>
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<td><strong>Ionizing Radiation</strong></td>
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N.D. not determined.
SUPPLEMENTARY REFERENCES


SUPPLEMENTARY FIGURES

**Supplementary Figure 1.** Exposure of cells to multiphoton laser beams at 15% of maximum laser output in the presence of Hoechst 33258 recruits the repair proteins (a) XPC and (b) Ku80 within 10 seconds of exposure.
Supplementary Figure 2. TRF2 is recruited to exposed nuclear regions following multiphoton laser beam exposure at 95% of maximum laser output, even in the absence of the photosensitizing dye Hoechst 33258.

Supplementary Figure 3. Quantitation of fluorescence intensity of TRF2 following longitudinal exposure to one or two α-particles. No increase in TRF2 fluorescence at DSBs is seen in primary cells (HDF) or ALT-positive cells (U2OS) 10 minutes post-exposure (mean ± s.d.; n=10). Large variability in telomere length, indicated by the deviation in TRF2 fluorescence, is characteristic of the recombination-based ALT phenotype.

Supplementary Figure 4. Protein response to localized exposure of 400 α-particles. (a) HeLa cell nuclei 10 minutes after perpendicular exposure to 400 α-particles shows no recruitment of TRF2 to irradiated region (yellow) (0 of 101 cells from 4 independent experiments). (b) γH2AX (green) and MDC1 (red) are robustly recruited to damaged sites 30 minutes after perpendicular exposure to 400 α-particles and show significant co-localization.