exists for the presence of differences between northern and southern Chinese.

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DNA double-strand breaks are not sufficient to initiate recruitment of TRF2

To the Editor:

The human telomere binding factor TRF2 is essential at telomeres, facilitating the formation and stabilization of t-loops[1] and suppressing local ATM-mediated damage response[2]. Bradshaw *et al.*[3] 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)[3], 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 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.

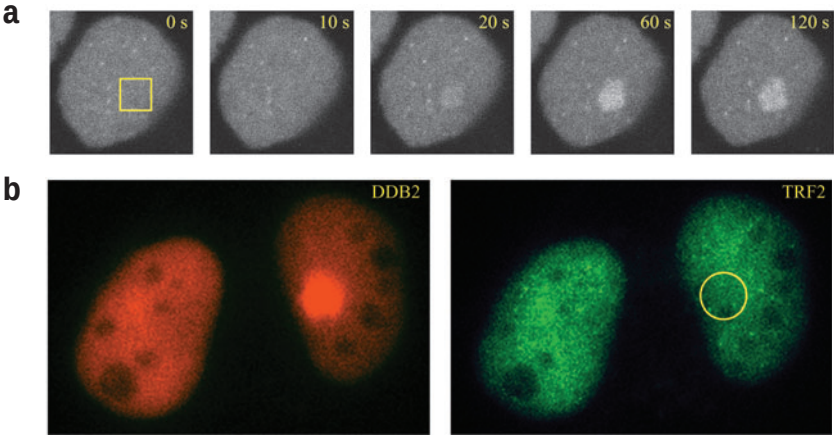

Figure 1 TRF2 response to photoinduced DNA damage. (**a**) Live-cell confocal images of a nucleus after high-intensity multiphoton laser exposure in the presence of Hoechst 33258 demonstrates recruitment of TRF2 to the exposed region. Representative time points are shown for images captured at 10-s intervals 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 m^{-2}, t = 5 min after illumination).

Hoechst 33258 promotes the precise photochemical reaction that produces DSBs after ultraviolet A exposure[4]. This reaction originally formed the basis for concluding that γ-H2AX foci were generated specifically in response to ultraviolet A laser–induced DSBs[5]. 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 DSBs[6]. α-particles deposit their energy along defined tracks that produce dense linear distributions of DSBs that are readily recognizable after detection of γ-H2AX by immunofluorescence[7]. In one series of experiments, an average of one to two α-particles from a ^{241}Am source

traversed HeLa cell nuclei in a longitudinal trajectory (**Supplementary Methods**). We observed significant accumulation of γ-H2AX 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 is independent of telomere maintenance mechanisms (**Supplementary Fig. 3** online). Also, consistent with previous observations[2], TRF2 did not colocalize with ionizing radiation–induced foci in response to 5 Gy of ^{137}Cs γ-rays (data not shown).

We considered the possibility that damage from one or two α-particle tracks might be insufficient to trigger TRF2 recruitment. To resolve this issue, we used a charged-particle microbeam to deliver defined numbers of α-particles to specified locations[8] (**Supplementary Methods**). Delivery of either 200 or 400 α-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 γ-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 dimers 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 though a polycarbonate filter (5 μm pore size) as described previously[9] (**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[10]. 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.

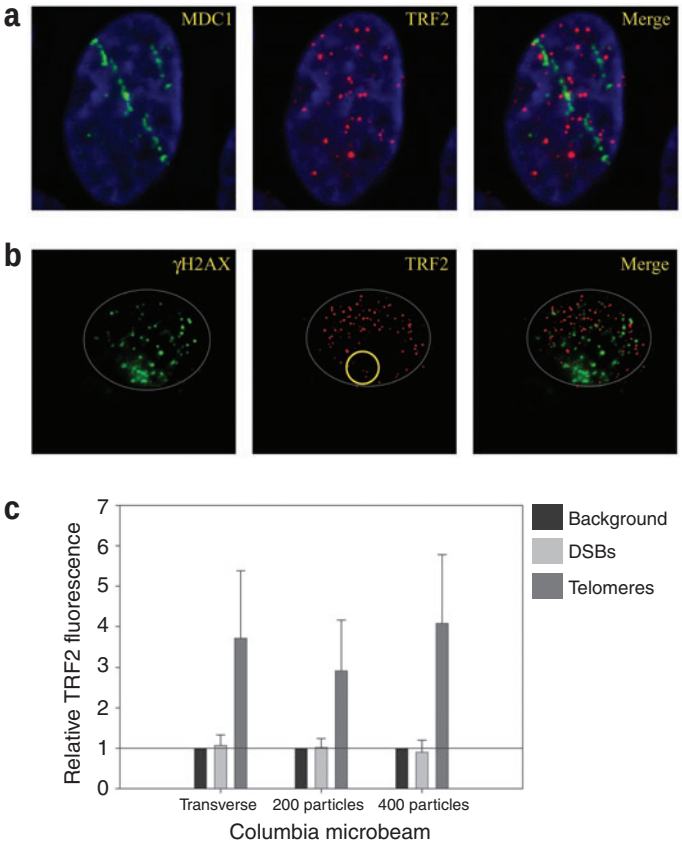

Figure 2 TRF2 fails to colocalize with DNA damage generated by α-particle irradiation. (**a**) Maximum intensity projection from a reconstructed stack of images of a U2OS nucleus 90 s after longitudinal transversal by two α-particles shows well-defined damage tracks (MDC1) with no change in TRF2 staining 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 γ-H2AX 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.

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Bradshaw and Meyn reply:
We thank Williams *et al.* for their work confirming our observations that in human cells, TRF2 rapidly associates with DNA damage induced by exposure to Hoechst 33258 plus ultraviolet A light. However, we disagree with their conclusion that TRF2 does not have a role in the cellular DNA damage response to DNA double-strand breaks (DSBs).

Our experimental approach of introducing a high concentration of DNA damage in defined subnuclear regions of chromatin by treating cells with Hoechst 33258 and then exposing them to an ultraviolet A laser microbeam (hereafter 'Hoechst + ultraviolet A')[1], was chosen based on a previous observation that TRF2 is not detectable by immunofluorescence at sites of damage induced by low-LET (linear energy transfer) γ-radiation[2]. We reasoned that isolated DSBs induced by γ-radiation might be insufficient to allow detection (by fluorescence microscopy) of proteins in low abundance at DSBs, those present at a subset of DSBs or those that bind to transient structures generated during the repair of DSBs. The results reported by Williams *et al.* confirm the previous observation and support our experimental rationale.

The conclusions drawn by Williams *et al.* are based on their inability to detect TRF2 accumulation by indirect immunofluorescence at sites of DNA damage induced by low-LET γ-radiation or high-LET ^{241}Am radiation. However, the ability to form visible foci at sites of ionizing radiation–induced DNA damage is not a universal property of DSB damage response proteins. The Ku proteins and the catalytic subunit of the DNA-dependent protein kinase (DNA-PKcs) are critical components of the nonhomologous end joining (NHEJ) DSB repair pathway that are known to interact directly with induced DSBs[3]. Although these NHEJ proteins can be visualized at sites of photoinduced DNA damage[4], they have not been detected by immunofluorescence techniques at DNA damage sites after exposure to low-LET γ-radiation. Further, others have reported that Ku70, Ku80 and DNA-PKcs do not form immunofluorescence-detectable nuclear foci at DNA damage sites after exposure to as much as 50 Gy of high-LET heavy ion radiation[5].

Although the inability to detect TRF2 at ionizing radiation–induced DSBs by immunofluorescence is weak evidence against the involvement of TRF2 in the cellular DSB response, other observations provide strong support for the idea that TRF2 is functionally involved in the response to ionizing radiation–induced DSBs. Like many classic DNA damage response proteins, TFR2 is rapidly phosphorylated in an ATM-dependent manner after exposure to γ-radiation[6]. We and others have also shown that TRF2 modulates γ-radiation–induced ATM-dependent phosphorylation of multiple DNA damage response proteins[7,8].

Williams *et al.* do raise an important point: we have an incomplete understanding of the range of DNA lesions induced by Hoechst + ultraviolet A and by low- and high-LET ionizing radiation. Significantly, DSBs induced by high-LET radiation are thought to occur at multiple damaged sites that also contain additional DNA lesions that may interfere with interactions between DSBs and DNA damage-response proteins[9]. In support of the idea that differing types of radiation generate distinct classes of DSBs that interact with cellular repair proteins in different ways, the repair kinetics of high LET–induced DSBs are markedly different from those created by low-LET γ-radiation[10].

Although our knowledge of the photochemistry of Hoechst + ultraviolet A is incomplete, we do know that, like ionizing radiation, Hoechst + ultraviolet A induces both single- and double-strand breaks in DNA[11]. In our own experimental system, we find that prior exposure to Hoechst 33258 is required for our ultraviolet A microbeam laser to induce a high enough local concentration of DSBs to be detected either by end-labeling with Cy3-dCTP or by the presence of γ-H2AX. We find that the same Hoechst treatment is required for detectable association of TRF2 with ultraviolet A laser–induced DNA damage. Williams *et al.* report essentially the same results: prior exposure to Hoechst 33258 greatly enhances both the production of DSBs by ultraviolet A and the association of TRF2 with ultraviolet A–induced DNA damage.

The DNA damage response proteins used by Williams *et al.* for their experiments are known to form large foci, easily visible by immunofluorescence, surrounding DSB-containing DNA. The accumulation of these proteins in large molar excess in the vicinity of a single DSB is thought to be due to complex protein-protein interactions with the modified chromatin that surrounds the DSB site rather than the DSB itself. Rather than form large nonstoichiometric protein aggregates bound to modified chromatin, we propose that TRF2, along with Ku and DNA-PKcs, may belong to a different class of DNA damage response proteins that directly interact with damaged DNA at low stoichiometric ratios and/or with distinct types of DSBs. Proteins that directly interact