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2007 MICHAEL FRY RESEARCH AWARD LECTURE
Telomeres and Double-Strand Breaks – All's Well that "Ends" Well. . . .

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Sometimes one's life (including one's science) makes a lot more sense when viewed from the perspective of time, reflected back on over a number of years. That has indeed been the case for me. Strangely enough, the story begins with chromosomes and "ends" with telomeres, both at Colorado State University. And, just as with chromosomes, a lot happened in between. Telomeres were first identified based on their function—they protected the physical ends of chromosomes from interaction with broken DNA ends created by ionizing radiation. While I was at Los Alamos National Laboratory, the sequence of human telomeres was discovered, making probes available that allowed us to re-examine and provide direct support of these early observations; thus began my fascination with telomeres. Chromosome orientation *in situ* hybridization (CO-FISH) also came onto the scene while I was in Los Alamos. This strand-specific modification of standard FISH, especially when combined with telomeric sequence probes, has proven to be a powerful approach that provides information not available by any other means. Applications have included pericentric inversion detection, distinction between leading- and lagging-strand telomeres, and identification of telomere-double-strand break (DSB) fusions. We also provided the first direct evidence that DSB repair proteins (DNA-PK in particular) are required for mammalian telomeric end capping, and we have been characterizing telomere dysfunction in NHEJ and HR repair-deficient backgrounds ever since. Cells must correctly distinguish between DNA ends represented by telomeres and DNA ends produced by DSBs if all is to end well. Just as these studies have provided new insight into the complex, often surprising, interactions at DNA ends, they also provoke new questions. Whereas it is now well established that DSB repair proteins associate with telomeres, most recently we've been asking whether the reverse scenario holds: Do telomere proteins interact with DSBs? We find that DSBs induced by ionizing radiations are not sufficient to recruit the essential telomere protein TRF2 as an early damage response, so perhaps this interplay is a one-way street. The rest of the story waits to unfold. © 2008 by Radiation Research Society

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Opening comments

It is indeed an honor to be receiving Michael Fry's namesake award, recognizing the contributions of a Junior Investigator to the field of radiation research. I imagine many of you may well be wondering Junior? Young? How can that possibly be? She's been around forever—and I'm afraid you'd be absolutely right. So I'm going to take this opportunity to share my personal journey, a stroll down memory lane if you will, to give you a glimpse of the road I've traveled that has led me here today.

A long time ago . . . in a land far away . . .

The tale begins in 1976 at Colorado State University, where Joel Bedford had just arrived from Vanderbilt, along with his Ph.D. student Jim Mitchell. I (a mere child at the time) had just finished a Bachelor's degree in biological sciences and had neither direction nor clue as to what I wanted to be when I grew up—but I did need a job. I went to see my advisor (for the first time) and he sent me to see this new professor on campus, setting into motion events that would shape my future. Joel and Jim introduced me to the world of radiation cytogenetics—and chromosomes! Some of the first I saw peering down the microscope weren't just any chromosomes—they were my chromosomes! It ignited a passion that continues to this day. I spent several very busy and exciting years doing lots of survival curves on lots of cell lines. Four publications in *Radiation Research* on which I was included as an author resulted from that work (*1–4*). Joel and Jim, I shall always be grateful to you for taking a chance on me—thank you so very much.

Well, life has a way of happening, and it sure did. Three children, a move back to Los Alamos, NM (my home town), and almost 10 years later (spent enjoying being a stay-at-home mom), there came a knock on the door and fate stepped in again. Michael Cornforth, a recent Ph.D. student of Joel's, was at Los Alamos National Laboratory (LANL) and was looking for a technician. He was willing to be flexible with my schedule so that I could work around family obligations, and I started back working part-time. Thank you, Michael, for providing the opportunity and optimism I needed to get back into the field. I quickly real-

ized, however, that I had a lot of catching up to do—a lot had happened—little things, like almost the entire field of molecular biology, for example. I also quickly realized that I had a lot of chromosome analyses to do—like one experiment that required scoring more than 10,000 cells—the dreaded LA069, which took years to complete. Surprisingly, this neither killed me nor cured me. I delighted in the fact that this work resulted in a beautiful, rock-solid demonstration of a limiting low dose rate below which there was no further decrease in the yield of chromosome aberrations (*5*).

Perhaps somewhat prophetically, another study during this time involved telomeres. The sequence of the human telomere $(TTAGGG)_n$ had just been discovered right upstairs by Julie Meyne and Bob Moyzis's group (*6*). So we had available to us telomere FISH probes, which we used to demonstrate that the breakpoint interfaces between chromosomes involved in radiation-induced dicentric formation did not maintain telomere sequence (*7*). Importantly, this study represented direct re-examination and support of Muller and McClintock's early observations using ionizing radiation (*8, 9*), which first identified the physical ends of linear chromosomes, termed telomeres by Muller, as special protective features that prevented inappropriate interaction with broken chromosome/DNA ends.

In the early 1990s, one chapter ends—Michael left LANL for UTMB Galveston—and another begins—Edwin Goodwin, Michael's post-doc at the time—inherited the lab, and me. Not long after this change in command, the University of New Mexico (UNM) School of Medicine Biomedical Sciences Program began a long-distance Master's program with LANL. I had long hoped to be able to return to graduate school (and perhaps even finish). This was a great opportunity to do just that. Thank you, Ed, for being willing to work with my situation and schedule, making it possible for me to go back to school and continue working, both part-time. Courses, one per semester, were videotaped on campus in Albuquerque, then shipped to UNM-LA/LANL, and a group of us watched them whenever we could, usually during lunch breaks.

Another defining development also quietly broke onto the scene during this time. . . . CO-FISH was born, working the very first time we tried it. Chromosome orientation fluorescence *in situ* hybridization (CO-FISH) was named for its ability to determine the orientation of repetitive sequences relative to one another (*10*). It is beautifully elegant in its simplicity, involving selective degradation of newly replicated, BrdU-substituted strands of DNA, thus producing single-stranded chromosomal target DNA for hybridization of single-stranded probes. The resulting single-stranded signals provide a wealth of information, not available by any other means.

And then there were telomeres!

The modern view of telomeres is one of complex and dynamic nucleoprotein structures (*11*) consisting of tandem arrays of short, repetitive G-rich sequences (species specific sequence and length) oriented 5′ to 3′ toward the end of the chromosome (*12, 13*). An essential feature of functional telomeres is a long 3′ single-stranded overhang (*14, 15*), which not only serves as a substrate for telomerase, the specialized reverse transcriptase that adds telomere repeats *de novo* (*16, 17*), but also facilitates t-loop formation (*18*). It was the property of the G-rich telomere sequence always being oriented 5′ to 3′, however, that gave CO-FISH a sense of direction; CO-FISH (i.e., removal of newly replicated strands) with the (single-stranded) C-rich telomere probe identifies the 3′ end of the chromosome, just as CO-FISH with the G-rich telomere probe identifies the 5′ end. So now, in addition to the relative orientation of repetitive sequences, CO-FISH could also reveal their absolute 5′-to-3′ direction, based on which chromatid they hybridized to. Thinking ourselves terribly clever, we named this approach COD-FISH [chromosome orientation and direction FISH; (*19*)], which never really caught on. CO-FISH, on the other hand, slowly but surely did.

Because telomeres also provide a point of reference, we used CO-FISH for detection of pericentric (involving centromere) inversions (*20*) as well as for detection of the obligate inversion that occurs during isochromosome formation (*21*). One of my favorite CO-FISH images (Fig. 1) is of a radiation-induced dicentric involving both homologues of chromosome 1 (red probe marks the centromeres); the 1p36 probe (green) marks the short arms of both chromosome 1s, one being on the associated acentric fragment. A synthetic oligomer to the heterochromatic region around the centromere of chromosome 1 (blue) detects both a pericentric inversion (flips signal to other side of centromere) and a paracentric inversion (flips signal to other chromatid). The ''CO-FISH family'' (Ed, Michael and I) recently reviewed the development and humble beginnings of CO-FISH, along with its various and more sophisticated subsequent applications (*22*).

Meanwhile, back at the ranch (LANL), I had finished a Master's degree and convinced UNM to continue the long-distance program through a Ph.D. (three of us continued). The first publication of my dissertation work provided the first direct evidence that DNA double-strand break (DSB) repair proteins are required for effective end-capping of mammalian chromosomes (*23*). This represented a very unexpected and unlikely liaison between two disparate fields and took both in new directions. What I again saw was that because telomeres normally do their job very well, they must be lost before chromosomal fusions can occur; i.e., no telomere sequence was visible at the points of fusion between chromosomes involved in Robertsonian-like translocations or dicentrics. This was true of wild-type control mouse cell lines and most of the mutant cell lines we examined as well, with a notable exception. Mouse knockout cell lines deficient in any of the subunits of DNA-dependent protein kinase (DNA-PK), Ku70, Ku80 or DNA-PKcs, displayed chromosomal fusions that maintained large blocks

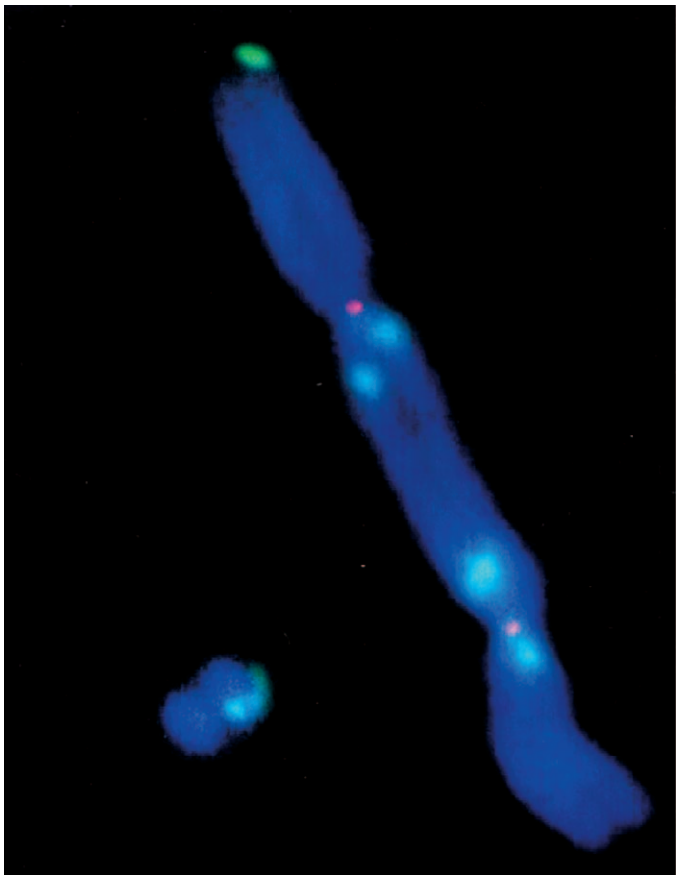

FIG. 1. Radiation-induced dicentric chromosome. One of my favorite CO-FISH images. This radiation-induced dicentric involves both homologues of chromosome 1 (red probe marks the centromeres). The 1p36 probe (green) marks the short arms of both, one being on the associated acentric fragment. A synthetic oligomer to the heterochromatic region around the centromere of chromosome 1 (blue) detects both a pericentric inversion (flips signal to other side of centromere) and a paracentric inversion (flips signal to other chromatid).

of interstitial telomere sequence. These end-to-end events were obviously not the result of significant telomere shortening (loss of sequence), nor were they merely telomere associations (telomeres close together). Rather, they represented failure of end-capping (loss of structure) due to deficiency of essential protein components of functional telomeres. Simon Bouffler graciously agreed to do some karyotyping of our mouse scid metaphases, and he found that the fusions could involve any of the chromosomes (end-capping failure was not chromosome-specific). He also noted clonal rearrangements, providing preliminary evidence that these telomere fusions were indeed covalent linkages.

We were fortunate enough to obtain, from Titia deLange, a human cell line expressing a dominant negative allele of TRF2, a critical telomere binding factor required to prevent end-to-end chromosomal fusion (*24*). We observed a remarkable cytogenetic phenotype; chromatids were fusing together, often stringing multiple chromosomes together, telomere to telomere. The exclusively chromatid-type fusions observed indicated that they occurred after replication in the cell cycle of collection. Additionally, CO-FISH facilitated discrimination between leading- (G-rich probe) and lagging-strand (C-rich probe) telomeres and revealed that the telomeres involved in the fusions, i.e., the ones experiencing end-capping failure due to TRF2 deficiency, were preferentially those produced via leading-strand synthesis (Fig. 2). Together, these results suggested strand-specific postreplicative processing of mammalian telomeres (*25*). Telomere-telomere fusion in DNA-PKcs-deficient backgrounds also involved end-capping failure of leading-strand telomeres, indicating that it, too, is necessary for proper processing and formation of a protective end-structure after replication.

We also demonstrated that dysfunctional/uncapped telomeres fuse not only to one another but also to ionizing radiation-induced DSBs (*26*). Ionizing radiation induced telomere-DSB misjoinings in a dose-dependent manner, creating novel chromosomal rearrangements, the consequences of which are currently not well understood. The dose response is basically linear (*27*), supporting a model in which telomeres become uncapped due to decreased DNA-PK function and compete with radiation-induced DSBs, essentially presenting as DSBs, increasing the opportunity for misrepair and contributing to the radiosensitivity seen in these repair-deficient backgrounds.

All this while, and all too quickly, my kids were growing up on me. Jake, Michelle and Matt all graduated from high school before I finished school—but Mom finally did finish, receiving my Ph.D. in December 2000. Thank you, dear children, for your understanding and support through many years of my holding on to a dream, of my pursuing a personal goal—and often juggling a few too many things.

With another turn of the page, a fateful and fortunate meeting with Bob Ullrich at the ICRR in Ireland led to our asking the question of whether BALB/c mice experienced telomere dysfunction. He and his group, including Brian Ponnaiya, Riuichi Okayasu and Yongjia Yu, had been working with the BALB/c mouse model, which is susceptible to radiation-induced breast cancer. They had demonstrated delayed chromosomal instability, decreased levels of DNA-PKcs expression and activity that was especially pronounced in mammary tissue, as well as two single-nucleotide polymorphisms in the *Prkdc* gene (*28–30*). The very first set of BALB/c mammary epithelial clones I analyzed provided evidence of telomere-telomere and telomere-DSB fusions in a clone that had been shown to be tumorigenic (manuscript in preparation). Thus began a beautiful relationship characterizing telomere dysfunction in repair-deficient backgrounds—that included a move back to Colorado State University. Thank you, Bob, for believing in me at a critical juncture in my life, both professionally and personally, and for opening the door of opportunity for my return to the same department where I had started so many years before.

Along the way, and in collaboration with Jac Nickoloff at UNM, a member of my dissertation committee, we found

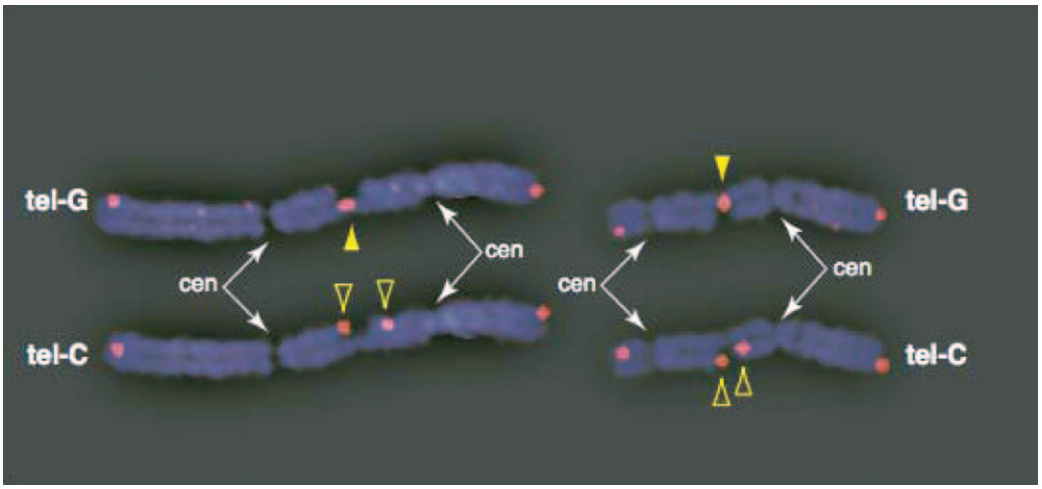

FIG. 2. Human cells expressing a dominant negative allele of TRF2. Exclusively chromatid-type telomere fusions observed indicated that they occurred after replication in the cell cycle of collection. CO-FISH facilitates discrimination between leading- (G-rich probe, solid arrowheads) and lagging-strand (C-rich probe, open arrowheads) telomeres and revealed that the telomeres involved in the fusions, i.e., the ones experiencing end-capping failure due to TRF2 deficiency, were preferentially those produced via leading-strand synthesis. (Image courtesy of Michael Cornforth, UTMB Galveston)

that the kinase activity of DNA-PK is required to protect mammalian telomeres (*31*); without it (one cell cycle in the presence of a specific DNA-PKcs inhibitor), multiple chromatids fuse together telomere-to-telomere. This fusion phenotype was much more pronounced in cells wild type for non-homologous end joining (NHEJ) than in scid cells, providing preliminary evidence that the fusions themselves are mediated by NHEJ. Additional supporting evidence was provided by mouse LigIV mutant cell lines provided by Penny Jeggo, in which telomere fusions were greatly reduced (manuscript in preparation). We were also very interested in whether DNA-PKcs autophosphorylation is important for its role at telomeres. In collaboration with Kathryn Meek and Susan Lees-Miller, and work done by the first graduate student in the lab, Eli Williams, we analyzed mouse mutants of the two autophosphorylation clusters around Thr-2609 and Ser-2056, as well as a kinase dead mutant. As expected, the kinase dead mutant displayed significantly elevated levels of telomere fusion events compared to the control, as did the Thr-2609 mutant, but the Ser-2056 did not (manuscript in preparation), consistent with the proposed reciprocal action of these two autophosphorylation clusters (*32*).

Studies up to this time had been restricted to mice, largely due to the availability of knockouts, but also due to the lack of human null conditions. With the advent of RNA interference (RNAi) (*33*), we extended our studies to human cells using siRNA knockdown of DNA-PKcs. With co-investigator Howard Liber and gratefully acknowledged support from NASA, we demonstrated similar telomeric end-capping failure, as well as increased radiation-induced mutagenesis, after knockdown of Ku80, DNA-PKcs or both (*34*). We have recently shown that partial deficiency (50% levels) of DNA-PKcs, a condition especially relevant to the human condition, produces similar phenotypes (*35*).

Let's stop and reflect for a moment on where we are: We have identified a new aspect of the genetic basis of mammalian telomere function; the NHEJ protein DNA-PK is essential for normal telomeric end-capping. In deficient backgrounds, telomeres become uncapped (not critically shortened) and inappropriately appear as DSBs; they activate the damage response factor γ-H2AX, they result in telomere fusion events that are mediated by NHEJ, and the kinase activity of DNA-PKcs is critical for its function at telomeres, perhaps being necessary for autophosphorylation of the Thr-2609 cluster. We have also identified a new potential source of ongoing radiation-induced instability, because uncapped telomeres in deficient backgrounds fuse not only to each other but also to radiation-induced DSBs. Uncapped telomeres, and therefore increased numbers of ends, combined with increased time (slowed kinetics of repair) in repair-deficient backgrounds, adds up to increased opportunity for misrepair after exposure, thus contributing to the observed radiosensitivity. This represents a new mechanism of telomere dysfunction that is not dependent on gradual or immediate telomere shortening or loss. In this scenario, it is of consequence to note that an open ''end'' remains after a telomere-DSB misjoining, providing a means of generating ongoing instability. In support of this view, Laure Sabatier and John Murnane demonstrated that the loss of a single telomere can result in instability of multiple chromosomes, until all ends are satisfactorily capped (*36*).

As we continue along, we ask the question of whether other DNA repair proteins are involved in telomere function. We found that siRNA reduction of the Nijmegen

breakage syndrome protein NBS1 increases radiation-induced mutation frequency and telomere association (distinctly different from telomere fusion) (*37*). There are so many proteins, and so little time—perhaps a good place to start, though, would be with other homologous recombination (HR) proteins.

Relevant in this regard, an application of CO-FISH that developed along the way involved detection of sister chromatid exchange (SCE)-like recombination within the telomeric repeats themselves, events that split the single-sided CO-FISH telomere signal and have been termed T-SCE (*38*). Michael Cornforth had shown that sub-telomeric repeats display highly elevated rates of mitotic recombination (*39*). We extended this observation into the telomere proper, demonstrating frequent recombination in telomeric DNA that was especially relevant in telomerase-negative, alternative lengthening of telomeres (ALT) backgrounds, a recombination-based mechanism of maintaining telomere length first proposed by John Murnane (*40, 41*). We proposed that unequal telomeric SCE may serve to extend the proliferative life of such cells (*38*).

Our search for genes that regulate T-SCE frequencies led us to the progeria syndromes Werner's and Bloom's. In collaboration with Sandy Chang at the M.D. Anderson Cancer Center in Houston, TX, we demonstrated highly elevated levels of telomeric recombination (significantly increased T-SCE frequencies) in mouse cells doubly deficient in WRN and telomerase (*42*). We also provided evidence that critically shortened telomeres in this HR repair-deficient background promote escape from senescence and engagement of the ALT pathway. Interestingly, increased frequencies of recombination were seen specifically within telomeric DNA, whereas SCE levels in the rest of the genome (G-SCE) and in another repetitive region (mouse major satellite) were not elevated above background. Since Bloom's syndrome is characterized by high levels of G-SCE, the next obvious question was what about T-SCE frequency? In work done by graduate student R. Tanner Hagelstrom, with both mouse mutants and human siRNA knockdowns, we found that BLM deficiency results in increased frequencies of both G-SCE and T-SCE, suggesting a more global role for BLM normally in repressing recombination. These studies are ongoing and are revealing intriguing relationships between DNA repair, premature aging and telomere instability.

Also intriguing was the report that the telomere protein POT1 [protection of telomeres 1 (*43*)] stimulates the RecQ helicases WRN and BLM to unwind telomeric DNA substrates (*44*). Through another enjoyable and productive collaboration with Sandy Chang, we demonstrated that POT1 is critical for maintenance not only of telomere integrity but also of overall genomic stability (*45*); without it, genome stability goes to pot, so to speak. POT1 deficiency resulted in preferential loss of lagging-strand telomeres [reminiscent of WRN deficiency (*46*)], as well as aberrant HR involving telomeric DNA (increased T-SCEs and telomere double minutes). POT1 deficiency also resulted in increased chromosomal instability, seen as novel aberrations (isochromatid rings) resulting from isochromatid breaks, as would be expected to occur in association with stalled replication forks. POT1-deficient cells also rapidly formed tumors in scid mice.

As the story continues to unfold, we see the dividing lines between chromosome ''ends'' becoming blurred. On the one hand, natural chromosomal termini, or telomeres, must be protected from end-joining and recombinational mischief. On the other hand, DSB ends, such as those created by radiation, must be rejoined quickly and correctly. Cells must distinguish between these two DNA ends and deal with them correctly if all is to end well, i.e., to maintain genomic stability and prevent cancer. Normal telomeric end-capping function suppresses both NHEJ and HR activities, yet requires both telomere proteins (e.g. TRF2 and POT1) and proteins more commonly associated with DNA repair (e.g. DNA-PK, WRN and BLM). The studies I've highlighted here have provided new insight into unexpected roles and complex interactions between telomeres and DNA repair that continue to provoke new questions. Most obviously, what about the reverse scenario? Do proteins typically viewed as being telomere-specific interact with DSBs?

This initially appeared to be the case with the report that TRF2 associated with DSBs as an early response to DNA damage (*47*), as seen in the co-localization of damage markers and TRF2 at sites of damage induced by high-intensity laser microbeams. We sought to characterize the damage spectrum responsible for TRF2 recruitment to proposed DSBs, embarking on a highly collaborative project, primarily involving Jacob Aten's laboratory in Amsterdam and Eli Williams, who was working on finishing his dissertation research. Our approach was straightforward: Generate localized DNA damage through a variety of sources and monitor TRF2 and damage marker recruitment by live-cell imaging or immunofluoresence. Damage-inducing sources included a dual photon laser microbeam, α particles (both transverse traversal of single particles and perpendicular delivery of large numbers of charged particles to defined regions with the Columbia/RARAF microbeam), γ rays, 254 nm UVC light, and a 405-nm UVA laser. Multiple damage markers were used, as were multiple cells lines with various telomerase statuses. While we did observe rapid TRF2 recruitment to sites of high-intensity laser microbeam damage, in no case did we find evidence of significant TRF2 recruitment to ionizing or UV-radiation-induced damage sites. We concluded that DNA DSBs are *not* sufficient to recruit TRF2 (*48*) and that TRF2 is unlikely to play a biologically relevant role in the early DNA damage response to DSBs.

Well, that brings us up to date. The road has brought me back to where I began—the circle is complete. As I look back, I realize anew that the journey has really been about the many wonderful and supportive people and mentors I've had the good fortune and pleasure of knowing along

the way, some of whom I've had opportunity to name, others I have not (I apologize). I thank you all.

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