Title
Laser ablation of single telomeres in mitosis: effects and consequences

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Publication Date
2014

Peer reviewed|Thesis/dissertation

UNIVERSITY OF CALIFORNIA,
IRVINE

Laser Ablation of Single Telomeres in Mitosis: Effects and Consequences

DISSERTATION

Submitted in partial satisfaction of the requirements
for the degree of

DOCTOR OF PHILOSOPHY

in Biological Sciences

by

Barbara Alcaraz Silva

Dissertation Committee:
Professor Michael W. Berns, Chair
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2014

DEDICATION

I want to dedicate all my work and studies to THREE special WOMEN in my life.

To my wonderful mommy. Ten years ago, I remember when we were still in Mexico, and you told me that we were going to move to the US, so I can be a Scientist and be with my "Bita." At the time, I couldn't understand why we were moving, but now I know that you wanted the best for my sister and myself. Thank you for all your dedication and support. You have worked very hard day and night with sacrificing so much to give us the best education and the best lifestyle. Through your hard work you taught me to have the best work ethic and to be humble. Thank you for your excellent mentoring and for your guidance. Thank you for being there in my joyful moments and for being my best friend during my struggles. Together we were able to move forward in our lives and be happy. There are no words to express how much I love you. You are my angel, my friend, my supporter and the best mother. I thank God everyday for giving me such an amazing role model. Thank you "mamita linda"!

To my grandma "Bita." She has been very supportive and inspirating. Bita, gracias por todo el cariño, amor y sobre todo por sus oraciones. No tengo palabras para agradecerle todo lo que ha hecho por nosotras. Recuerdo como nos cuidaba a mi hermana y a mi cuando éramos pequeñas. Todos los veranos que pasamos en Pajacuarán fueron los mejores momentos de mi vida y fué porque estabamos con usted. La quiero mucho Bita!

To my sister Jennifer. Little sis, I want to thank you for all your support. You are a strong woman and you taught me the best lesson in life. I wish I were as adventurous as you, and had that amazing memory of yours! Even though sometimes we disagree or have arguments, we still know how much we love one another. I remember how much you helped while I was in college and during Gradute School. You were there helping me with my assisments and staying with me everytime that I was going through something difficult. There are no words to express my gratitude! Love you Jepesito and thank you for taking care of Osiris!

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LIST OF ABREVIATIONS

53BP1	p53-binding protein 1
ATM	Ataxia-telangiectasia mutated
ATR	ATM- and Rad3-related
BRCA1	Breast cancer 1
DDR	DNA damage response
DNA	Deoxyribonucleic acid
DSB	Double- stranded break
FISH	Fluorescence in situ hybridization
GFP	Green flourescence protein
HR	Homologous recombination
IR	Ionizing radiation
MDC1	Mediator of DNA damage checkpoint 1
Mre11	Meotic recombination 11
MRN	Mre11/RAD50/Nbs1
Msp1	Monopolar spindle 1 protein
NBS1	Nijmegen breakage syndrome
NHEJ	Non-homologus end-joining
PCNA	Proliferating cell nuclear antigen
RNF168	RING-finger ubiquitin ligase 168
RNF8	RING-finger ubiqutin ligase 8

siRNA	small interfering RNA
SSB	Single strand break
TRF2	Telomere repeat binding factor 2
XRCC1	X-ray repair cross-complementing protein 1
YFP	Yellow fluorescent protein

ACKNOWLEDMENTS

I would like to express my gratitude to my supervisor Professor Michael W. Bern for giving me the opportunity to work in his laboratory and for teaching me how to be an independent scientits. I want to thank you for believing in me. Also, for allowing the opportunity to expand my expertise by building collaborations with others. Last but not least, for giving me the opportunity to attend various conferences throughout my PhD.

Furthermore, I would like to thank Professor Jagesh V. Shah for being a wonderful mentor and great collaborator. Thanks for all your guidance throughout my studies, especially for keeping such a positive attitute and great enthusiam on our project.

Also, I would like to thank Professor Kyoko Yokomori for all her support. Thank you very much for all your help and for teaching me how to be a better scientist.

I want to thank all my friends, collegues and family who helped me during the challenging writing journey of my dissertation. Without you, I wouldn't be able to finish my dissertation. Thank you for all your support during this stressful stage and for staying with me late at night while I worked. Thank you for teaching me how to be a better person and for making me smile everyday.

I thank the Journal of Biomedical Optics to include copyrighted figures as part of my dissertation. Financial support was provided by the National Institutes of Health Laser Microbeam and Medical Program (RR01192), the Air Force Office of Scientific Research (FA9550-04-1-0101), the Beckman Laser Institute Inc. Foundation (to Michael, W.Berns), and the Ford Foundation Fellowship program from the National Academy of Sciences (to Barbara Alcaraz Silva).

CURRICULUM VITAE

Education

2009 – 2014	Ph.D., Molecular and Cell Biology, University of California, Irvine.
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Professional Experience

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2008	NIH Summer Research Internship. Department of Cognitive and Behavioral Sciences. Universidad Autónoma de México (UNAM). México.
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Academic Honors and Awards

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Research Experience

Graduate Research

2010 – present. Beckman Laser Institute. Department of Developmental and Cell Biology. UC Irvine. Research advisor, Michael W. Berns. Thesis title: Characterization of specialized telomere responses to DNA damage in mitosis.

Undergraduate Research

Aug – Dec 2007. Department of Earth System Sciences. UC Irvine. Research advisor, Michael Goulden Ph.D. Project: understand effect of different concentrations of carbon in the soil, based on the difference of plant morphology.

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Research Interests

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- Chromatin and chromatin modifications
- DNA replication and single molecule analysis
- Telomere role and function in aging and cancer
- Translational research: cancer therapeutics

Invited Lectures

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2012 Characterization of specialized telomere responses to DNA damage in mitosis. Department of Developmental and Cell Biology. Research in progress seminar. UC Irvine.

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Teaching/Mentoring Experience

2013 – present	Graduate mentor for international students at the School of Biological Sciences, UC Irvine.
2011 – present	Biology mentor for undergraduate students at the School of Biological Sciences, UC Irvine.
2010 – 2013	Mentored several undergraduates in my research lab, one of which went on to win a Excellence in Research Award from UCI for her research project entitled, "Cancer and Aging: telomere microirradiation during mitosis results in damage checkpoint activation in eukaryotes."
2011 – 2012	Teaching assistant: Developmental and Cell Biology laboratory course (upper division). Lectured and proctored labs on in-vitro fertilization, genetics, plant biology, SDS-PAGE, western blotting, mitochondria and chloroplast isolation. Held office hours, graded lab reports and exams.

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American Society of Biochemistry and Molecular Biology (ASBMB)
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Women in Biology

Publications

Silva BA, Stambaugh JR, Berns MW. Targeting telomere-containing chromosome ends with a near-infrared femtosecond laser to study the activation of the DNA damage response and DNA damage repair pathways. J Biomed Opt. 2013 Sep;18(9):095003.

Silva BA, Stambaugh JR, Kyoko Yokomori, Shah JV, Berns MW. "Damage to mitotic telomeres delays anaphase onset." (Under review).

Silva BA, Berns MW. "Inhibition of ATM delays phosphorylated TRF2 at double strand breaks (DSBs)" (In advanced preparation).

Poster presentations

1. Barbara Alcaraz Silva, Jessica R. Stambaugh, Kyoko Yokomori, Jagesh V. Shah, Michael W. Berns. Damage to mitotic chromosome ends results in the activation of the DNA damage response (DDR), cell delay and micronucleation. GRC, DNA repair and mutation meeting. March 16-21. Ventura, CA. **Founded by *The National Academy of Sciences*.**
2. Barbara Alcaraz Silva, Jessica R. Stambaugh, Jagesh V. Shah, Michael W. Berns. Damage to mitotic chromosome ends results in the activation of the DNA damage response (DDR), cell delay and micronucleation. The American Association for Cell Biology (ASCB), Dec 13-18. NOLA. **Travel Award.**
3. Barbara Alcaraz Silva, Jessica R. Stambaugh, Jagesh V. Shah, Michael W. Berns. Damage to mitotic telomeres results in DDR and SAC activation that leads to cell delay and micronucleation**.** Keystone symposia, Genome Instability and DNA Repair, March 3-8. Banff, Alberta, Canada**. Travel Award.**
4. Barbara Alcaraz-Silva, Michael W. Berns. *Activation of Homologous Recombination (HR) and Non-Homologous end joining repair on Double-strand breaks of mitotic telomeres*. 2011 Annual Meeting of The American Society of Cell Biology, December 15-19. Denver, Co. **Travel Award.**
5. Barbara Alcaraz-Silva, Michael W. Berns. *Activation of Homologous Recombination (HR) and Non-Homologous end joining repair on Double-strand breaks of mitotic telomeres*. 6th LFD Workshop in Advanced Fluorescence Imaging and Dynamics. University of California, Irvine. Irvine, CA. October 24-28. **Attendance Award.**
6. Barbara Alcaraz-Silva, Michael W. Berns. *Live Recruitment of Double Strand Break DNA damage signaling and Repair Factors on Mitotic Telomeres.*

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7. Barbara Alcaraz-Silva, Michael W. Berns. *Live Recruitment of Double Strand Break DNA damage signaling and Repair Factors on Mitotic Telomeres*. The annual meeting of the American Association for the Advancement of Science (AAAS), Feb 17-21, 2011 at Washington D.C. **Travel Award.**
8. Barbara Alcaraz-Silva, Julian V. Reyes-Lopez, Maria-Isabel Miranda. *Chromatin Remodeling in Taste Aversive Memory Due to Sodium Butyrate (NaBu*). The annual meeting of the American Association for the Advancement of Science (AAAS), February 14-18, 2009 at Chicago IL. **Founded by the Minority Science Program (MSP).**
9. Barbara Alcaraz-Silva, Julian V. Reyes-Lopez, Maria-Isabel Miranda. *Chromatin Remodeling in Taste Aversive Memory Due to Sodium Butyrate (NaBu)*. Sigma Xi Annual Conference, November 20-23, 2008 Washington, D.C. **Founded by MSP.**
10. Barbara Alcaraz-Silva, Julian V. Reyes-Lopez, Maria-Isabel Miranda. *Chromatin Remodeling in Taste Aversive Memory Due to Sodium Butyrate (NaBu)*. Annual Biomedical Research Conference for Minority Students (ABRCMS), November 5-8, 2008 at Orlando FL. **Founded by MSP.**

ABSTRACT OF THE DISSERTATION

Laser ablation of single telomeres in mitosis: effects and consequences

By

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Doctor of Philosophy in Biology Sciences

University of California, Irvine, 2014

Professor Michael W. Berns, Chair

Telomeres are essential for protecting chromosome ends not only from the replication-associated DNA loss, but also from unwanted DNA damage response (DDR) and repair by the damage machinery. Uncapping of telomeres during interphase elicits a DDR mechanism that results in cell cycle arrest. However, it is unclear how chromosome ends are normally protected from the DNA damage machinery, and how DDR is regulated at telomeres during mitosis.

With that in mind, the goal of my thesis is to investigate the consequences of DNA damage occurring at specific chromosomal domains. Laser microirradiation was used in combination with dual fluorescent labeling to monitor the co-localization of DDR factors in PtK_2 (*Potorous tridactylus*) chromosomes.

The results of my thesis show that laser-induced DNA break in chromosome ends as well as in chromosome arms of anaphase cells result in recruitment of the following: poly (ADP-ribose) polymerase 1 (PARP1), checkpoint sensors (p-Chk1, p-Chk2), DNA repair protein Ku70/Ku80, and proliferating cell nuclear antigen (PCNA). However, p53 phosphorylated at serine 15 was detected only at chromosome ends, and not at chromosome arms of anaphase cells.

Furthermore, my experiments show that damage to a single mitotic chromosome end, but not on a chromosome arm, results in specific DDR factor recruitment, damage and spindle checkpoint-dependent mitotic delay, and subsequent micronuclei formation in G1. Together these findings reveal mitosis-specific DDR uniquely associated with chromosome ends.

Furthermore, we found the laser parameters used to induce telomeric TRF2 (repeat-binding factor 2) recruitment. We introduced double-strand breaks (DSBs) and assayed for the recruitment of TRF2. We found that a laser dose of $2.43e^{+11}W/cm^2$ is sufficient to form DSBs, based on the recruitment of repair factor 53BP1. Nevertheless, at this laser dose, TRF2 fails to accumulate at damage sites. In contrast, at an irradiance of $2.65\ e^{+11}\ W/cm^2$ or higher, TRF2 accumulates at damage sites, which is independent of ATM. We also found that phosphorylation of TRF2 on threonine 188 occurs at both low and high irradiance laser-induced DSBs in both interphase and mitotic cells in an ATM-dependent manner. By contrast, Phosphorylated TRF2 on threonine 188 did not form foci by using γ-irradiation.

Chapter 1 Introduction

The Mechanism of DNA damage Response

In order to ensure long-term cell survival, eukaryotic DNA needs to have a protection mechanism against DNA damage. Cellular DNA is susceptible to endogenous factors such as collapse of the replication fork, reactive oxygen species (ROS), alkylating agents such as S-adenosylmethionine, and exogenous DNA damage such as chemotherapeutics, UV radiation, ionizing radiation (IR), just to name a few (1-7). Damage by these agents leads to the formation of a variety of lesions, including base modifications, single strand breaks (SSBs) and double strand breaks (DSBs) (3, 5, 8-13). DSBs, which result from the breakage of two phosphates of the DNA backbone, are the most deleterious types of damage, and can lead to chromosomal fragmentation and genomic rearrangements (11, 14, 15). DSBs that are not repaired in a timely manner can lead to tumorigenesis.

The first mechanism activated in response to DSBs during interphase is the DNA damage response (DDR) pathway. This is a surveillance mechanism that coordinates the detection of damage by activating the repair machinery, ultimately leading to cell delay for timely repair to occur, or apoptosis. The DNA damage response pathway consists of sensors (proteins that detect the damage, and initiate the signaling response), effectors (protein kinases that amplify the signal), and mediators (proteins involved in DNA repair and checkpoint activation) (16). All of these proteins collaborate to sense and repair the damage (Figure 1.1).

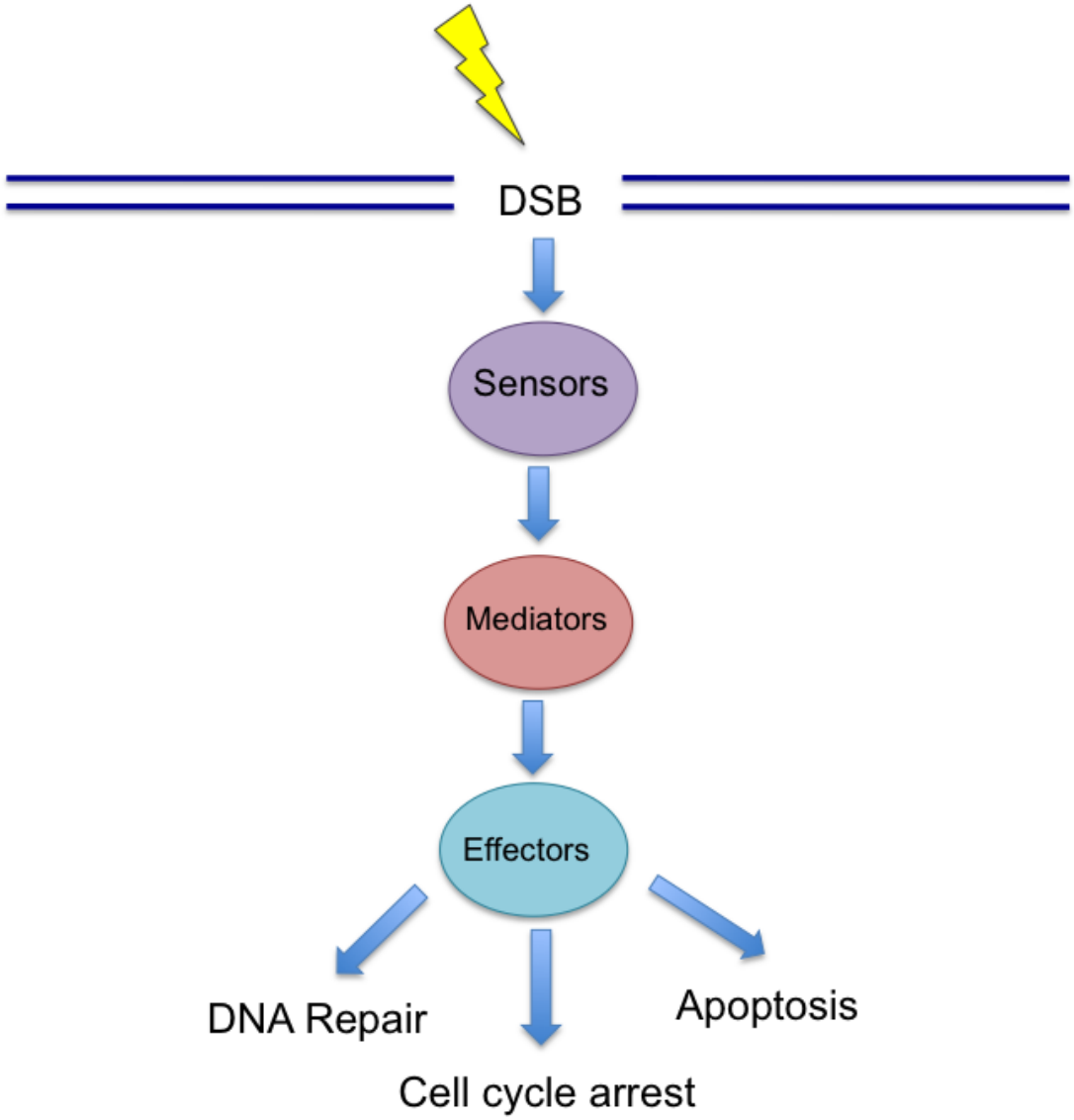

Figure 1.1 DNA damage response DDR
DSBs are recognized by sensor proteins followed by mediator proteins, which amplify the signal to effectors leading to DNA repair, cell cycle arrest or apoptosis.

Double Strand Break Repair in interphase cells

In mammalian cells, DSBs are mainly repaired by two mechanisms: homologous recombination (HR) and non-homologous end joining (NHEJ) (Figure 1.2). HR is an error free mechanism that uses the undamaged sister chromatid as a template to copy the missing information at the break and therefore, it only repairs DNA lesions occurring after DNA synthesis (late S/G2). In contrast, NHEJ is an error prone mechanism that consists of the direct ligation of the two broken DNA ends, which can produce short deletions leading to mutations. NHEJ occurs at any stage of the cell cycle (17, 18) (Figure 1.2).

HR is initiated by the MRN complex (MRE11, RAD50, Nbs1) and CtIP (19), which results in a 5' to 3' resection forming a single 3' overhang (19). Replication protein A (RPA) will rapidly bind to the single stranded DNA (ssDNA) forming a filament. RAD51 will displace RPA and bind to the ssDNA to promote invasion and displacement using an undamaged template (20). Repair will be followed by ligation using the undamaged sister chromatid as a template. In contrast, NHEJ results in the direct ligation of the broken ends. The first protein complex to bind to DSBs is the heterodimer Ku70/80. This protein complex serves as an anchoring site for additional repair proteins. Ku70/80 then recruits DNA-dependent protein kinase, catalytic subunit (DNA-PKcs), forming the DNA-PK complex. This complex facilitates the phosphorylation, activation and recruitment of DNA ligase IV (LigIV), and X-ray cross complementing group 4 (XRCC4) resulting in the direct ligation of the broken DNA ends (21).

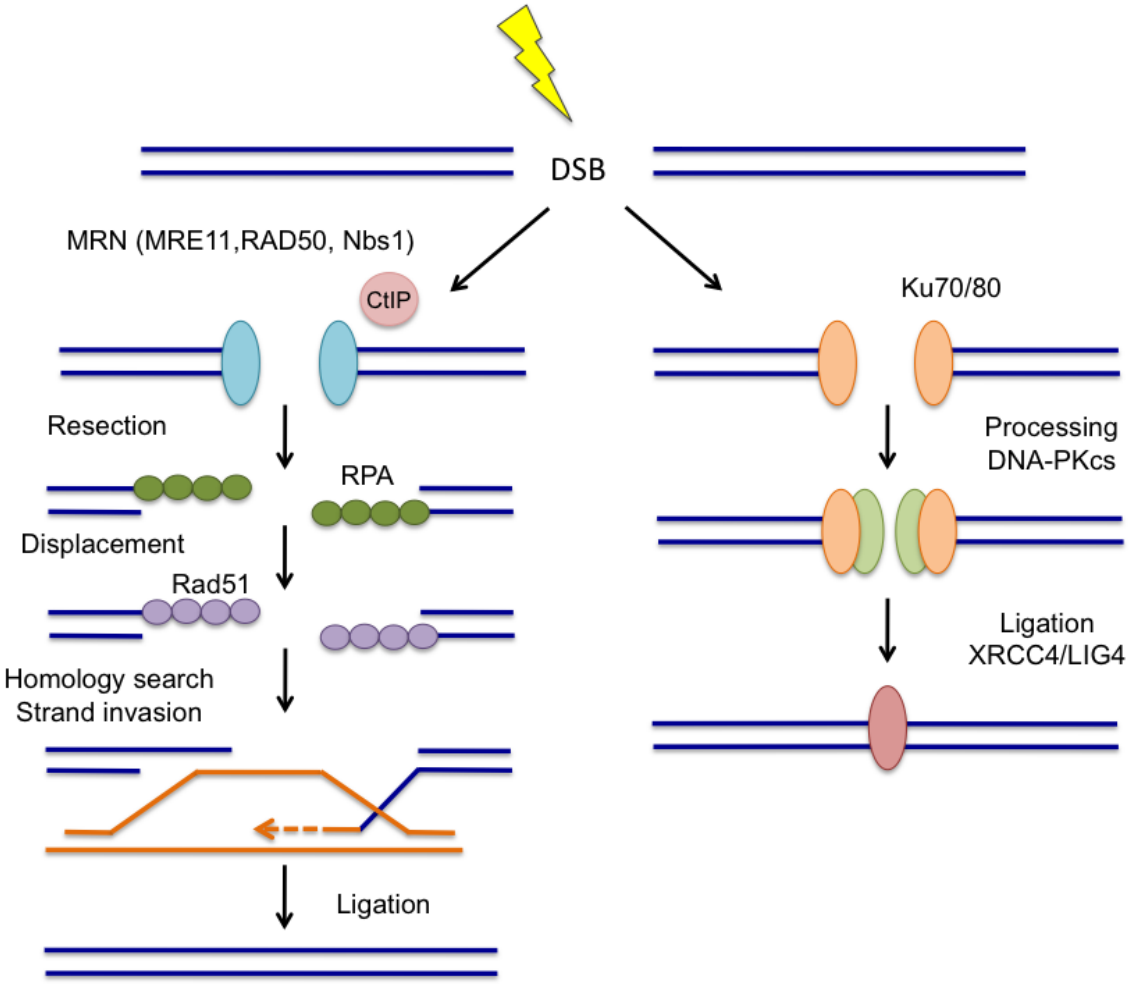

Figure 1.2 Double strand break (DSB) repair. DSBs are repaired by two pathways, homologous recombination (HR) and non-homologous end joining (NHEJ)**.**
HR uses a non-damage sister chromatid as a template to repair the damage site, while NHEJ involves the direct ligation of the broken ends.

Telomere DNA Damage Response

Telomeres are nucleoprotein structures found at the ends of chromosomes (Figure 1.3A). They are composed of TTTAGGG repeats (Figure 1.3B), and a 3' overhang that folds and invades one of the strands to form a t-loop structure. Telomeres are protected by a complex of six proteins known as shelterin (TIN2, TRF1, RAP1, TRF2, TPP1 and POT1) [Figure 1.3B]. The role of the shelterin complex is to protect chromosomes ends from damage, degradation,

recombination, non-homologous end joining reactions, as well as from being recognized as DSBs (22-25). This complex is essential for the stability of telomeres, and removing any part of this complex (either by sequence repeat shortening or by artificial uncapping), leads to the activation of the DDR, cell cycle arrest, and/or apoptosis (26-28) [Figure 1.3C]. This highly conserved DDR mechanism evolved to prevent the damage produced by DSBs at chromosome ends. Uncapped interphase telomeres, known as telomere induced dysfunctional foci or TIFs, (26) are sensed by the DDR, and accumulate γH2AX and checkpoint mediator protein 53BP1 at damage foci (26).

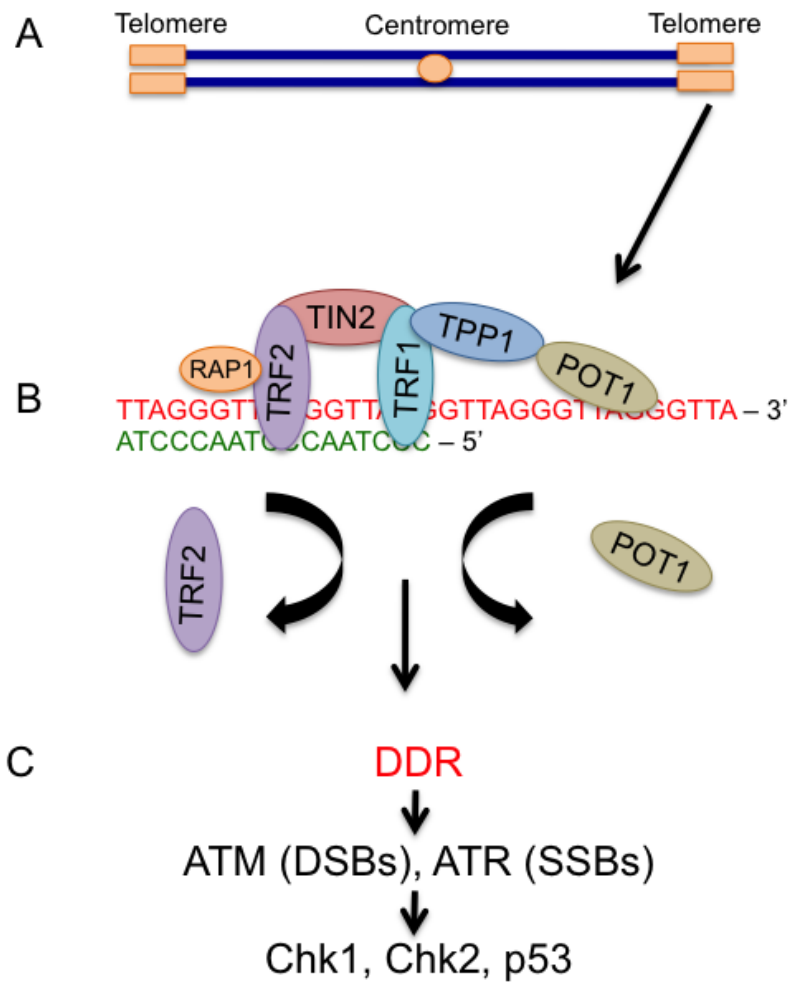

Figure 1.3 Telomere shelterin complex and the activation of the DNA damage response (DDR).

The shelterin nucleoprotein complex protects telomeres. Removal of the shelterin protein components leads to the activation of the DDR.

Previous studies have shown that when the DDR is activated, this leads to the activation of two phosphatidylinositol 3-kinase-related protein kinase (PIKKs): ATM (ataxia telangiectasia mutated), which responds primarily to DSBs, and ATR (ataxia telangiectasia and Rad3 Related), which senses SSBs (26, 29). The ATM kinase will get phosphorylated on the serine 1981 residue, and then phosphorylate the MRN sensing complex (MRE11, RAD50 and NBS1), which is known to be one of the first DSB sensor protein complexes to recognized DSBs. ATM will later phosphorylates histone variant H2AX at the serine 139 residues to becomes γH2AX (30). The activation of ATM will result in phosphorylation of downstream targets, which are involved in cell cycle control, such as effector kinases (Chk2 and Chk1), and will lead to the activation of p53 (tumor protein 53) resulting in cell cycle arrest (31). This produces a cascade of signals leading to the recruitment of DNA repair proteins factors such as NHEJ factor Ku70/80 (26, 27, 32). On the other hand, the ATR pathway is activated by the formation of SSBs due to the induction of UV-damage. SSBs are recognized by a specific RPA repair factor, and recruits an ATRIP/ATR DNA repair complex (33, 34), leading to phosphorylation of downstream components. This also leads to cell cycle arrest in order to allow for timely repair.

Uncapped telomeres of interphase cells activate both kinases regardless of the type of damage. This results in the recruitment of damage recognition and repair factors to the damage telomeres (35, 36).

DNA damage response of telomeres during mitosis

Evidence suggests that during mitosis the DDR is attenuated. While some factors form damage foci, the main proteins required for repair such as 53BP1 do not co-localize to DSBs (37). Recent evidence suggests that telomeric lesions can occur throughout the cell cycle, and are sensed by specific checkpoint pathways during interphase (38). However, little is known about the DDR in telomeres during mitosis.

Recent studies have shown that mammalian telomeres are naturally uncapped at G2 and can continue into mitosis with damaged ends. These uncapped telomeres are sensed as DSBs resulting in the phosphorylation of H2AX (γH2AX), recruitment of MDC1 and activation of the ATM pathway (39). Additionally, mitotic telomeres can be uncapped by prolonged mitotic arrest using depolymerizing drugs such as nocadozole (40). This results in the activation of the DDR, and is based on the phosphorylation of H2AX. However, there is still not enough evidence to conclude that damaged mitotic telomeres are able to activate and recruit downstream components of the DDR.

An alternative method to uncap mammalian telomeres during mitosis is by the loss of function of one of the telomeric components, TRF2 (41). It was recently shown that partial deletions of TRF2 do not alter cell division but result in phosphorylation of H2AX at damaged chromosome ends (41). However, there is neither a method nor technique to show whether damage telomeres can recruit downstream components of the DDR. One of the main obstacles is the time that cells spend in mitosis. Mitosis is the shortest phase of the cell cycle (1-2 hrs, while in interphase last 18-20 hrs), and studies have shown inhibition of DDR in this phase (37).

Laser microirradiation and the activation of DDR during mitosis

There are several methods to study the recruitment of DDR at DSBs. Recently, biophotonic approaches such as scanning laser microscopy, and single molecule analysis have been used to study the dynamics of the recruitment of DDR and repair factors. This allows the *in vivo* study of DDR factors and the assessment of the kinetics at DSBs using either immunofluorescence as a detection method, or real-time fluorescence microscopy when producing DBSs in transfected cells. Also, highly focused short-pulsed lasers have been used to induced DNA damage by multiphoton processes without the use of exogenous photosensitizers (42).

It has been well characterized that different lasers produce unique types of damage (43). Near-infrared (NIR) lasers are known to produce localized submicron DNA lesions in cells with no collateral damage. We have shown, as well as others, that a focused 800 nm femtosecond (fs) NIR laser is capable of producing mostly DSBs (43-46). These short-pulsed NIR lasers can produce DNA lesions at specific chromosomal sites, such as the tips of chromosomes (46).

We have also shown recently that the fs NIR laser, at a threshold-for-damage irradiance of 2.43 e^{+11} W/cm^2 (which is lower than other published studies), is capable of producing DNA breaks such as DSBs and SSBs, significant enough to activate the DDR at chromosome ends (containing the telomeres) in anaphase cells (46). NIR laser microirradiation can be used as an alternative method to create submicron DNA lesions in dividing chromosomes (46-48). The objectives of this thesis are (a) to demonstrate the activation of the DDR on telomere containing mitotic chromosome ends after laser microirradiation, and (b) to study the consequent molecular and cytological effects compared to focal NIR laser elsewhere on the chromosomes.

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Chapter 2 Targeting telomere-containing chromosome ends with a Near Infrared (NIR) Femtosecond laser to study the activation of the DNA damage response (DDR) and DNA damage repair pathways[1]

Abstract

Telomeres are at the ends of chromosomes. Previous evidence suggests that laser-induced DNA breaks at chromosome ends during anaphase results in delayed cytokinesis. A possible explanation for this delay is that the DNA damage response (DDR) mechanism has been activated. In this study, we describe a live imaging method to study the effects of DDR activation following focal point near infra-red (NIR) femtosecond laser microirradiation either at a single chromosome end or chromosome arm in mitotic anaphase cells. Laser microirradiation was used in combination with dual fluorescent labeling to monitor the co-localization of double-strand break (DSB) marker γH2AX along with DDR factors in PtK_2 (*Potorous tridactylus*) cells. Laser-induced DNA breaks in chromosome ends as well as in chromosome arms resulted in recruitment of the following: poly(ADP-ribose) polymerase 1 (PARP1), checkpoint sensors (p-Chk1, p-Chk2), DNA repair protein Ku70/Ku80, and proliferating cell nuclear antigen (PCNA). However, phosphorylated p53 at serine 15 was detected only at chromosome ends, and not at chromosome arms. Full activation of DDR on damaged chromosome ends may explain previously published results that showed the delay of cytokinesis.

[1] (Some of the material in this chapter has been published. Bárbara Alcaraz Silva, Jessica R. Stambaugh and Michael W. Berns. Targeting telomere-containing chromosome ends with a Near Infrared (NIR) Femtosecond laser to study the activation of the DNA damage response (DDR) and DNA damage repair pathways. Journal of Biomedical Optics, 18(9):095003, 2013).

Introduction

Previously this lab has shown that a 200 fs near infrared (NIR) laser induced focal-point-specific damage in the genomic DNA of live cells, and such damage led to the activation of the DNA damage response (DDR) (1). Further studies on fully condensed chromosomes during cell division (mitosis) demonstrated the activation of the DDR when NIR laser focal-point-damage was produced on chromosome arms in mitotic PtK_2 (*Potorous tridactylus*) cells (2). When a second harmonic green ps Nd:YVO4 laser was used to expose the ends (tips) of PtK_2 chromosomes as they separated during mitotic anaphase, a significant percent of cells either delayed cytokinesis, or did not divide at all. This occurred even when a single chromosome tip was damaged (3). The present study was designed to determine if the damage to a single chromosome end, which is known to contain the telomere, activates the DDR and repair pathways by looking at the phosphorylation of DDR factors. We also wanted to understand whether induced-laser microirradiation at different chromosomal locations leads to distinct recruitment of DDR and repair factors. Analyzing the recruitment of factors at different chromosomal locations will determine if laser microirradiation, particularly with the commonly used NIR fs lasers, can be used to study the activation of DDR and repair pathways regardless of the chromosome location, and as well, shed light on the functionality of the telomere during mitosis.

It is well established that telomeres occur at the ends of chromosomes. They are nucleoprotein structures composed of DNA double-stranded 5′-TTAGGG-3′ repeats and a single-stranded G-rich 3′ overhang (4). The ends of linear chromosomes structurally resemble

double-stranded DNA breaks (DSBs), but are protected by a nucleoprotein structure called shelterin (4-6). Interphase mammalian telomeres can be "uncapped" by the loss of function of shelterin components, which result in the activation of the DDR (4). While there have been studies on the activation of DDR at uncapped telomeres in the interphase stages of the cell cycle, there are no studies on the activation of DDR at the telomere-containing chromosome ends while the cell is in mitosis, especially during anaphase. Most existing methods used to study mitotic DDR activation and the recruitment of repair proteins at mammalian telomeres (telomere-dysfunction induced foci [TIFs](7) utilize microtubule depolymerizing drugs, which result in uncapping of chromosome ends leading to long-time mitotic arrest (8). DNA breaks can also be produced in mitotic chromosomes by incubating cells with depolymerizing drugs, but only recruitment of DDR factors to DNA breaks has been observed and not recruitment of repair proteins (9). Recent evidence also shows that DSBs persist at telomeres in interphase and are unable to be repaired, while DSBs in chromosomal DNA are efficiently repaired (10).

Earlier studies have shown that laser-induced DNA damage results in the activation of the DDR in interphase mammalian cells (11-13). Additionally, based on the observed accumulation of phosphorylated histone H2AX (γH2AX), a well known DSB marker, it has been shown that NIR lasers without the use of sensitizing agents are capable of producing DSBs on a sub-μm scale without unwanted structural damage to the nucleus of living cells (14-19). Our goal in this study is to determine if NIR laser induced-DNA breaks at telomere-containing chromosome ends in mitotic anaphase, results in the activation of the DDR and recruitment of repair proteins. An additional goal is to determine if this correlates with the delay in mitotic cytokinesis reported previously (3), and in particular, whether there is a difference in the DDR factors recruited to DNA breaks when a single chromosome end is damaged as opposed to a

chromosome arm. The flat morphology, small number, and large size of PtK_2 chromosomes (N = 12) as well as the close sequence identity of these cells with those of humans, mice and rats (76-90%) make this cell type ideal to study the DDR processes using selective short pulse NIR laser microirradiation (20-22).

Materials and Methods

Cell line and cell culture

Potorous tridactylus (PtK_2-male) kidney epithelial cells (American Type Culture Collection ATCC, CCL 56) were grown in Gibco advanced minimum essential medium (MEM) (Invitrogen) supplemented with L-Glutamine, 4% fetal bovine serum (FBS), and antibiotics. Cells were incubated at 37°C with 5% CO_2 (3). Three days before experiments, cells were trypsinized (0.03 tripleEX) and plated on 35 mm gridded imaging dishes (MatTek) at approximately 20,000 cells per dish.

Laser exposure and dosimetry

The custom RoboLase ablation system uses a 200 femtosecond 76 MHz Ti:Sapphire Near-infrared (NIR) 800 nm laser (Coherent Inc., Santa Clara, CA) coupled to a motorized inverted Zeiss microscope (Axiovert 200 M) with a 37°C 35 mm culture dish stage **(**Warner Instruments, LLC**)**. Custom LabView software was developed for the use of the automated microscope system and laser (23). Single telomere-containing chromosome ends and chromosome arms of live anaphase cells were irradiated with a diffraction-limited (0.7 μm diameter) focal spot (2). The laser was focused by front-surfaced mirrors to a Zeiss 63X/1.4 NA

phase contrast oil objective. To determine the irradiance at the focal spot, the transmission of the objective at 800 nm was measured using the double-objective method (2). Physical measurement of the beam power was made prior to beam entry into the back aperture of the microscope objective using a FieldMaxII-TOP power laser meter (Coherent Inc). Based upon measurement of the entry power into the objective, the objective transmission, and the estimated focal spot size, the laser irradiance at the focal point was determined to be 2.43 e^{+11} W/cm.^{2} To study the recruitment of DDR and repair proteins at microirradiated focal spots, individual microirradiated anaphase cells were monitored after laser microirradiation (minutes) and fixed for subsequent fluorescence *in situ* hybridization (FISH), terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL), or antibody staining.

FISH and TUNEL labeling assays

Microirradiated single chromosome ends and chromosome arms of individual cells cultured on gridded dishes were fixed after laser focal-point microirradiation with 3.7% formaldehyde in tris-buffer saline (TBS) for 10 min at room temperature (RT). Dishes were washed three times with phosphate buffered saline (PBS), and were left at 4°C overnight. Telomeres were visualized using FISH with a Cy3-congugated (TTAGGG)-PNA probe as described in the manufacturer's instructions (DAKO, Carpinteria, CA). Cells were later permeabilized twice with PBS/0.2% Triton X-100 for 10 min at RT and then washed three times in PBS-EDTA for 5 minutes followed by one wash with PBS. To label DNA breaks on laser microirradiated samples, cells were incubated with 1:10 enzyme/label solution mix (TUNEL, Roche) in a humidified chamber at 37°C for 1 hr. After the reaction, cells were washed three times on a shaker in PBS-EDTA for 5 minutes to reduce background staining. Samples were

visualized and images acquired using a 63X objective on a Zeiss inverted microscope (Axiovert 200M) equipped with a Hamamatsu Orca ccd camera. Images were analyzed using ImageJ software (NIH, Bethesda, MD).

Immunofluorescence and imaging

To observe the recruitment of DDR factors and repair proteins at single microirradiated chromosome ends or internal chromosome arms, cells grown on gridded culture dishes were fixed with 3.0% formaldehyde tris-buffer saline (TBS) for 10 min at RT and placed on ice. Cells were permeabilized with 0.5% Triton X-100 for 10 min at RT, washed twice with PBS for 5 min at RT, and incubated with blocking solution (10% calf serum, 1% BSA/PBS) for 1 hr at RT. Cells subsequently were washed once in PBS for 5 min at RT and then stained with a primary antibody solution of 3% BSA/PBS overnight at 4°C. The following primary antibodies were used: anti-γ-H2AX (07-164; Millipore) anti-Nbs1 (NB100-143, Novus Biological), phospho-Chk1Ser345 (2348, Cell Signaling), phospho-Chk2Thr68 (2661, Cell Signaling), anti-PCNA (2586, Cell Signaling), phospho-p53Ser15 (sc-101762, Santa Cruz Biotechnlogy, INC.), anti-Ku70/Ku80 (sc-71471, Santa Cruz Biotechnology, INC.), anti-Rad51 (sc-53428, Santa Cruz Biotechnology, INC.). After antibody incubation, cells were washed twice in PBS/0.05% Tween-20 for 5 min at RT, and incubated with secondary antibodies (Invitrogen; 1:1,000) for 1hr at RT. Cells were washed twice with PBS/0.05% Tween-20 for 5 min at RT and the DNA was stained with 4, 6-diamidino-2-phenylindole (1:1000 in PBS) for 5 min at RT. Samples were imaged as described in the previous section.

Results

Laser microirradiation to single chromosomes during anaphase

We examined the DDR proteins recruited to either a single chromosome end, or chromosome arm (distant from the telomere-containing end), during anaphase onset after DNA breaks are produced with the NIR laser (2, 12). Two methods were used to verify that the telomere of a single anaphase chromosome end was damaged: (1) FISH using a Cy3-conjugated (TTAGGG)-PNA probe against telomeric DNA, and (2) TUNEL to visualize DNA breaks (Fig. 2.1). The results demonstrate that DNA breaks can be induced by focal point laser NIR microirradiation at either chromosome ends, or chromosome arms. A previous study has shown that laser microirradiation of mitotic chromosome DNA results in 'paling' at the site of damage followed by the gradual formation of phase-dark material—demonstrated to be the result of the accumulation of DDR factors (2). To verify that we were obtaining the same response at chromosome ends, either individual chromosome ends, or chromosome arms, were microirradiated at anaphase and monitored by phase-contrast microscopy for several minutes until the presence of phase-dark material was detected. Following laser microirradiation (10 seconds post-laser), phase paling was evident at microirradiated chromosome ends and microirradiated internal chromosome arms (Fig. 2.2A [ii], 2.2B [ii]). After 120 seconds, phase-dark material was visible at the damage site corresponding to the accumulation of DSB marker phosphorylated histone H2AX (γH2AX), and the early modification enzyme known to facilitate DNA repair of single-stranded breaks (SSBs): poly(ADP-ribose) polymerase 1 (PARP1) (Fig. 2.2A' [iii], 2.2B' [iii]).

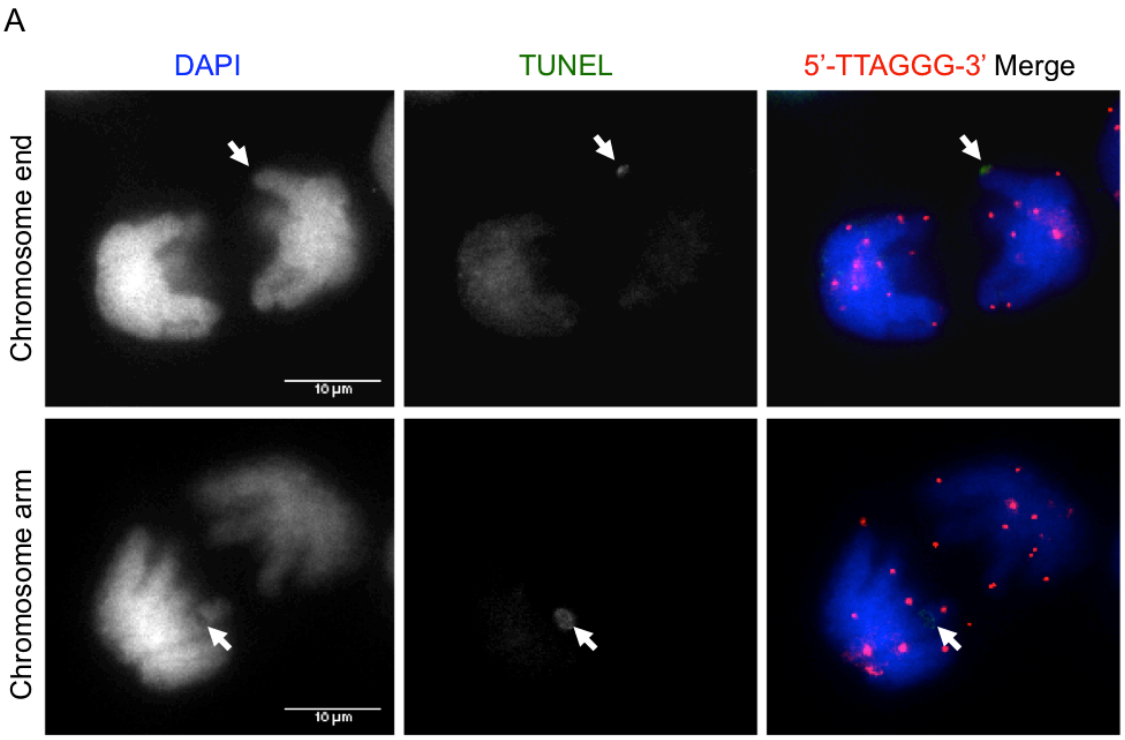

Figure 2.1 Single point laser microirradiation induces localized DNA breaks.
(A) TUNEL-FISH to detect DNA breaks produced by laser microirradiation at a single chromosome end and chromosome arm in anaphase PtK_2 cells. Green, TUNEL staining to label DNA breaks; red, Cy3-5'-TTAGGG-3' FISH probe to label the telomeric sequence; blue, DAPI to stain DNA. Arrow points to laser-induced DNA breaks. Scale bar 10 µm. N = 3 of independent experiments.

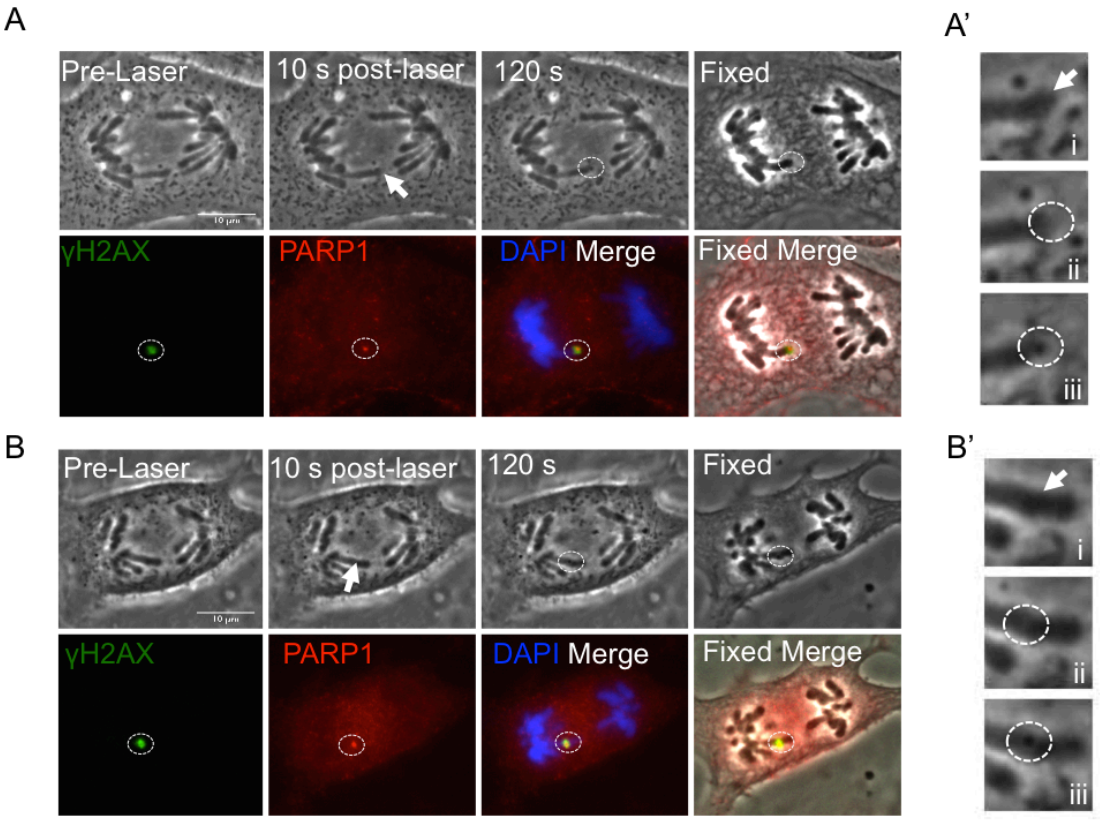

Figure 2.2 Single point laser-induced DNA breaks activate the DDR.
(A) Phase image of PtK_2 cell prior laser microirradiation at telomere-containing chromosome end (Pre-laser), 10 s after laser microirradiation (post-laser), and 120 s post-laser. (A') Inset of (A) of a single chromosome end before microirradiation/pre-laser (i), 10 s post-laser (ii), and 120 s post-laser (iii). (B) Phase image of PtK_2 cell prior to laser microirradiation at chromosome arm (Pre-laser), 10 s after laser microirradiation (post-laser), and 120 s post-laser. (B') Inset of (B) of a single chromosome arm before microirradiation/pre-laser (i), 10 s post-laser (ii), and 120 s post-laser (iii). Cells were fixed at 120 s and stained with anti-γH2AX do detect DNA damage (green), anti-PARP1 (red), and co-stained with DAPI (blue). Dashed circle shows microirradiated DNA and foci accumulation. Scale bar 10 μm. N = 5 independent experiments.

Activation of DDR at laser-induced DNA breaks

Recent work has demonstrated that telomeric damage by long-time mitotic arrest leads to checkpoint activation leading to cell cycle arrest (8). To determine whether proteins involved in cell cycle arrest are recruited to laser-induced DNA breaks in chromosome ends or chromosome arms at the onset of anaphase, the recruitment of DNA-damage checkpoint kinases Chk1, Chk2

and p53 were assessed by immunofluorescence. Cells were fixed 5 minutes post-laser exposure either to a chromosome end, or a chromosome arm distant from the end (internal chromosome arm). Immediate recruitment of Chk1 phosphorylation on serine 345 (Ser345) and Chk2 phosphorylation on threonine 68 (Thr68) was observed at damaged chromosome ends and at damaged chromosome arms (Fig. 2.3A, 2.3B, lane 1 and 2, N = 5). In addition, DNA breaks on chromosome ends showed immediate foci accumulation of p53 phosphorylation on serine 15 (Ser15), (Fig. 2.3A*, lane 3, N = 5). Laser-induced DNA breaks at the control internal chromosome arms failed to recruit p53 phosphorylation on serine 15 (Ser15) (Fig 2.3B, lane 3, N = 5). The recruitment and activation of checkpoint proteins Chk1, Chk2 and p53, which are involved in the DDR (Table 1, page 27), confirms the activation of a DDR that is likely responsible for the previously observed delay in cytokinesis (3).

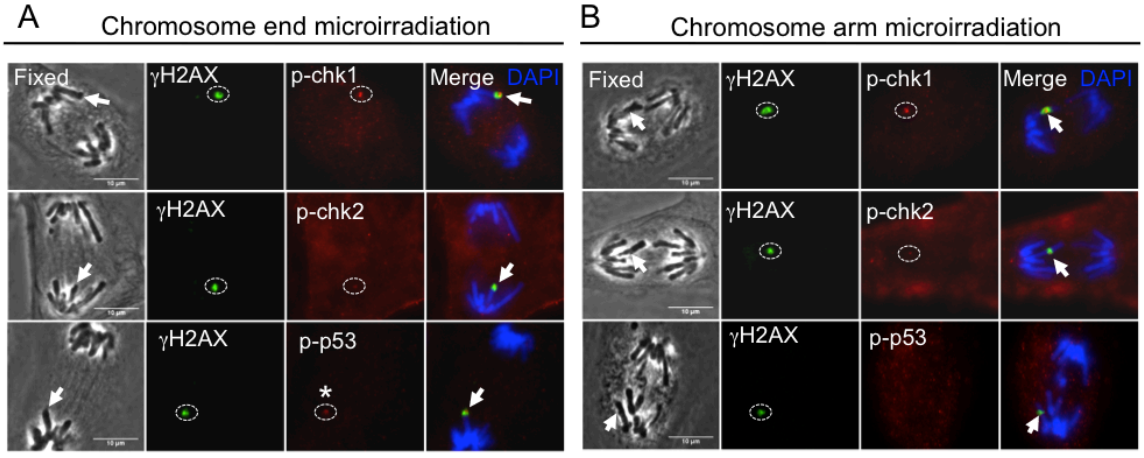

Figure 2.3 Recruitment of checkpoint DDR proteins to single point laser-induced DNA breaks during anaphase onset.
(A) Fixation performed 5 min after chromosome end laser microirradiation (fixed) of anaphase PtK_2 cells. (B) Fixation performed 5 min after chromosome arm laser microirradiation (fixed). Endogenous DNA damage recognition factors accumulate at laser-induced DNA breaks. DNA breaks are detected with anti-γH2AX, checkpoint response proteins detected with anti-p-Chk2(Ser345), anti-p-Chk1(Thr68), anti-p-p53(Ser15). Arrows on fixed cells point to laser-

induced DNA breaks. Scale bar 10 μm, N = 5 independent experiments. White asterisk shows the phosphorylation of p53 in response to chromosome end damage.

Recruitment of repair proteins at laser-induced DNA breaks

Having established the activation of the DDR by inducing DNA breaks at chromosome ends and internal chromosome arms, we next examined whether repair proteins from the two major repair pathways, non-homologous end joining (NHEJ) and homologous recombination (HR), are able to form foci at laser-induced DNA breaks at specific chromosomal sites. Using an anti-Ku70/Ku80 endogenous antibody, induced DNA breaks at chromosome ends and chromosome arms showed foci accumulation of endogenous NHEJ repair Ku70/Ku80. This complex co-localized with DDR sensing factor Nbs1, which served as a control for damage (Fig. 2.4A, 2.4B, lane 1, N = 5). To investigate whether additional factors accumulate at laser-induced DNA breaks at chromosome ends or chromosome arms, we examined the recruitment of proliferating cell nuclear antigen (PCNA), which is known to be involved in replication and DNA repair (24-26). Endogenous PCNA foci accumulated at damaged chromosome ends and damaged chromosome arms (Fig. 2.4A, 2.4B, lane 2, N = 5). Furthermore, antibody staining for Rad51, a mammalian HR repair factor (27), revealed no detectable fluorescence at localized DNA breaks of anaphase chromosome ends or arms as previously shown in mitotic cells [Fig. 2.5A] (28). Altogether, these results reveal that DNA lesion result in the accumulation of DDR factors and repair proteins at both chromosome ends and arms (Table 1, page 27).

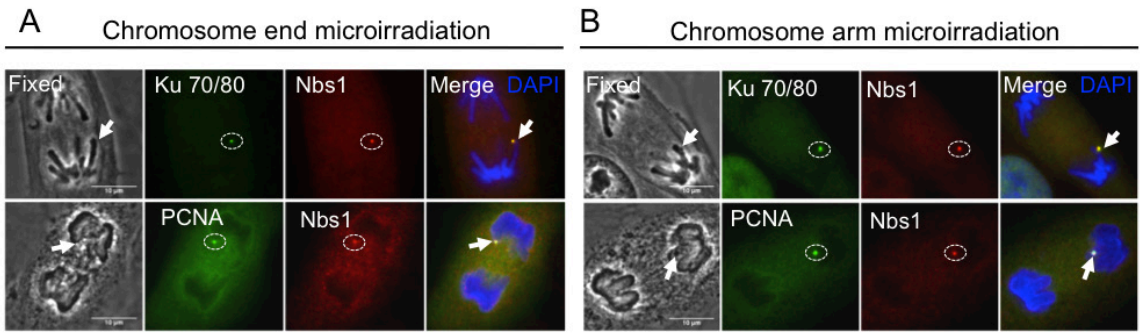

Figure 2.4 Recruitment of DNA damage repair factors to single point laser-induced DNA breaks during anaphase onset.
(A) Fixation performed 5 min after chromosome end laser microirradiation (fixed) of anaphase PtK_2 cells. (B) Post-fixation performed 5 min after chromosome arm laser microirradiation (fixed). Endogenous DNA damage recognition factors accumulate at laser-induced DNA breaks. DNA breaks are detected with anti-γH2AX, anti-Ku70/Ku80, anti-Nbs1, anti-PCNA antibodies and counterstained with DAPI (blue). Arrows on fixed cells point to laser-induced DNA breaks. Scale bar 10 μm, N = 5 independent experiments.

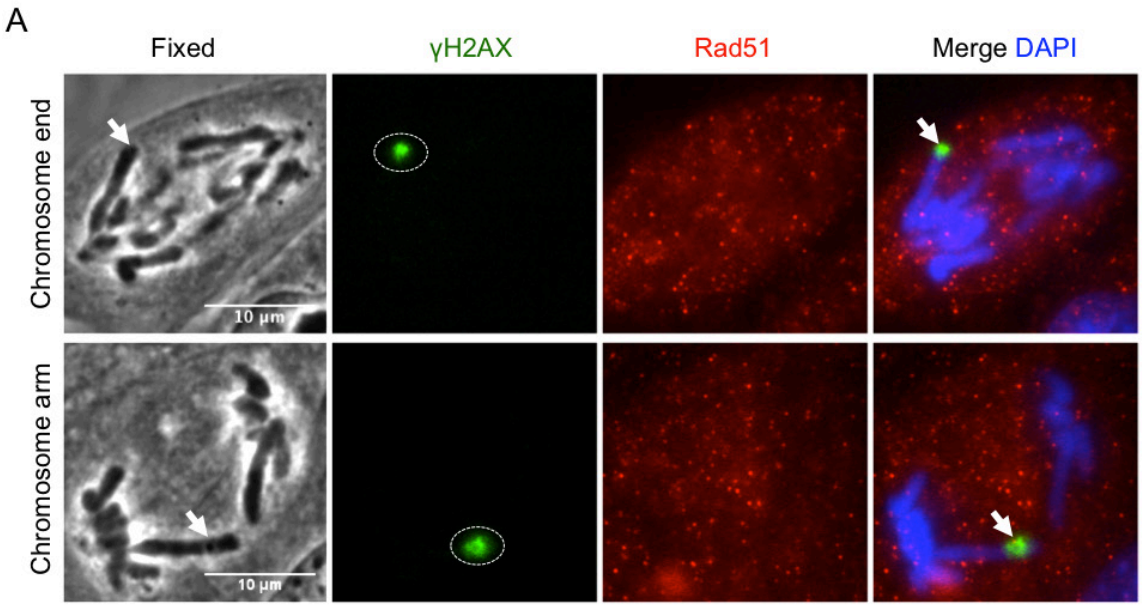

Figure 2.5 Homologous repair protein, Rad51 does not get recruited to anaphase DNA breaks.
(A) Post-fixation performed 5 min after laser irradiation to a single chromosome end and chromosome arm. Repair proteins are detected with anti-γH2AX (green), anti-Rad51 (red) and co-stained with DAPI (blue). Arrow points to microirradiated chromosome site. Scale bar 10 μm, N = 5 independent experiments.

Table 1 Number of cells that show accumulation of DNA damage response (DDR) and repair proteins at a single damaged chromosome end or chromosome arm. Experiments were repeated three times (N=3).

	No. of cells with foci at chromosome ends	**No. of cells with foci at chromosome arms**
Double-stranded break (DSB) factor		
Phosphorylated histone H2AX (γH2AX)	5	5
DNA damage response (DDR) factors		
Nbs1	5	5
Chk2 phosphorylation on threonine 68 (Thr68)	5	5
Chk1 phosphorylation on serine 345 (Ser345)	5	5
Phosphorylated p53 on ser 15 (p-p53)	5	0
Single-stranded break (SSB) repair proteins		
poly(ADP-ribose) polymerase 1 (PARP1)	5	5
Non-homologous end joining (NHEJ) repair protein		
Ku70/Ku80 complex	5	5
Homologous recombination (HR) repair protein		
Rad51	0	0
DNA replication and repair factor		
PCNA	5	5

Induced DNA damage at chromosome ends delays cytokinesis

Since DNA breaks at telomere-containing chromosome ends recruit factors that are involved in cell cycle arrest (Fig 2.3A, 2.3B, Table 1, page 27), it is important to address whether localized DNA breaks at chromosome ends of cells undergoing anaphase are able to activate cell cycle arrest: a delay in the transition from anaphase to cytokinesis. Previous evidence suggests that laser microirradiation at chromosome ends during anaphase onset is capable of eliciting cell delay. However, these experiments were done at RT, a higher irradiance was used, and with a

visible green picosecond laser (535 nm) as opposed to the fs NIR laser used here [12]. To test the effect of NIR laser-induced DNA breaks at single telomere-containing chromosome ends, cells were monitored from anaphase to cytokinesis. The results show a significant difference ($p < 0.05$, $p = 0.0009$) in the timing of cell division as a result of laser microirradiation of a single anaphase telomere-containing chromosome end (24.7 min, +/- 4.1 s.d, N = 16, Fig. 2.6A, 2.7A) when compared to cells that were damaged only on a single chromosome arm (19.4 min, +/- 4.7 s.d, N =16, Fig 2.6B, 2.7A), or non-irradiated control cells (19.6 min, +/- 4.4 s.d, N = 21, Fig 2.6C, 2.7A). These results suggest that DNA damage induced at chromosome ends is not only able to activate a DDR but also induces a delay in cytokinesis.

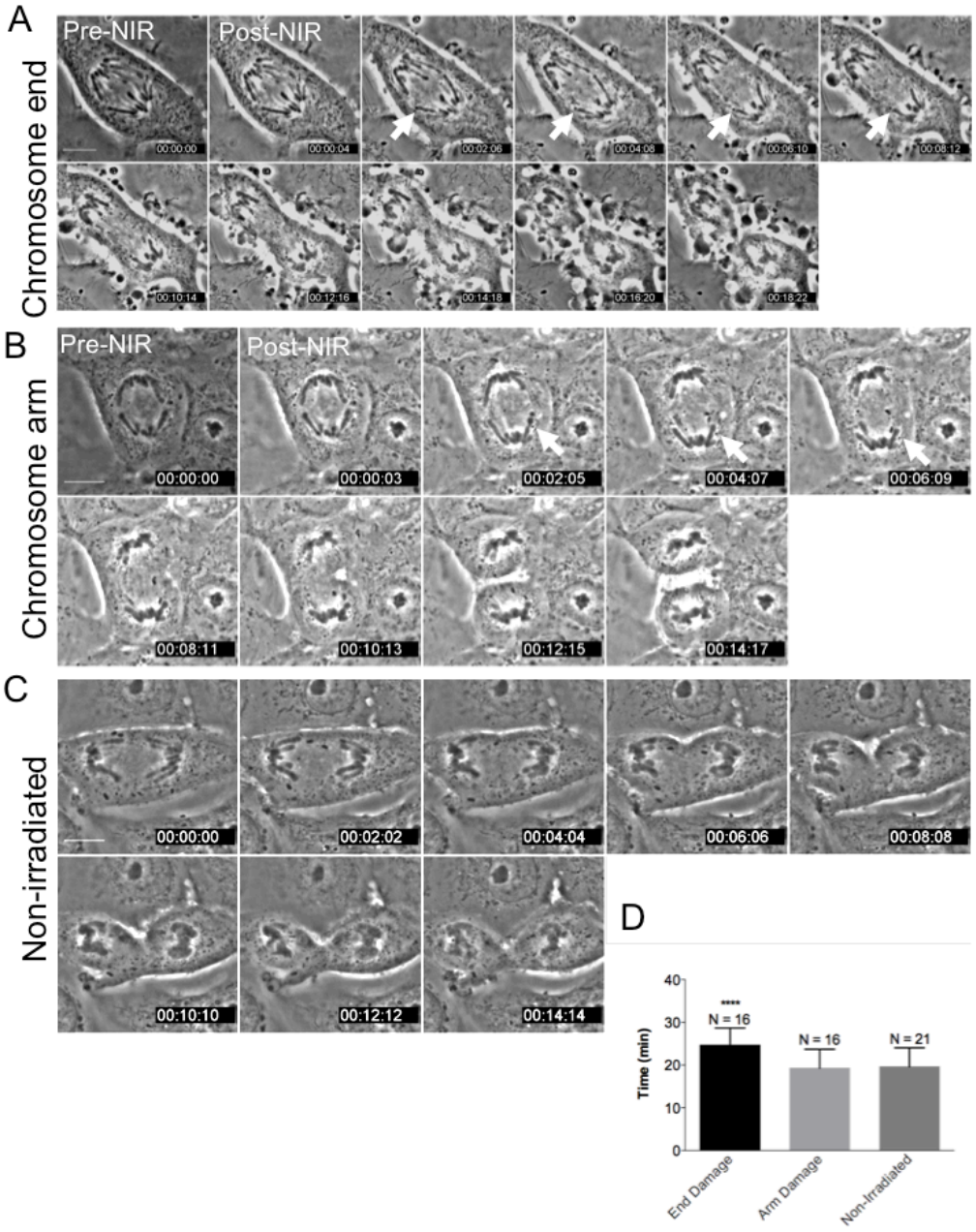

Figure 2.6 Chromosome end damage induces cytokinesis delay.
(A) Elapsed time in minutes of a single anaphase cell prior to microirradiation (Pre-laser), and after microirradiation (post-laser) at a chromosome end and chromosome arm (B) and Non-Irradiated control cell (C). Arrow points to a single microirradiated chromosome. (D) Division time graph of microirradiated chromosome end (End Damage), microirradiated chromosome arm (Arm Damage), and non-irradiated cells ($p = 0.0009$, $p < 0.05$). Scale bar 10 μM.

Discussion

Recent evidence indicates that chromosome ends of interphase cells lack a repair mechanism compared to the rest of the chromosome (10). Despite this evidence, few studies have examined the accumulation of DDR factors at damage chromosome ends during mitosis (8). In fact, there are neither studies nor methods that show the localization of additional DDR factors when the damage is produced while the cell is in mitosis. Furthermore, it remains unclear whether damaged chromosomes ends of mitotic cells can activate a full DDR by recruiting proteins from repair pathways such as homologous recombination (HR) and/or non-homologous end joining (NHEJ).

In the present study, DNA breaks at telomere-containing chromosome ends and chromosome arms in vertebrate PtK2 cells were induced at the onset of anaphase through the use of a NIR femtosecond laser. The irradiance used to produce DNA breaks such as DSBs and SSBs was 2.43 e^{+11} W/cm^2 in the focal spot. At this irradiance, the mechanism of damage was likely a non-linear multiphoton optical process, or possibly a threshold microplasma event localized to the laser focal volume (12). It is unlikely that a significant plasma event occurred because the cell has survived which is indicative of an intact cell membrane. Because these cells stay relatively flat during mitosis the outer cell membrane is within a couple of microns of the focal plane of the laser as shown in previous transmission electron microscope (TEM) images (2). If a major plasma event occurred at the focal point, the generated shock wave would likely have been sufficient to rupture the outer cell membrane, resulting in cell death.

Previous evidence shows the activation of the DDR using high irradiance of UV microirradiation at 10 e^{+11} W/cm^2 without any plasma formation, and 9.3 e^{+11} W/cm^2 using a NIR (12, 29). We cannot ignore the damage being produced to the cell using these high

irradiances previously published. In our studies we demonstrate that a lower irradiance of 2.43 e^{+11} W/cm^2 is enough to activate the DDR and repair pathways at chromosome ends and chromosome arms. Additionally, by using TEM it was estimated that the NIR microirradiation produces damage in 10-100 mega base pairs (Mbp) of DNA (2). However, the irradiance used in this study (3.81 e^{+11} W/cm^2) was slightly larger than our studies, which we believe is at the threshold of the DDR response. Therefore, we believe the damage being produced in our study is less than in previous studies, but above the threshold to activate the DDR.

Our results help explain the previously reported inhibition of cytokinesis induced by laser microirradiation at chromosome ends (3). Here we show that laser-induced DNA breaks (DSBs and SSBs) at a single chromosome end, is capable of activating a DDR during anaphase onset via phosphorylation of H2AX, and inhibit cytokinesis. Furthermore, proteins involved in the DDR such as PARP1, Nbs1 and cell cycle delay proteins Chk1 and Chk2 are recruited to laser-induced DNA breaks at single chromosome ends as well as internal chromosome sites in anaphase cells. However only NIR focal point laser-induced DNA breaks at chromosome ends phosphorylated p53 at serine 15, whereas internal chromosomal breaks do not. It is unclear as to why p53 is not phosphorylated at internal chromosomal breaks; however, one possibility is a lack of full DDR in mitosis, which has been shown to occur during mitosis (30). In the case of the telomere-containing chromosome ends, this result may be consistent with the unique activity of the telomere as a protection from degradation processes, recombination, and chromosome fusion events. In addition, induced DNA breaks at chromosome ends and chromosome arms recruit endogenous NHEJ repair Ku70/Ku80 complex and PCNA during anaphase onset, suggesting a processing role in DNA repair, but DNA lesions at both chromosome ends and chromosome arms fail to recruit HR repair protein Rad51, Furthermore, our unpublished data

suggest damaged chromosome ends continue into G1 phase with unresolved repair as previously shown in interphase cells (10).

In this study we combine focal point femtosecond NIR laser microirradiation with immunofluorescence to understand whether additional DDR factors are recruited to DNA breaks at specific chromosome sites. This approach provides the opportunity to study DNA repair in single cells. Collectively, our data suggest that damaged anaphase chromosome ends and damaged chromosome arms activate DDR, and may be processed by NHEJ based on the recruitment of Ku70/Ku80 protein complex. Our results also demonstrate that inhibition of cytokinesis previously shown (3), is due to the activation of a DDR at laser induced DNA breaks on single anaphase chromosome ends. However, additional studies need to confirm whether activaion of the DDR at chromosome ends is sufficient to activate a delay on cytokinesis. These results are significant because of the cell's ability to protect chromosome ends in order to prevent the activation of the DDR, which is consistent with the protective role of telomeres in maintaining genomic stability. Further studies should address the possible recruitment of additional DDR factors between damage chromosome regions, and determine if there are differences in the kinetics of the DDR at both damage sites.

Conclusion

A focal point 800 nm femtosecond NIR microirradiation system can be used to study the effect of DNA break induction by the production of DSBs and SSBs at either a chromosome end, or an internal chromosomal site during early anaphase of PtK_2 cells. Our results demonstrate that DNA breaks induced at either site are able to activate the DDR that results in the recruitment of cell cycle response factors Chk1, Chk2 and repair proteins Ku70/Ku80. There appears to be

uniqueness in the response of the chromosome ends in that they also recruit p53 phosphorylation on serine 15 (Ser15), whereas the damaged chromosome arms do not. Due to its high temporal and spatial resolution, laser microirradiation can be used to study the activation of the DDR at a single cell level, the effect of DNA breaks at different chromosome regions during mitosis, and in subsequent stages of the cell cycle.

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Chapter 3 DNA Damage to a single chromosome end delays anaphase onset[2]

Abstract

Chromosome ends contain nucleoprotein structures known as telomeres. Damage to chromosome ends during interphase elicits a DNA damage response (DDR) resulting in cell cycle arrest. However, little is known regarding the signaling from damaged chromosome ends (defined here as "TIPs") during mitosis. In the present study we investigated the consequences of DNA damage induced at a single TIP in mitosis. We used laser microirradiation to damage mitotic TIPs or chromosome arms (Non-TIPs) in PtK2 kidney epithelial cells. We found that damage to a single TIP, but not Non-TIP, delays anaphase onset. This TIP-specific checkpoint response is accompanied by differential recruitment of DDR proteins. While phosphorylation of H2AX and the recruitment of several repair factors, such as Ku70/Ku80, occurs in a comparable manner at both TIP and Non-TIP damage sites, the recruitment of other DDR factors is either not detectable (ATM, MDC1, WRN and FANCD2) or delayed (Nbs1 and BRCA1 as well as ubiquitin signal) at Non-TIPs compared to TIPs. ATR and 53BP1 are not detected from both TIPs and Non-TIPs in mitosis. The observed delay in anaphase onset is dependent on the activity of DDR kinases ATM, Chk1, and the spindle assembly checkpoint (SAC) kinase Mps1. Cells damaged at a single TIP or Non-TIP eventually exit mitosis with unrepaired lesions. Damaged TIPs are segregated into micronuclei at a significantly higher frequency than damaged Non-TIPs. Together these findings reveal mitosis-specific DDR uniquely associated with chromosome ends.

[2] This work is submitted to JBC; authors of this work: Bárbara Alcaraz Silva, Jessica R. Stambaugh, Kyoko Yokomori, Jagesh V. Shah, Michael W. Berns.

Introduction

Unrepaired DNA damage can lead to mutation, chromosomal fragmentation, and genomic rearrangements (1-3). DNA damage, in the form of double-strand breaks (DSBs) or single-strand breaks (SSBs), can be generated by many processes, such as the collapse of the replication fork, reactive oxygen species (ROS) and radiomimetic compounds (4-8). The DNA damage response (DDR) pathways recognize DNA lesions and recruit proteins to these sites to promote repair. The ends of linear chromosomes, which contain telomeres (also referred to as "TIPs" in this manuscript), can also be recognized as damaged DNA. Telomeres are normally protected from recognition as DNA damage or inappropriate repair processes by a nucleoprotein-structure composed of repetitive DNA and the shelterin protein complex (9-13). Removal of this protein complex from chromosome ends results in local activation of the DDR and recruitment of repair proteins to the telomeres (6,12,14,15).

Shelterin proteins TIN2, TRF1, RAP1, TRF2, TPP1 and POT1 act to prevent activation of the canonical DDR. Specifically, TRF2 binds to double-stranded DNA ends to prevent its recognition as a DSB and subsequent activation of the ATM (ataxia telangiectasia mutated) kinase. On the other hand, POT1 binds to single-stranded DNA in the telomere preventing ATR kinase (ATM and Rad3-related) activation (16). DNA lesions or uncapping of the telomere during interphase of the cell cycle both activate ATM and ATR kinases, phosphorylation of downstream kinases Chk1 and Chk2, and the transcription factor p53, which leads ultimately to cell cycle arrest with a persistent DDR (17-19). In vertebrate cells, uncapped telomeres induce G2 arrest through the inactivation and degradation of the Cyclin B by activating phosphatase Cdc25C (20) as well as the upregulation of the Cdk inhibitor p21 (17). In addition to activation

of DDR kinases, unprotected or damaged telomeres are also marked by phosphorylation of H2AX (γH2AX) and recruit a number of repair proteins such as MDC1 and 53BP1, which form telomere dysfunction-induced foci (TIFs) (17,18).

Our understanding of how the cell responds to telomere damage in mitosis is limited. Cells with defective ATM or p53 escape from G2 arrest and enter mitosis with persisting TIFs on mitotic chromosomes (20). However, recent studies indicated that DDR is attenuated in mitosis compared to interphase (21-23), and thus cells may respond differently to telomere damage induced during mitosis (24). A recent study demonstrated that the forced mitotic arrest results in telomere uncapping with γH2AX focus formation and ATM activation (25). However, the cellular response to telomere damage in mitosis under normal cell cycling condition has not been explored in detail.

In this study, we asked how cells respond to damage induced specifically at telomere-containing chromosome ends (TIPs) in comparison to damage induced at chromosome arms (Non-TIPs) in mitosis using laser microirradiation. We performed systematic comparison of the DDR and repair factor recruitment at TIP and Non-TIP damage sites and the effect on the progression of mitosis. Laser microirradiation has been shown to produce predominantly DSBs, akin to ionizing radiation, eliciting the DDR in mammalian interphase cells, mitotic chromosomes, and anaphase telomeres of PtK2 cells (26-33). Previous studies have shown that laser-induced DNA breaks on mitotic chromosomes do not result in chromosome fragmentation, and DSBs are only introduced to the focal spot (27,33). The spatio-temporal control of laser ablation permits the generation of damage chromosome tips without genetic perturbation or long-term mitotic arrest. Moreover cells from the rat kangaroo (Potorous tridactylus kidney, PtK2) are ideal systems to study cell division and checkpoint signaling because they have a small

number of large chromosomes [2N = 12] (34), and close sequence identity with humans, mice and rats (80%-90%) (35). Additionally, a single chromosome tip-containing telomere can be targeted by laser microirradiation in these cells (33,36). This facilitates the investigation of signaling and protein recruitment on a single damaged chromosome tip. Here, we report that laser-induced damage at TIPs recruits a distinct set of DDR factors compared to damage at Non-TIPs. Remarkably, the damage to a single TIP results in a delay in the transition from metaphase to anaphase. This delay was found to be dependent on the DNA damage checkpoint kinases ATM, Chk1 and the spindle-assembly checkpoint (SAC) kinase, Mps1. Despite the damage-induced delay, cells with a single damaged chromosome tip eventually exited mitosis with persistent DNA lesions forming micronuclei in the G1 phase. Thus, our results uncover a mitotic DDR specifically associated with telomere-containing chromosome ends.

Materials and Methods

Cell lines and cell culture

Long-nosed potoroo (rat kangaroo), *Potorous tridactylus* (PtK2-male, and PtK1-female) kidney epithelial cells (American Type Culture Collection ATCC, CCL 56 and CCL 35), and TRF2-AID-EYFP PtK2 (AID – auxin-inducible degron that has been shown to degrade the AID-tagged target protein upon addition of a plant hormone. In these experiments, activation of AID was not induced) (37), were grown in Gibco Advanced Minimum Essential Medium (MEM) (Invitrogen) supplemented with L-Glutamine, 4% fetal bovine serum (FBS), and antibiotics. PtK1 cells stably expressing a green fluorescent protein (GFP)-tagged Nbs1 previously generated (27), were incubated with Advanced F12/DMEM supplemented with L-Glutamine, 4% fetal bovine serum (FBS), and antibiotics. All cell types were incubated at 37°C

with 5% CO_2. Three days before experiments, cells were trypsinized (TrypLE™ Express, Life Technologies) and plated on round 35 mm gridded imaging dishes (MatTek, Ashland, MA) at approximately 20,000 cells per dish as previously shown (33). Before laser microirradiation, the medium was replaced with Hanks' Balanced Salt Solution (HBSS, 1X) to prevent the absorption of the laser light by the phenol red, and to facilitate the monitoring of the cells after damage via live fluorescence imaging.

Generation of stable PtK cell lines

To generate PtK2 cells stably expressing eGFP-53BP1 or TRF2-AID-EYFP, we transiently transfected retroviral plasmids eGFP-53BP1 pLPC (kindly donated by the Denchi Lab, Scripps Research Institute) and pBABEneo TRF2-AID-EYFP (kindly donated by the Cleveland Lab, UC-San Diego) (37) into Phoenix amphotropic packing cell line, using Effectene Transfection Reagent (Qiagen) according to the manufacturer's instructions. Viral particles were generated using a modified protocol (38,39). For 53BP1 and TRF2 infections, marsupial PtK2 cells were plated in growth media containing 4 μg ml^{-1} polybrene (Sigma) and viruses. Cells were infected at a multiplicity of infection of 3. Forty-eight hours after infection, cells were split, and were incubated with media containing 2 mg ml^{-1} puromycin for 53BP1 and 2 mg ml^{-1} neomycin for TRF2. Cells were selected for 5 days. Stable cell lines were further selected using fluorescence-activated cell sorting (FACS, City of Hope, Duarte CA).

SiRNA transfection and sequences

The partial PtK2 ATM sequence was identified by high-throughput sequencing (Illumina) of a commercially generated PtK2 cDNA library (Express Genomics). Duplexes targeting ATM

PtK protein were designed and synthesized (Invitrogen). The sequences of the duplexes for ATM siRNA are (1) sense: 5'-GCAGCUUGGUUAAAUACUUTT-3', anti-sense: 5'-AAGUAUUUAACCAAGCUGCTT-3'. ATM siRNA (2) sense: 5'-GCUACUUAUGGAGCGGAUUTT-3', anti-sense: 5'-AAUCCGCUCCAUAAGUAGCTT-3'. Scramble siRNA was used as a control. Both siRNA's (1 and 2) were transfected into PtK2 cells using Lipofectamine RNAiMAX (Invitrogen). To obtain 70% transfection efficiency, two consecutive rounds of two-siRNA duplex (1 and 2) transfection were carried out according to the manufacturer's protocol. Transfected PtK2 cells were assayed by Immunoblotting 72 h after transfection for ATM depletion.

Immunoblotting

PtK2 cells, transfected with siRNAs, were grown on a 6-well plate and lysed with 100 μl hot 2X Laemmli buffer (Bio-Rad) supplemented with 6% SDS, followed by sonication. Proteins were resolved by 5% - 10% SDS-PAGE and transferred to a PVDF membrane. The membrane was blocked with 5% BSA for 1 h and incubated overnight with primary rabbit ATM antibody (1:1000), and rabbit secondary HRP using a 1:1000 mix (Promega) and detected by chemiluminescence.

Laser exposure and dosimetry

A previously described custom RoboLase ablation system was used in these studies (27,33,40). Briefly, the system uses a femtosecond pulsed Ti:Sapphire Near-infrared (NIR) laser (Coherent Inc., Santa Clara, CA) coupled to a motorized inverted Zeiss microscope (Axiovert 200 M) with a 37°C culture dish stage (Warner Instruments, LLC). LabView software was developed for use with the automated microscope system and laser (41). Single chromosome tips

(TIPs) and chromosome arms (Non-TIPs) of live unsynchronized metaphase cells were microirradiated using the 200 femtosecond (fs) NIR emission wavelength of 800 nm with a diffraction-limited (0.7 μm diameter) focal spot as previously shown (33). Due to the ultra short fs time exposure, damage was confined to the focal spot (26,27). Individual laser exposure (irradiation) to single TIP and Non-TIP of cells was performed at a dose range of 2.43 – 2.65 e^{+11} W/cm^2. DNA breaks were assayed by using DSB marker H2AX that becomes phosphorylated on serine 139 (γ-H2AX) upon damage. In addition, to check the recruitment of DDR and repair factors at microirradiated DNA, eGFP-53BP1 PtK2 cells were used as controls (unpublished data). DNA breaks were created at interphase cells, however, at irradiances above 2.65e^{+11} W/cm^2, 53BP1 protein was not recruited, which may be due to optical breakdown and microscopic thermoelastic stress waves (28). Thus, the lower irradiance (2.43 e^{+11} W/cm^2) was used for optimal mitotic protein recruitment and kinetics, and has recently been shown to produce DSBs (33).

Immunofluorescence and imaging

Cells grown on gridded dishes were fixed with 3% formaldehyde tris-buffer saline (TBS) buffer for 10 min at room temperature (RT) and placed on ice after microirradiation. Cells were washed three times in PBS and permeabilized with 0.5% Triton-X 100 for 10 min at RT. Cells were later washed twice with PBS for 5 min at RT, and incubated with blocking solution (10% calf serum, 1% BSA/PBS) for 1 hr at RT. Cells were later washed once in PBS for 5 min at RT. Next, cells were incubated with primary antibodies in 3% BSA/PBS. After the incubation, cells were washed twice in PBS/0.05% Tween-20 for 5 min at RT, and incubated with secondary antibodies (Invitrogen; 1:1,000) for 1 hr at RT. Cells were washed twice with PBS/0.05%

Tween-20 for 5 min at RT and DNA was stained with 4, 6-diamidino-2-phenylindole (1:500 in PBS) for 5 min at RT. A final wash was performed with PBS for 5 min. Samples were visualized on a Zeiss inverted microscope (Axiovert 200M) equipped with a Hamamatsu Orca cooled CCD Camera. Images were analyzed using ImageJ software (NIH, Bethesda, MD). Immunofluorescent staining was repeated at least five times for each antibody in TIP and Non-TIP damaged cells, and the consistent localization results were obtained with all the cells examined (N > 5). Localization of the DDR signal at damage TIPs was observed in all the cells tested.

Antibodies

The following antibodies were used: anti-γ-H2AX (07-164; Millipore) anti-Nbs1 (NB100-143, Novus Biological), anti-Mre11 (NB1000-142, Novus Biologicals), anti-ATM (NB100-104, Novus Biologicals), anti-FANCD2 (NB100-182, Novus Biologicals), anti-MDC1 (NB100-395, Novus Biologicals), anti-XRCC1 (NB100-532, Novus Biologicals), anti-WRN (ab200, abcam), anti-CtIP (ab70163, abcam), RNF8 (ab4183, abcam), RNF168 (ab58063, abcam), anti-γH2AX (9718, Cell Signaling), phospho-Chk1ser345 (2348, Cell Signaling), phospho-Chk2Thr68 (2661, Cell Signaling), anti-PCNA (2586, Cell Signaling), anti-GFP (2956, Cell Signaling) anti-Ku70/Ku80 (sc-71471, Santa Cruz Biotechnology, INC), phospho-p53ser15 (sc-101762, Santa Cruz Biotechnology, INC), anti-actin (A4700, Sigma), anti-PARP1 (42), anti-Ubiquitin (spa-205, StressGen), and anti-BRCA1 (GTX50692, GeneTex).

Nbs1 kinetics

GFP-Nbs1 PtK1 mitotic cells grown in cover glass bottom 35 mm dishes (World Precision Instruments, Inc. Sarasota, FL) were microirradiated at chromosome ends (TIPs) and

chromosome arms (Non-TIPs) as described above. GFP-Nbs1 recruitment in individual mitotic cells was monitored by taking fluorescent images at 10 s intervals for 120 s with a Hamamatsu Orca cooled CCD Camera. Images were analyzed using ImageJ software (NIH, Bethesda, MD).

TUNEL labeling assay

Microirradiated mitotic PtK2 cells on gridded dishes were fixed with 3.7% formaldehyde in TBS for 10 min at RT. Cells were permeabilized twice with PBS/0.2% Triton-X 100 for 10 min at RT. Cells were later washed three times in PBS-EDTA for 5 minutes and washed once with PBS. Cells were incubated with 1:10 enzyme/label solution mix (TUNEL, Roche) in a humidified chamber at 37°C for 1 hr. After the reaction, cells were washed three times on a shaker in PBS-EDTA for 5 minutes to reduce background staining.

IMMUNO-Fluorescence in situ hybridization (FISH) assay

Microirradiated mitotic PtK2 cells on gridded dishes were fixed and stained with DDR antibodies as previously described in this paper. Samples were fixed with 3.7% formaldehyde in TBS for 2 min, and telomeres were visualized with a Cy3-congugated (TTAGGG)-PNA probe (DAKO, Carpinteria, CA) as explained in manufacturer's instructions.

Cell division progression

To monitor individual mitotic PtK2 cells aligned at the metaphase plate, individual prophase cells were followed until the alignment of chromosomes at metaphase plate was complete (1 hr approximately). DNA breaks were introduced either at single chromosome end (TIP), or on a chromosome arm (Non-TIP) when the chromosomes were aligned at the metaphase plate (30 min after prophase). Cells were monitored using a Zeiss microscope coupled to a CCD camera

as previously described in this thesis. Cells were monitored via time-lapse microscopy imaging at 120-second intervals. Time-lapse images were processed with ImageJ and the durations of mitotic progression was quantitatively measured until the onset of chromosome separation in anaphase.

Inhibitor studies

PtK2 cells grown on gridded dishes were incubated 1 hr prior to microirradiation with either one of the following inhibitors: (ATMi, ku-60019) or (ATMi, ku-55993) at 10 μM; Chk1 inhibitor (Chk1i, AZD7762) was used at 1 μM. Immediately after irradiation progression through division was microscopically monitored. For experiments with Mps1 inhibitor (Msp1i), cells were incubated with Mps1i at 1 μM (Reversine, R3904) immediately after microirradiation, and subsequent progression through division was monitored microscopically (43). To identify the ideal concentration of all the inhibitors, cells were incubated with concentrations ranging from 100 nM-50 μM; the readout was division time of irradiated and non-irradiated samples.

Criteria for scoring micronuclei

Damaged mitotic cells were monitored 8-10 hrs post-microirradiation microscopically using the laser and microscope system described above. After 8-10 hrs post-damage, cells were fixed with 3% formaldehyde in TBS, stained with DDR antibodies, co-stained with DAPI, and imaged with Hamamatsu Orca cooled CCD Camera. Structures that were morphologically identical but smaller than the nucleus were classified as micronuclei as previously described (44).

Data analysis

The statistical analysis was performed using a two-tailed, unpaired Student's t-test to obtain p values and to determine the significance of the kinetics of DDR protein recruitment at damage chromosome ends (TIPs) versus damage chromosome arms (Non-TIP). ANOVA tests were used to determine significant differences ($p < 0.05$) in division time when cells were incubated with inhibitors, and alone. A Chi-Squared test was used to determine the significance of the presence of micronuclei due to the production of DNA breaks at TIP and Non-TIP sites. Tests were performed with GraphPad Prism version 6.00 (GraphPad Software Inc.).

Results

Damage to a single chromosome tip results in anaphase onset delay

Localized DNA breaks were induced in metaphase cells by laser microirradiation using a focused femtosecond laser source as previously described (27,28,33). Recently, we have shown that focal point laser microirradiation can be used to produce sub-micron DNA lesions. This results in the activation of the DDR at single chromosome tips (containing telomeres) and internal chromosomal sites (chromosome arms) of anaphase PtK2 cells (33). To further characterize the response of DDR at chromosome tips, we created a stable PtK2 cell line expressing TRF2-YFP. Telomere damage in the irradiated PtK2 cells was monitored by the loss of TRF2-YFP signal (Fig. 3.1A). Laser microirradiation was used to specifically ablate a single telomere while leaving the sister telomere intact (Fig. 3.1A and 3.1B). To verify that the loss of signal represents generation of DSBs and not photobleaching, the appearance of γH2AX was examined in relationship to telomeres (detected by a 5'-TTAGGG-3' FISH probe) (Figure 3.1B, TIP). Damage at a chromosome arm (Non-TIP) and TIP induced both γH2AX and the

recruitment of the non-homologous end joining (NHEJ) factor Ku70/Ku80 in a comparable fashion (Fig. 3.1B and 3.1C). Fluorescent measurement of the γH2AX signal at TIP and Non-TIP damage sites indicates that comparable amounts of damage were induced at both locations (Fig. 3.1D).

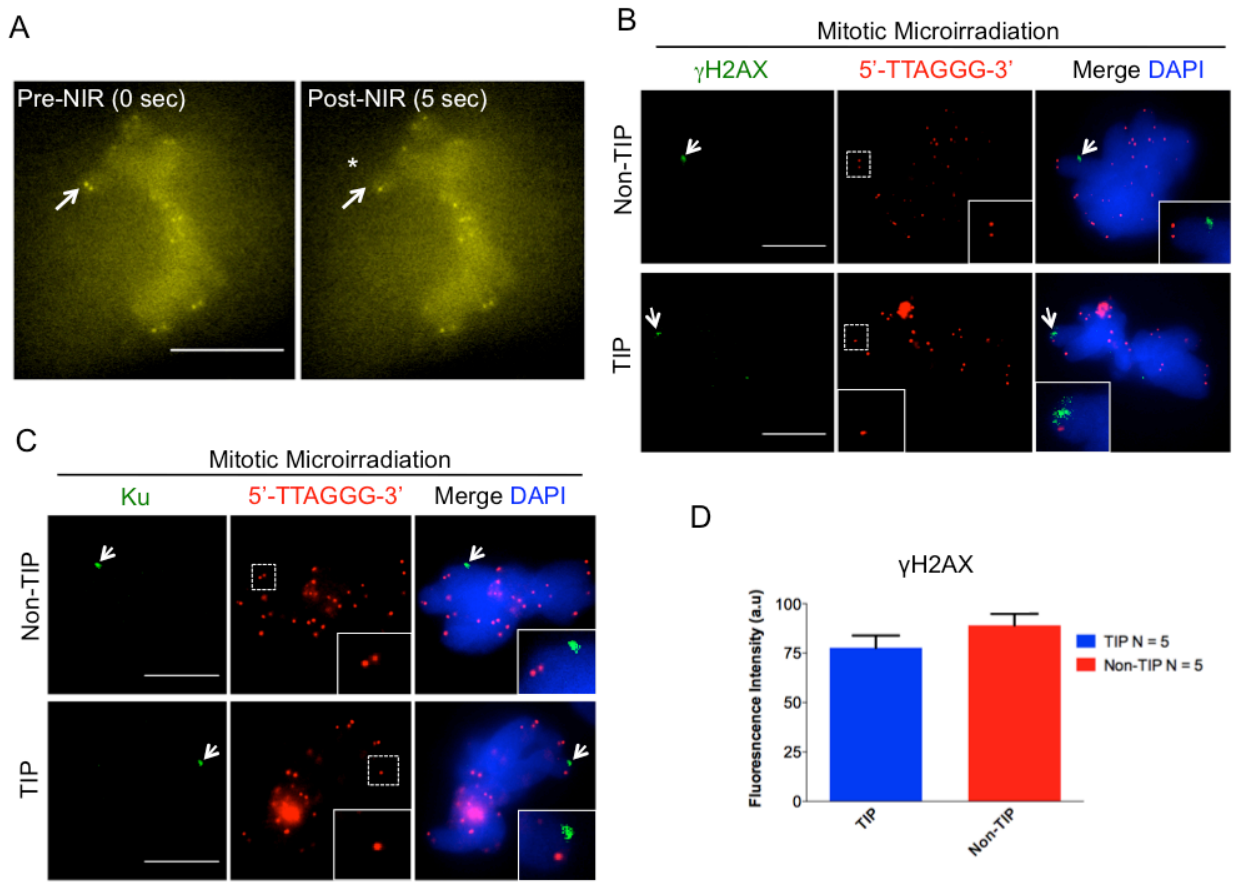

Figure 3.1 Laser-induced damage activates the DDR in mitotic chromosomes.
(A) Mitotic PtK2 cell stably expressing YFP-TRF2. Images were taken before microirradiation (pre-NIR) and at 5 sec post-microirradiation (post-NIR). White arrows point to a single telomere before (0 sec) and after laser microirradiation (5 sec) (also indicated with an asterisk). (B) Arrows point γ-H2AX to detect damage (green) at damaged Non-TIP and TIP sites at 5 min after laser microirradiation. Telomeres are detected by the 5'-TTAGGG-3' FISH probe (red). (C) Arrow points to the accumulation of Ku70/Ku80 complex at Non-TIP and TIP damage sites. Insets show twofold magnification of the images in the dotted boxes. Scale bar 10 μm. (D) Quantitative measurements (mean ± s.d. $p = 0.08$) after 120 s accumulation of γH2AX at TIP and Non-TIP damage sites. Statistics are for five independent values with three biological replicates ($N = 5$).

Having established the activation of the DDR at both TIP and Non-TIP damage sites on metaphase chromosomes, we investigated their potential effects on mitotic progression. Damage at a Non-TIP site failed to cause any significant delay in anaphase onset, as determined by the initiation of chromosome separation, compared to no damage (29.7 min, +/- 16.2 s.d, N = 24, and 26.4 min, +/- 8.94 s.d, N = 18, respectively) (Figure 3.2B). In contrast, anaphase onset was significantly delayed by induction of damage at a single TIP (73.2 min, +/- 29.3 s.d, N = 17, $p < 0.0001$) (Figure 3.2B). There was no apparent chromosome breakage or segregation defect (Figure 3.2A). Thus, the results indicate that chromosome TIP-specific DDR triggers delay in anaphase transition.

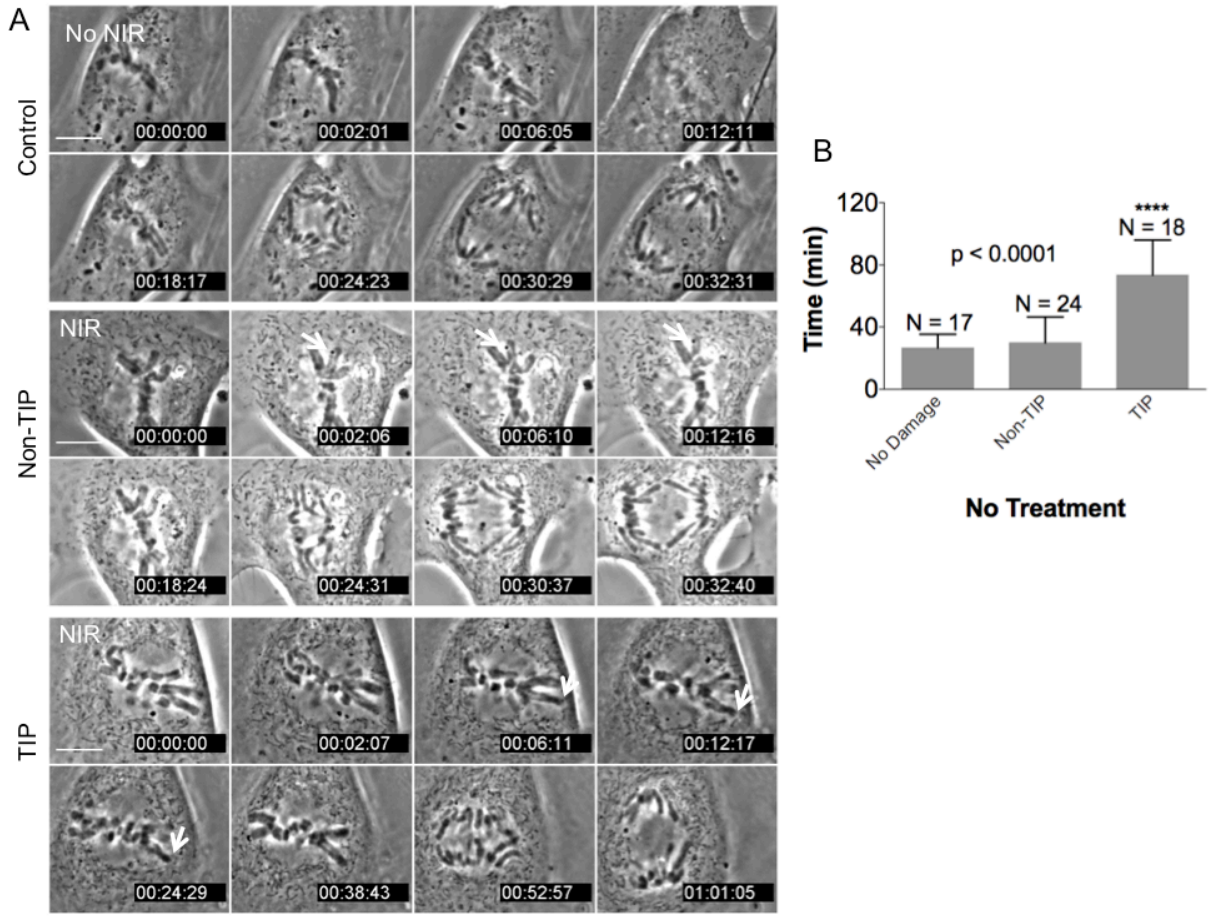

Figure 3.2 TIP damage results in delay of anaphase onset.
(A) Time-lapse microscopy images (in minutes) of mitotic non-irradiated cell (control), cells irradiated at Non-TIP and TIP. White arrow points to the irradiated chromosome. Scale bar 10 μm. (B) Duration of metaphase progression to anaphase was measured in individual cells (non-irradiated, microirradiated at Non-TIP, or at TIP) using the time-lapse microscopy imaging with 120-sec intervals (see Materials & Methods for details) ($p < 0.0001$ TIP compared to the control).

Damage to a single TIP recruits a unique set of DDR proteins

We next examined whether there are any differences in the recruitment of DDR factors between damaged TIP and Non-TIP sites, which may explain the TIP damage-specific mitotic delay. We detected γH2AX, Mre11, Ku70/Ku80, XRCC1, proliferating cell nuclear antigen (PCNA), phospho-Chk1, and phospho-p53 at both TIP and Non-TIP damage sites (Figure 3.3, Table 2). We found, however, that ATM and MDC1 are detected only at damaged TIPs but not

Non-TIPs (Figure 3.4). Both ATM and MDC1 were detectable at damaged TIPs as early as 15 min after damage induction (Figure 3.4A, N = 10) while no recruitment was observed at damaged Non-TIPs even after 30 min post-irradiation (Figure 3.4B, N = 10) (data not shown). Fanconi anemia protein FANCD2 and the RecQ (Werner syndrome) DNA helicase (WRN) were also recruited exclusively to damaged TIPs, but were undetectable at Non-TIP damage sites, in all the cells tested (N = 10). In contrast, ATR and 53BP1 were not detected at either TIP or Non-TIP damage sites (Figure 3.4, and data not shown). Lack of 53BP1 recruitment to uncapped telomeres and DNA damage sites in mitotic cells is consistent with previous studies (21,45).

Table 2 DNA damage response factors and repair proteins that form foci at DNA breaks of mitotic cells.

	Foci at TIPs	Foci at Non-TIPs
Damage-induced posttranslational modifications		
Phosphorylated histone H2AX (γH2AX)	✓	✓
Ubiquitin *	✓	✓
Chk1 phosphorylation on serine 345 (Ser345) *	✓	✓
Chk2 phosphorylation on threonine 68 (Thr68)	✓	✓
Phosphorylated p53 on serine 15 (p-p53) *	✓	✓
DNA damage signaling and repair factors		
Mre11	✓	✓
Nbs1 *	✓	✓
Poly(ADP-ribose) polymerase 1 (PARP1) *	✓	✓
Ataxia telangiectasia mutated (ATM)	✓	×
Ataxia telangiectasia and Rad3 related (ATR)	×	×
MDC1	✓	×
53BP1	×	×
BRCA1 *	✓	✓
CtIP *	✓	✓
Non-homologous end joining (NHEJ) repair proteins		
Ku70/Ku80 complex	✓	✓
Homologous recombination (HR) repair proteins		
Rad51	×	×
Other DNA repair-related proteins		
XRCC1	✓	✓
PCNA	✓	✓
FANCD2	✓	×
Werner syndrome (WRN) helicase	✓	×

* DDR and repair factors that show altered kinetics.

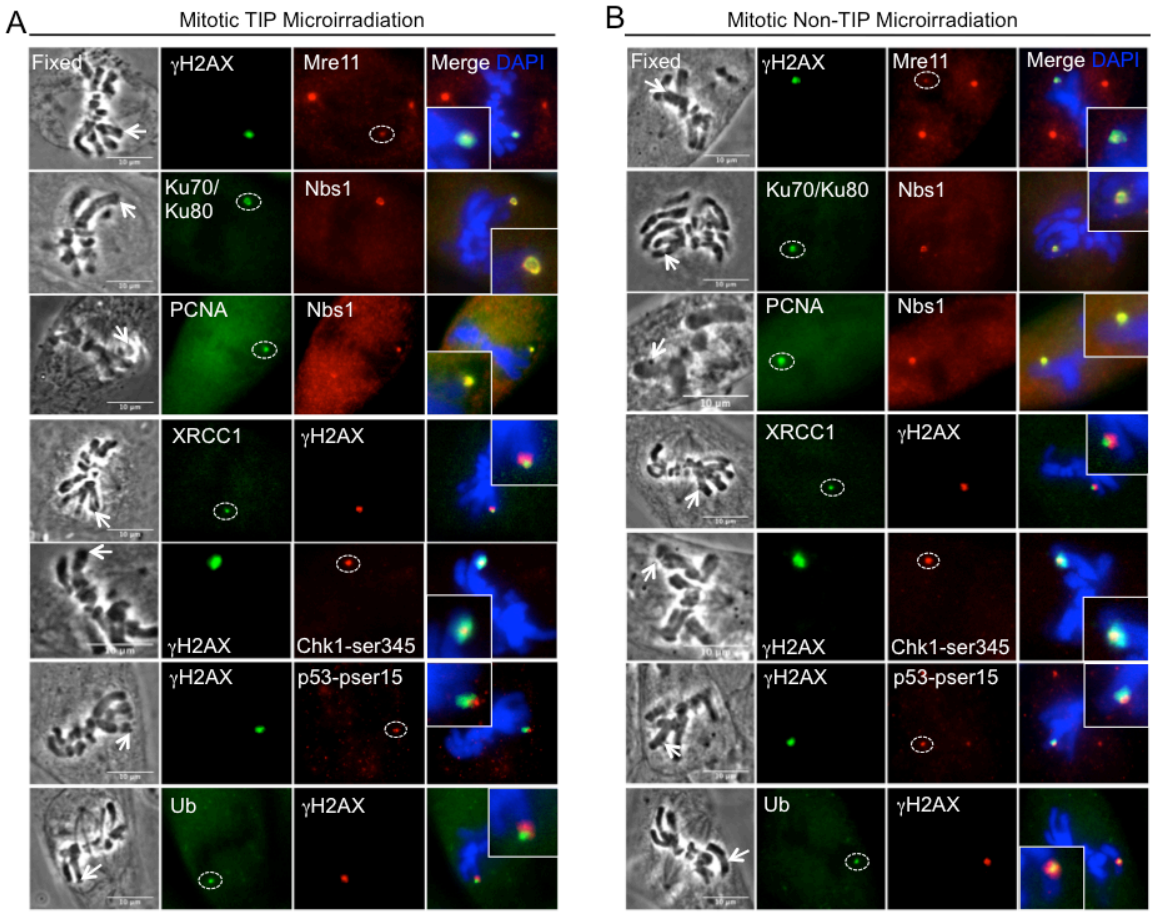

Figure 3.3 Recruitment of DDR and repair proteins to damaged TIP and Non-TIP sites. Recruitment of DDR factors along γH2AX at damaged TIP (A) and Non-TIP (B) sites. Dashed circles show DDR foci at TIP and Non-TIP damage sites. Cells were fixed at 15 min after irradiation and were stained with antibodies specific for Mre11, Ku70/Ku80, PCNA, XRCC1, phosphorylated Chk1 at serine 345 (pser345), phosphorylated p53 at serine 15 (pser15) and counterstained with DAPI (blue). Nbs1 and γH2AX antibodies were used as controls to detect DNA breaks. Insets show twofold magnification of damage signal at TIP and Non-TIP sites. Scale bar 10 μm. The results are summarized in Table 2.

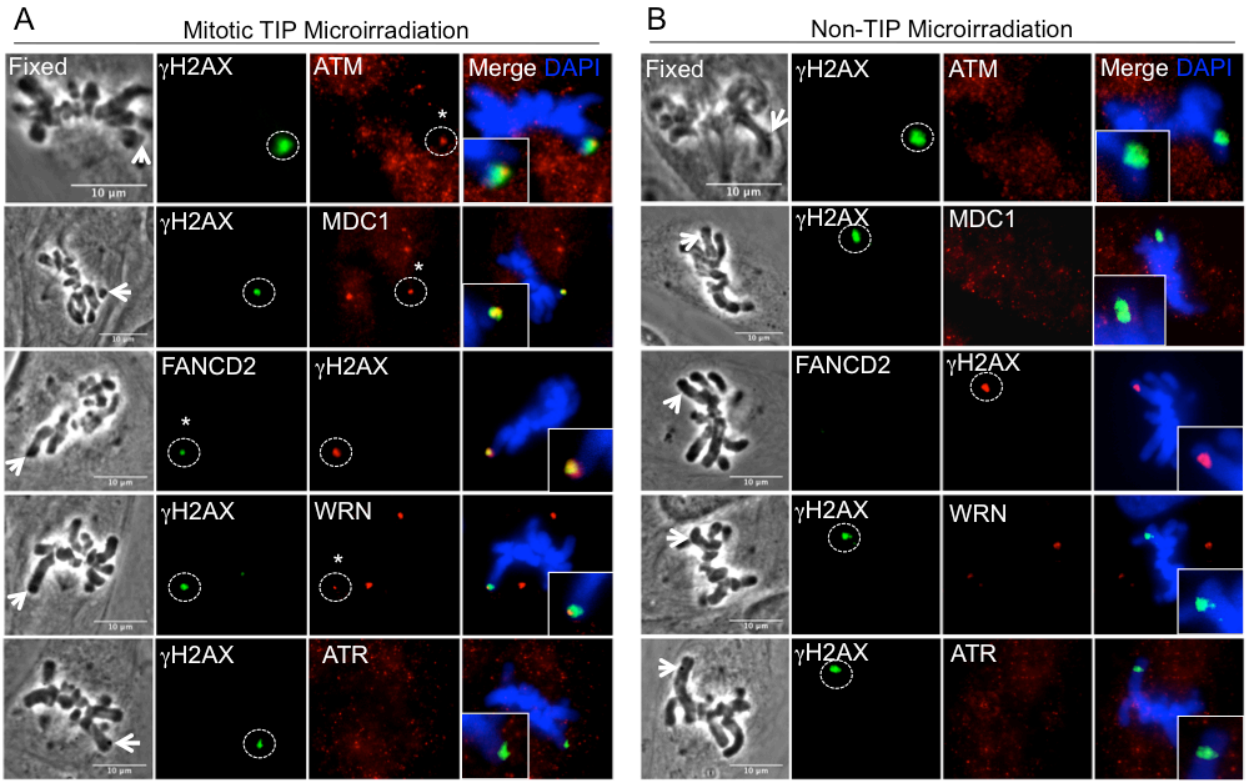

Figure 3.4 ATM, MDC1, FANCD2 and WRN are preferentially recruited to damaged TIPs. (A) Cells were damaged at a single TIP (A) or Non-TIP (B) and fixed at 15 min after damage induction. Damaged samples were stained with DDR factors and DSB marker γH2AX as indicated. Damage sites are indicated with white arrows in the phase image and dashed circles indicate corresponding immunofluorescent signals. Asterisks indicate the presence of DDR factors at damaged TIPs. Insets show two-fold magnification of damage signal at TIP and Non-TIP sites. Scale bar 10 μm. The results are summarized in Table 2.

We also found that some DDR proteins are recruited to TIP and Non-TIP damage sites with different kinetics. PtK2 cells fixed at similar time points after laser damage induction demonstrated that the recruitment of PARP1 (Figure 3.5A, $p = 0.005$, $N = 8$) and TUNEL (Figure 3.5B, $p < 0.003$, $N= 5$) signals persisted for longer periods of time at damaged TIPs compared to Non-TIP sites, suggesting the different repair efficiencies. CtIP foci accumulated faster at damaged Non-TIPs sites compared to damage TIPs (Figure 3.5C, $p = 0.05$, $N = 8$).

Interestingly, phosphorylation of Chk2, a target of ATM kinase, showed an increased signal at Non-TIP sites compared to TIP sites (Figure 3.5D, p = 0.006, N = 8).

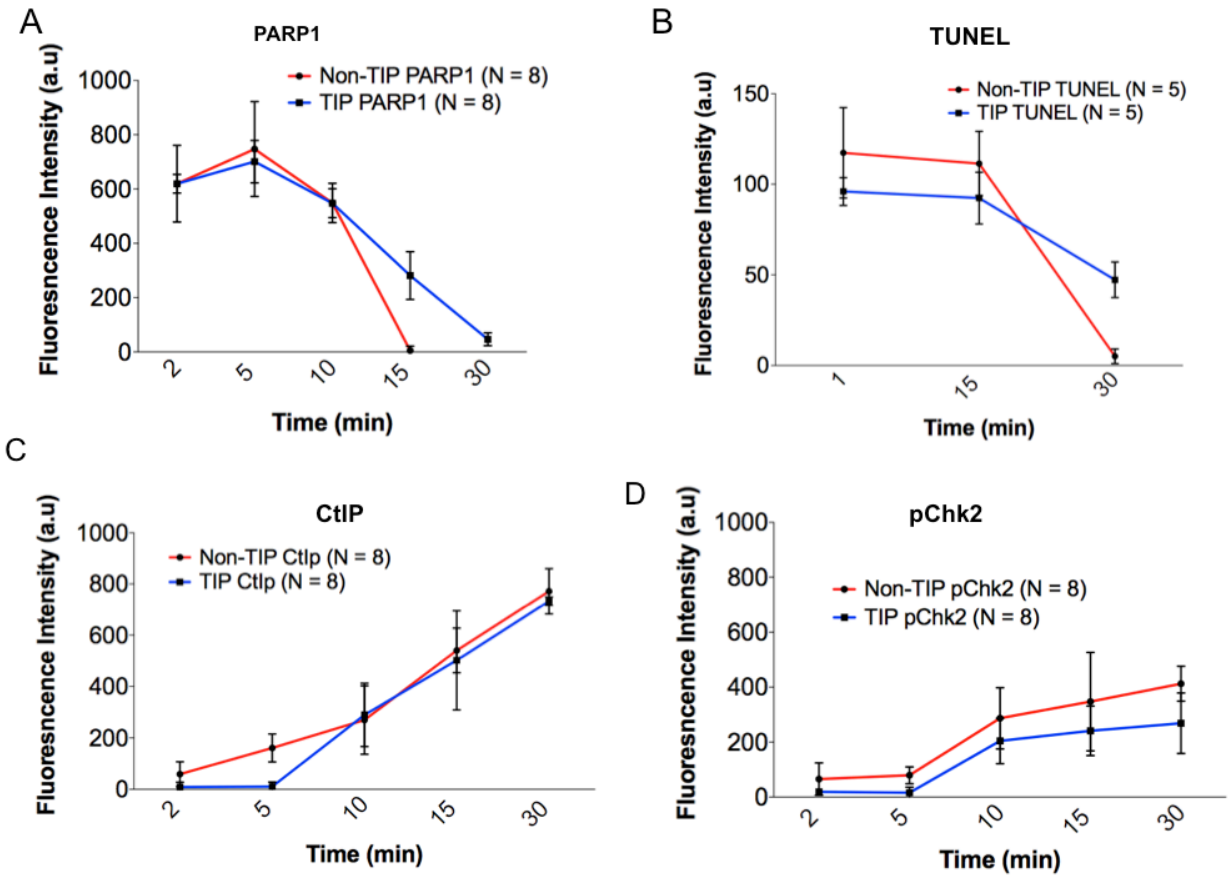

Figure 3.5 DDR factors and repair proteins show altered kinetics at damaged TIPs and Non-TIP. After microirradiation at a single Non-TIP or TIP site, cells were fixed at indicated time points and were subjected to immunofluorescence staining using antibodies specific for (A) PARP1, (C) CtIP, (D) phosphorylated Chk2 (pChk2) or to a TUNEL assay (B). Quantitative measurements (mean ± s.d.) of the fluorescent signals at damaged Non-TIP (red) and TIP (blue) sites are plotted. Two-tailed, unpaired Student's t-test was used to obtain P values. (A) PARP1 persists longer at damaged TIP compared to Non-TIP sites. Statistics are for eight independent experiments (N = 8, p = 0.005). (B) TUNEL persist at DNA breaks of mitotic TIP compared to Non-TIP sites (N = 5, p < 0.003). (C) CtIP accumulates more rapidly at Non-TIP breaks than TIP sites (N = 8, p = 0.05). (D) Phospho-Chk2 accumulates faster at Non-TIP breaks than TIPs (N = 8, p = 0.006). The fluorescence was normalized using undamaged cells stianined with the respective DDR factors.

In contrast, GFP-Nbs1 (27) began to accumulate at DNA damage sites 17 seconds +/- 4.5 s.d after microirradiation to a damaged TIP (N = 16), whereas its accumulation was delayed at a Non-TIP damage site (27 secs +/- 3.3 s.d, $p < 0.005$, N = 10) (Figure 3.6). The results reveal a faster recruitment and increased accumulation of GFP-Nbs1 at damaged TIPs compared to Non-TIPs (Figure 3.6B, $p < 0.005$). Similarly, we found that BRCA1 accumulates more efficiently at damaged TIP sites compared to Non-TIPs (Figure 3.7A, 3.7B and 3.7C, $p < 0.05$, N=5). Since BRCA1 recruitment to damage sites is intimately linked to ubiquitylation (46-48), we also examined the Ub signal at TIP and Non-TIP damage sites. Previous work has shown that ubiquitylation at IR-induced DNA breaks is absent in mitosis (21). In contrast, we found that the Ub signal is present at laser-induced damage sites in mitosis though with different kinetics at TIP and Non-TIP sites (Figure 3.3 bottom, Figure 3.7D). We failed, however, to detect RNF8 and RNF168, which are the major Ub E3 ligases at damage sites (23,48), at either TIP or Non-TIP damage sites (data not shown). This suggests an alternative mechanism of Ub induction in mitotic PtK2 cells. Taken together, the results reveal mitosis-specific differential recruitment of repair and checkpoint proteins to TIP and Non-TIP damage sites.

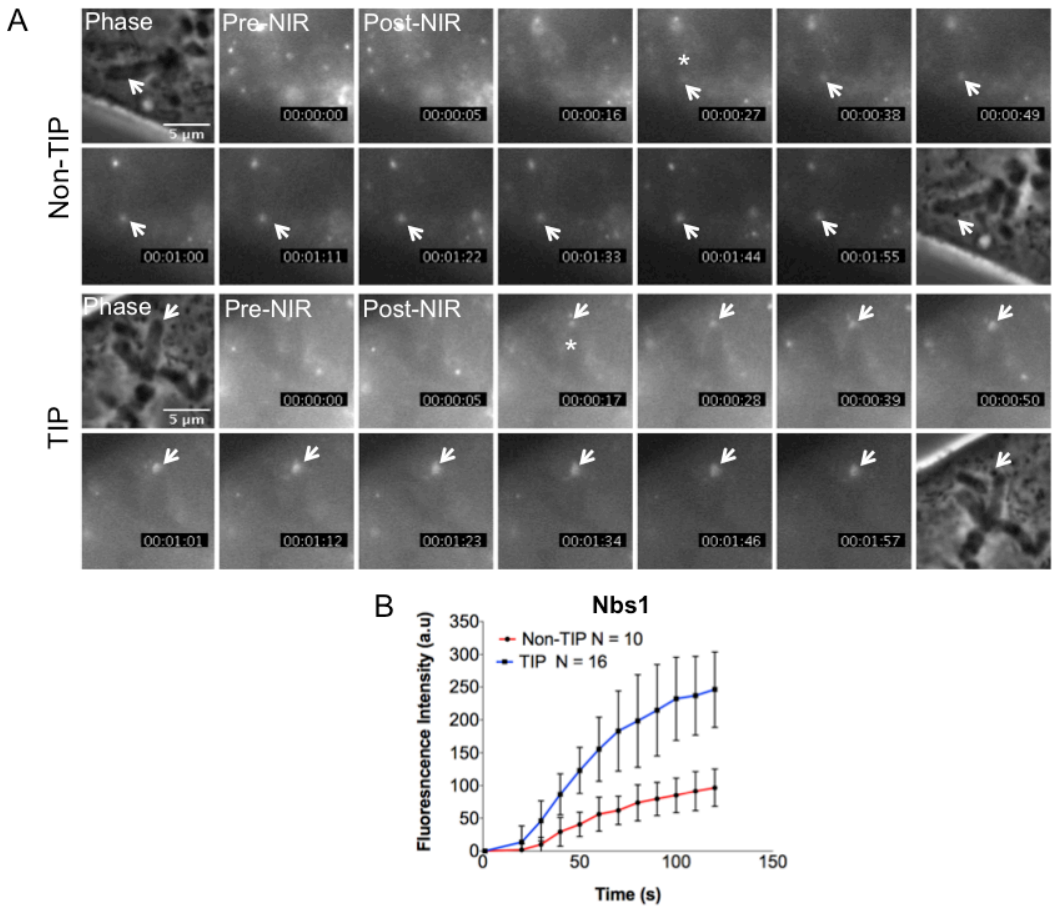

Figure 3.6 Nbs1 accumulates faster at damaged chromosome tips.
(A) Live cell imaging of GFP-Nbs1 accumulation (asterisk shows when Nbs1 starts to accumulate, and white arrow points to the accumulation of Nbs1) at Non-TIP, and TIP sites of PtK2 cells after microirradiation. Image taken before microirradiation at time 0-s (Pre-NIR). Image taken immediately after microirradiation (Post-NIR). White arrows indicate where damage was induced on a single chromosome (Phase image). Fluorescent Nbs1 recruitment at Non-TIP and TIP sites was captured at 10s intervals for 120 s after irradiation. The numbers at the bottom right of each image indicate elapsed time after damage induction (s). (B) Quantitative measurements (mean ± s.d.) at 10-s intervals of GFP-Nbs1 accumulation at TIP (N = 16) and Non-TIP (N = 10) sites ($p = 0.0048$). Two-tailed, unpaired Student's t-test was used to obtain p values.

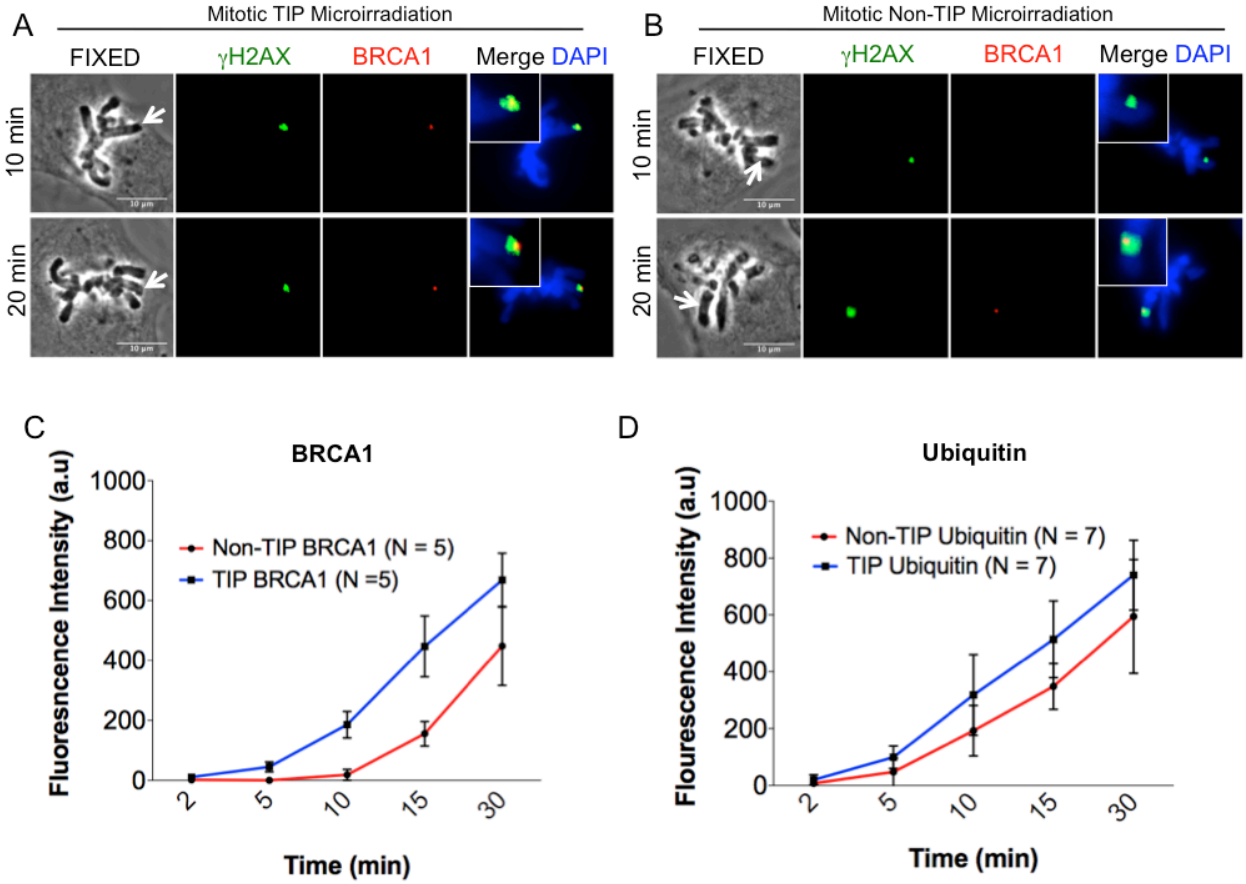

Figure 3.7 BRCA1 and Ubiquitin accumulate faster at damaged chromosome tips.
(A). Cells were damaged at a single TIP (A) or Non-TIP (B), and were fixed and stained with BRCA1 (red) and γ-H2AX (green). White arrows indicate damage sites. Insets show twofold magnification of damaged regions at TIP and Non-TIP sites. (C) Quantitative measurements (mean ± s.d.) of BRCA1 accumulation at damaged Non-TIP (red) and TIP (blue) sites. BRCA1 accumulates faster at damaged TIPs ($N = 5$, $p < 0.05$). (D) Cells were damaged at a single TIP (A) or Non-TIP (B), and were fixed and stained with ubiquitin and γ-H2AX. Quantitative measurements (mean ± s.d.) of Ubiquitin accumulation only at damaged Non-TIP (red) and TIP (blue) sites. Ubiquitin accumulates faster at damaged TIPs ($N = 7$, $p = 0.026$). Two-tailed, unpaired student's t-test was used to obtain p values.

Damage-induced anaphase delay requires ATM, Chk1 activity and the SAC

During interphase, ATM and Chk1-mediated checkpoint activation can delay cell cycle progression (49). Given the specific localization of ATM at damaged TIPs, we investigated

whether the anaphase delay is due to ATM activation. Administration of small-molecule inhibitors specific to ATM prior to laser microirradiation prevented recruitment of ATM to TIP damage sites (Figure 3.8A). ATM and Chk1 inhibitions resulted in the loss of TIP damage-induced anaphase delay (24.9 min, +/- 10.1 s.d. ($p < 0.0001$) and 20.5 min, +/- 7.9 s.d. ($p < 0.001$), respectively when compared to untreated cells with TIP damage (73.2 min, +/- 29.3 s.d, ($p < 0.0001$) (Figure 3.8C). ATM of Chk1 inhibition did not cause a significant change in timing of anaphase onset in cells with Non-TIP damage (Figure 3.8D) and in the absence of damage (data not shown). Moreover, ATM depletion by siRNAs resulted in the similar loss of TIP damage-induced anaphase delay (23.9 min, +/- 5.72 s.d. ($p < 0.0001$); Figure 3.8C). SiRNA transfection had no effect on chromosome segregation (data not shown). ATM depletion was verified by western blot analysis of siRNA-transfected cells (Figure 3.8B).

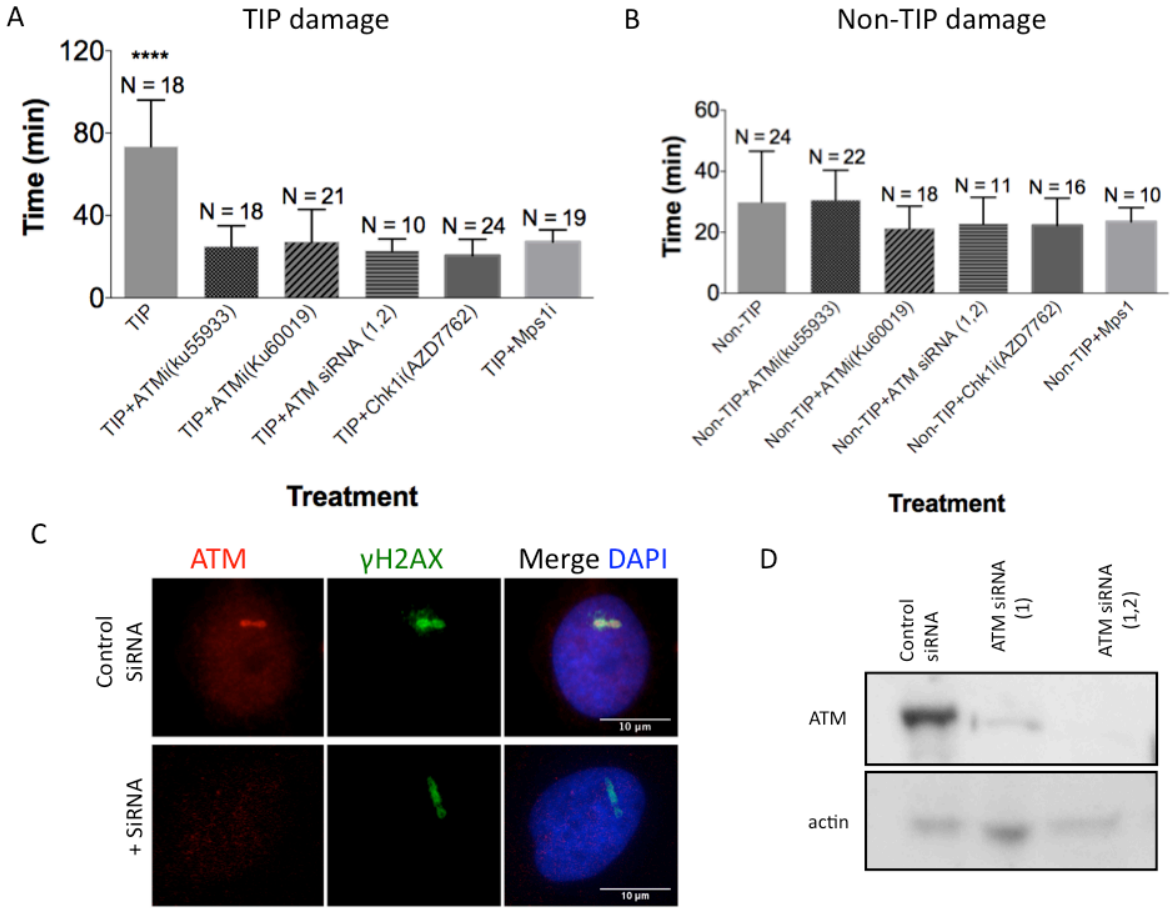

Figure 3.8 Damaged TIPs activate an ATM-dependent anaphase onset delay.
(A) The effect of the inhibition of ATM, Chk1 and Mps1 on the anaphase onset delay in the cells with TIP damage. (B) The effect of the inhibition of ATM, Chk1 and Mps1 on the anaphase onset delay in the cells with Non-TIP damage. Cells were either treated with or without inhibitors for one hour or transfected with siRNAs for 48 hrs before the TIP damage induction in metaphase. Damaged cells were monitored and the duration of metaphase progression to anaphase was measured as in Figure 3.2. Cells were treated with 1μM Msp1i (reversine) immediately after microirradiation. ANOVA test revealed a significant difference among TIP damage treated cells with inhibitors and siRNAs ($p < 0.0001$). (C) Transfected cells with control siRNA and siRNA (1 and 2) duplexes were stained with stained with ATM (red) and DSB marker γH2AX (green) and counterstained with DAPI (blue). (D) Western blot analysis of cells transfected with control scramble siRNA, with one ATM (ATM siRNA 1) siRNA duplex, or two ATM siRNA (1 and 2) duplexes (refer to materials and methods for sequences). Cell extracts were subjected to SDS-PAGE and immunoblotted using ATM antibody. Actin serves as a loading control.

It is well established that anaphase onset is mediated by the spindle assembly checkpoint (SAC) (50). Administration of a small molecule inhibitor of the SAC kinase Mps1, which results in abrogation of the SAC (51), also suppressed the anaphase delay caused by laser-induced TIP damage (23.9 min, +/- 6.3 s.d, ($p < 0.0001$) (Figure 3.8C). We found no significant acceleration of anaphase onset by the inhibitor treatment in cells with Non-TIP damage (Figure 3.8D). The inhibitor did not affect the anaphase onset of undamaged cells (data not shown). Taken together, these results indicate that the anaphase delay caused by TIP damage is dependent on the activity of ATM, Chk1 and Mps1.

Damage at chromosome tips results in the formation of micronuclei

Cells with the damaged TIPs eventually undergo anaphase, albeit with delayed kinetics. By monitoring PtK2 cells with a damaged TIP and Non-TIP until G1, we observed a significant increase in the presence of micronuclei in cells that were damaged at a single chromosome TIP (54.8 %, N = 31) compared with cells that were damaged at a Non-TIP (19.2 %, N = 26, $p < 0.002$) or non-irradiated control cells (0 %, N = 26, $p < 0.002$) (Figure 3.9A). Damaged TIPs were consistently found within micronuclei as confirmed by the presence of the telomere repeat sequence using FISH (Figure 3.9B, inset b'). Damaged TIPs continue to be marked by γH2AX, CtIP (Figure 3.9C, TIP panel, inset c'), and PCNA (Figure 3.9D, TIP panel, inset d'). Unlike during mitosis, however, 53BP1 associates with damaged TIPs in the micronuclei in G1 (Figure 3.10, TIP panel, inset a'). The results indicate that damaged TIPs marked by DDR factors are preferentially encapsulated into micronuclei in the subsequent G1 phase.

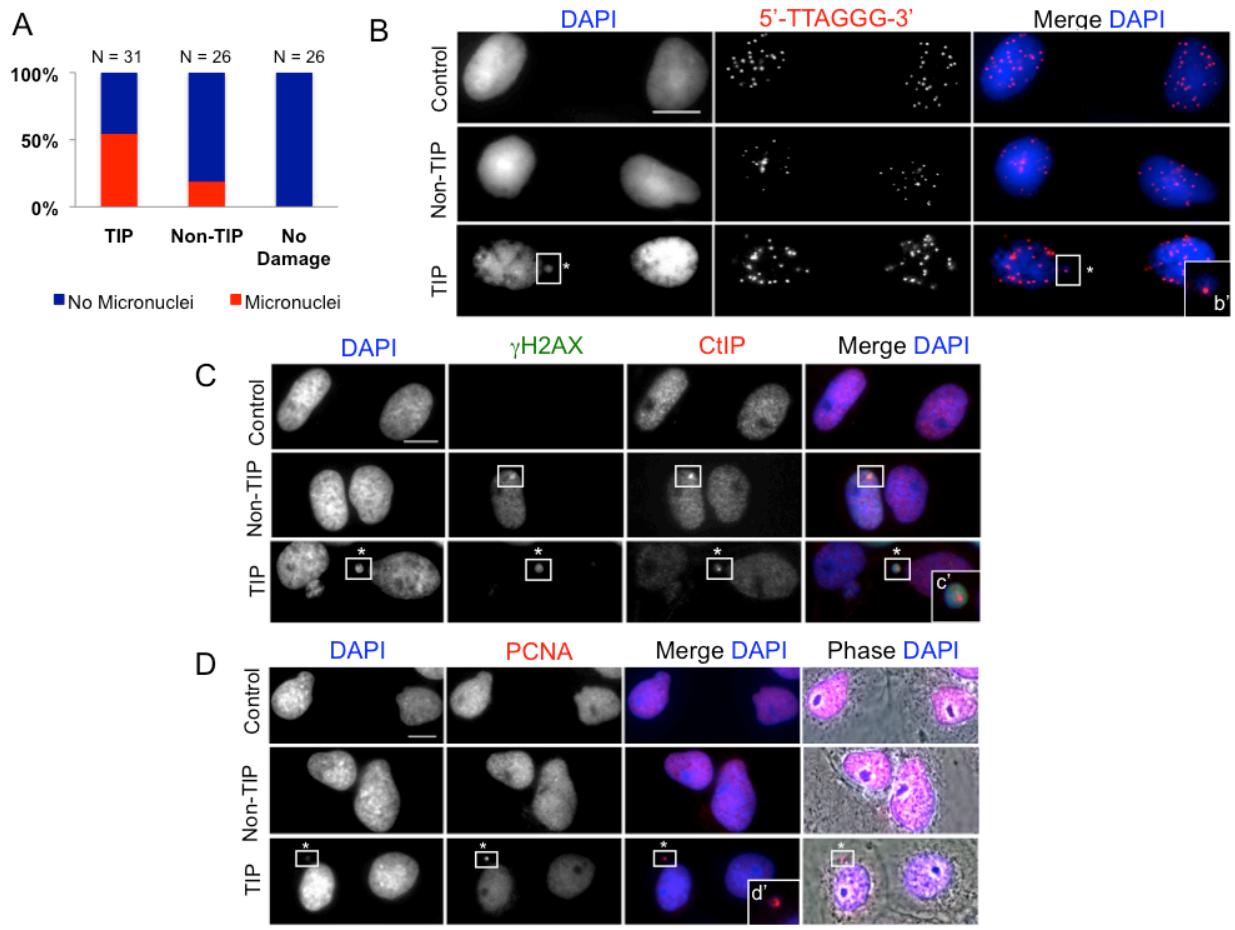

Figure 3.9 Damaged TIPs segregate into micronuclei at G1.
(A) Mitotic cells (damaged at a TIP or Non-TIP, or no damage) were monitored until G1, fixed and stained with DAPI to score micronuclei. Chi-squared test revealed a significant difference between DNA breaks produced at TIP compared to Non-TIP and non-irradiated control cells ($p = 0.002$). Damage TIP cells showed a higher percentage of micronuclei (54.8%). (B) Microirradiated mitotic cells were monitored until G1, fixed and stained with Cy3-5'-TTAGGG-3' probe along with DAPI. Damaged TIP containing telomere repeats is found inside micronuclei (inset b', white square, *). (C) Irradiated mitotic cells were monitored until G1, fixed and stained with antibodies against γH2AX and CtIP counterstained with DAPI. CtIP persists into G1 at both Non-TIP and TIP DNA breaks (indicated by white squares). Damaged TIP forms a micronucleus colocalizing with CtIP and γ-H2AX (indicated by white squares and asterisks) (two-fold magnification in the inset c'). N = 3 of cells tested. (D) Microirradiated mitotic cells were monitored until G1, fixed and stained with anti-PCNA and counterstained with DAPI. PCNA is present inside the micronuclei of TIP breaks (inset d', *). N = 3 of cells tested.

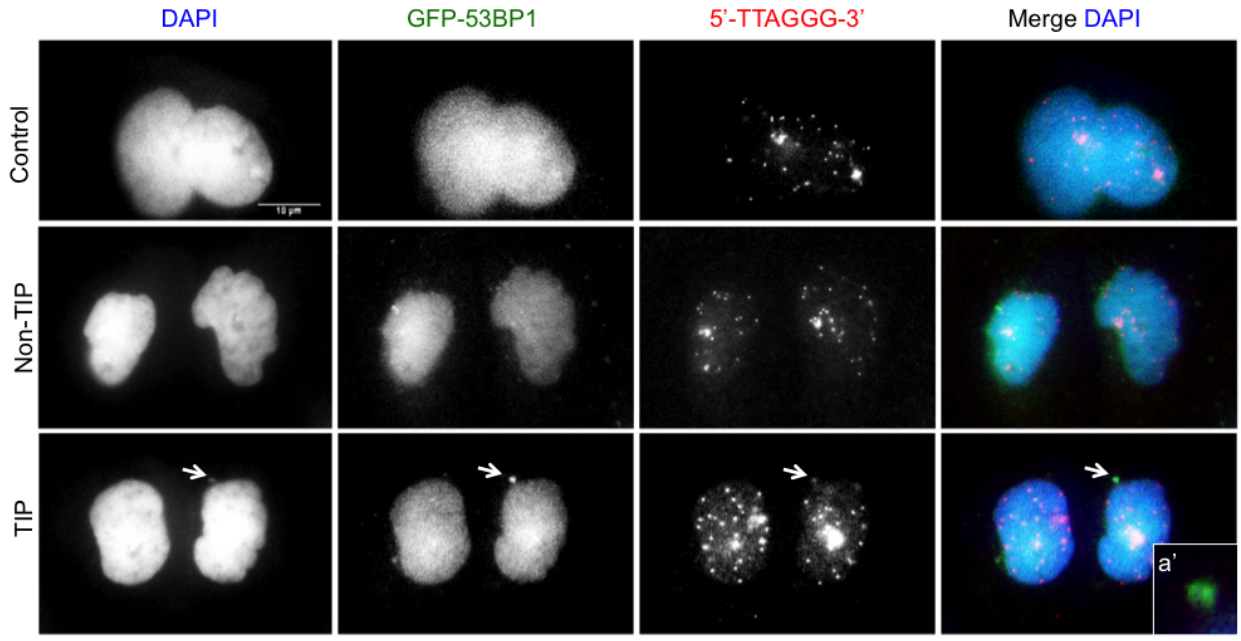

Figure 3.10 Damaged chromosome tips accumulate 53BP1 in micronuclei.
(A) PtK2 cells stably expressing GFP-53BP1 were irradiated at TIP and Non-TIP during mitosis, and monitored until G1. Non-irradiated cells served as controls. Cells were fixed and stain with anti-GFP, Cy3-5'-TTAGGG-3' probe along with DAPI. Persistent accumulation of 53BP1 colocalizing with Cy3-5'-TTAGGG-3' is detected inside micronuclei in response to TIP damage (white arrows and inset a'). Insets represent two-fold magnification. Scale bar 10 μm.

Discussion

In this study, we show that induction of damage to a single mitotic telomere-containing chromosome tip, leads to an ATM and Chk1-dependent anaphase delay that may act through the spindle assembly checkpoint (SAC). In addition, we found that a single damaged chromosome tip specifically recruits ATM, MDC1, FANCD1 and WRN proteins, which are not detected at damaged chromosome arms (Table 1). The mitotic cells with damaged TIPs eventually exit mitosis with an increased occurrence of micronuclei that encapsulate the DNA lesions. Altogether, these results reveal a unique signaling mechanism associated with damaged chromosome ends in mitosis.

ATM and SAC-dependent mitotic delay in response to TIP damage

In this study, damage to a single telomere-containing chromosome TIP in metaphase cells, as opposed to Non-TIP damage sites, delays progression into anaphase and the subsequent separation of chromosomes into daughter cells. A telomere deprotection-induced damage in interphase initiates an ATM/Chk1-dependent damage checkpoint pathway, culminating in cell cycle arrest or delay in G2/M (20) or p53-dependent G1 arrest (24). In mitosis, we also found that ATM and phosphorylated Chk1 at damage sites in the telomere-containing chromosome TIP, and subsequent mitotic delay can be suppressed by ATM inhibition, indicating that the similar DDR signaling remains active during mitosis. The delay in anaphase onset caused by TIP damage can be suppressed by inhibition of Mps1, indicating the involvement of the SAC in this process. ATM activation is known to be involved in the phosphorylation of many SAC components including SAC kinases, such as Bub1 and core SAC signaling proteins such as Mad1 and $p31^{comet}$ (52). A recent study has shown that ATM and MDC1 modulate the assembly of SAC components at the kinetochores (53), further supporting the role of ATM signaling in SAC regulation. Recent evidence indicates that the uncapping of telomeres in mitosis results in activation of ATM in human cells (25), and an ATM and SAC-mediated delay in anaphase onset in Drosophila embryos (49,54). Telomere uncapping-induced damage also results in inhibition of mitotic exit in *Saccharomyces cerevisiae* (55). Thus, although our laser microirradiation may also damage the neighboring subtelomeric regions, the observed ATM/SAC-dependent TIP-specific damage checkpoint signaling is consistent with the disruption of telomere integrity as also evidenced by the loss of TRF2 and TTAGGG sequence.

Lack of checkpoint activation by Non-TIP damage in mitosis

We failed to observe any significant mitotic delay after Non-TIP damage in PtK cells. This is consistent with the previous study in which random high irradiance laser-mediated DNA damage evoked no mitotic delay in PtK cells (56). In contrast, in mammalian cells, Plk1 kinase deactivation and subsequent Cdc25C degradation has been reported in response to DNA damage in mitosis (57). Mitotic reversal into a G2-like state, with high Cyclin A levels, has also been reported in response to DNA damage (58). In both cases, however, damage was induced genome-wide during nocodazole-induced prometaphase block with SAC activation. Previous work in Drosophila also demonstrated that mitotic damage indirectly activates the SAC (59,60). A similar mitotic delay was seen in budding yeast, where mitotic DNA damage resulted in Pds1/securin stabilization preventing Esp1/separase activation and anaphase onset (61). This was recently shown to rely on SAC proteins (62,63). Additionally, high irradiance laser-mediated ablation to prophase cells reported by Mikhailov and colleagues demonstrated a SAC-mediated delay in cell cycle progression in human cells (56). Taken together these studies indicate the presence of mitotic damage-induced DDR mechanisms. In these studies, damage was induced randomly and thus, DDR signaling may be originated from telomere damage. It is important to note, however, that PtK cells, like many rodent cells, do not have a strong SAC (64). Thus, it is possible that SAC-dependent DDR against Non-TIP damage may be attenuated in PtK cells. Further investigation to distinguish TIP and Non-TIP damage responses in human cells is important. Nevertheless, our results highlight the distinct mechanism of mitotic surveillance of the telomeres.

Damage at chromosome tips recruits a distinct set of proteins

Our results indicate the selective recruitment of MDC1 at telomere-containing TIP damaged sites, but not Non-TIP sites of metaphase cells. Although MDC1 focus formation was previously observed following IR damage in mitosis (21), it was unclear whether MDC1 foci are restricted at telomeres. The failure of MDC1 to accumulate at Non-TIP damage sites in the current study is consistent with previous studies that suggest DDR protein recruitment is attenuated in mitosis (65). In addition, our results also show the accumulation of WRN helicase, FANCD2 and ATM is found specifically at the mitotic TIP, but not Non-TIP damage sites. WRN is a RecQ helicase involved in DNA repair and is specifically implicated in telomere DDR (66,67). WRN recruitment to telomeres is also associated with an alternative lengthening pathway of telomeres (ALT) (68), and requires the activity of FANCD2 (69). Thus, our results suggest that these factors make unique contributions to telomere DDR even in mitosis.

It was reported previously that Ub signal and 53BP1 accumulation are inhibited during mitosis following IR damage in human cells (21). A recent study demonstrated that mitotic kinases phosphorylate RNF8 Ub ligase and 53BP1 to specifically inhibit their damage site recruitment and the NHEJ pathway in mitosis, preventing the telomere fusion in human U2OS cells following IR damage (23). While we also failed to observe RNF8 and 53BP1 at both TIP and Non-TIP damage sites, while othere factors were able to accumulate (Table 2), significant Ub signals were detected at laser-induced damage sites in mitotic PtK cells. This raises the possibility that RNF8-independent Ub signaling is activated by laser-induced damage in mitosis. Furthermore, despite the lack of MDC1, both Ub and BRCA1 were also detectable at Non-TIP damage sites, suggesting that Ub signaling and BRCA1 recruitment can occur in an MDC1-independent manner in mitotic PtK cells. This apparent discrepancy may be explained by the

species difference (PtK vs. human cells), and/or the different method of damage induction. Multiple DNA breaks are induced by laser microirradiation at the focal spot of the chromosome, resulting in the rapid detection of DDR factors not observed by other systems (33,70). Interestingly, a recent study indicated that the Ub response and BRCA1 recruitment can be mediated by BBAP Ub ligase independent of ATM and MDC1 at laser-induced damage sites in human cells (71). Thus, an Ub ligase other than RNF8 may also be responsible for Ub and BRCA1 signals at Non-TIP damage sites in mitotic PtK cells. The fact that the Ub and BRCA1 signals are stronger at TIPs suggests that the presence of MDC1 further enhances Ub and BRCA1 signals specifically at TIPs. Taken together, the Ub and BRCA1 accumulation at damage sites at TIPs and Non-TIPs are complex and may involve multiple mechanisms.

Damage to chromosome tips results in micronucleus formation

Despite the recruitment of some of the DDR and repair factors and cell cycle delay, most of the damaged cells eventually enter the G1 phase still carrying unrepaired DNA lesions. Even though we observed Ku70/Ku80 recruitment to both TIP and Non-TIP damage sites, damage persists in G1 phase, suggesting the repair activity is impaired most likely due the absence of 53BP1, which facilitates NHEJ. This is consistent with the inhibition of the NHEJ pathway in mitotic U2OS cells (23). There is no discernable recruitment of Rad51 at mitotic damage sites, indicating that the HR pathway is also not active. Interestingly, the single damaged TIP is encapsulated to form a single micronucleus in the subsequent G1 in high frequency compared to damaged region at a Non-TIP site. Persistent damage in the micronucleus in G1 is marked by 53BP1, which was absent during mitosis. Micronuclei are the hallmark of chromosome instability (44). They are often present in cells with defective DDR, or in cells with a disrupted

cell cycle checkpoint machinery (72). While micronuclei are often formed as the results of spindle abnormality and chromosome segregation defects (23,72,73), telomere erosion during senescence have also been reported to form micronuclei (23). Interestingly, re-activation of DDR response at bulk chromatin in mitotic cells, accomplished by the expression of unphosphorylated 53BP1 and RNF8 mutants also resulted in whole chromosome micronuclei (23). Currently, it is unclear how the specific chromosome or the sub-region of the chromosome containing the damaged telomere (or more generally DNA lesions) is selectively encapsulated into a micronucleus. It is also not known whether DNA lesions in the micronuclei are eventually repaired. Inefficient repair of DNA contained in micronuclei can contribute to genome instability (72,73). It is possible, that formation of damaged telomere-containing micronuclei may lead to genome instability. Alternatively, segregation of damaged telomeres from the rest of the nucleus may help to protect the cell. Further study is necessary to follow the fate of these cells.

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Chapter 4 Inhibition of ATM delays Phosphorylated TRF2 Recruitment at Laser-Induced Double Strand Breaks (DSBs) of interphase and mitotic cells[3]

Abstract

Evidence suggests that one of the telomeric proteins, telomeric repeat binding factor 2 (TRF2) plays a role in the DNA damage response (DDR) by binding to non-telomeric laser-induced DNA damage. However, there is conflicting evidence about the laser parameters used to induce TRF2 recruitment. In this study we used a near infrared (NIR) femtosecond laser to induce DNA damage. Specifically we introduced double-strand breaks (DSBs) and assayed for the recruitment of TRF2. We found that a laser dose of $2.43e^{+11}W/cm^2$ is sufficient to form DSBs, based on the recruitment of repair factor 53BP1. However, at this laser dose, TRF2 fails to accumulate at damage sites. In contrast, at an irradiance of $2.65\ e^{+11}\ W/cm^2$ or higher, TRF2 accumulates at dmage sites, which is independent of ATM. Thus, TRF2 accumulation is detectable only when damage is induced with higher laser irradiances. Furthermore, we found that phosphorylation of TRF2 on threonine 188 occurs at both low and high irradiance laser-induced DSBs in both interphase and mitotic cells in an ATM-dependent manner. In contras, Phosphorylated TRF2 on threonine 188 did not form foci by using γ-irradiation. These results suggest that different irradiation doses result in the recruitment of specific DDR factors. In addition, the NIR femtosecond laser microirradiation as opposed to γ-irradiation can be an effective tool to study the kinetics of DDR factors.

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Introduction

Telomeres are nucleoproteins structures that protect the ends of chromosomes from degradation processes and end-to-end fusion events (1-3). The ends of linear chromosomes resemble double-strand breaks (DSBs), but are protected by shelterin, a six-protein complex (4).

Telomeric repeat binding factor 2 (TRF2), one of the shelterin protein components, directly binds to duplex telomeric (TTAGG) repeats. Its function is to stabilize the T-loop structure, and to prevent the activation of the DNA damage response (DDR) pathway by suppressing ataxia-telangiectasia-mutated (ATM) protein kinase (1, 5-7). TRF2 is also known to interact with proteins from the DDR pathway, which together are responsible of maintaining the integrity of telomeres (3). When telomeres become dysfunctional (e.g., due to shortening or loss of function from shelterin protein complex TRF2 and POT1) they activate the DDR pathway (8, 9). This results in the phosphorylation of histone H2AX at serine 139 (γH2AX) and subsequent accumulation of repair protein 53BP1 visualized as telomere dysfunction-induced foci (TIF) (10).

Studies are conflicting in regards to the role of TRF2 in DDR. Early studies linked TRF2 as a DDR factor that facilitates homologous recombination (HR) repair (11), by binding to laser-induced non-telomeric DSBs of interphase cells (12). However, this finding has not always been reproduced when laser DNA breaks are introduced at low irradiances (13, 14). Further evidence suggests that TRF2 accumulates only to high irradiance laser-induced DNA breaks, and its accumulation is independent of ATM (14). Despite this, phosphorylated TRF2 at threonine 188 does localize to ATM-dependent laser induced DNA breaks (15) via the nonhomologous-end-joining repair pathway (16). However, there's no evidence that suggests that phosphorylated TRF2 at threonine 188 binds specifically to either low irradiance laser-induced DNA breaks (such as DSBs) or high irradiance laser-induced DNA breaks (known to produced multiple types

of lesions). Although accumulation of phosphorylated TRF2 has been studied in interphase cells, there is no evidence that phosphorylated TRF2 accumulates, or forms damage foci in mitosis in response to DNA lesions.

In the present study, we show that a near-infrared (NIR) short-pulsed femtosecond laser is able to produced DSBs at an irradiance (dose) of 2.43 e^{+11} W/cm^2, by assessing the recruitment of 53BP1 at interphase cells. However, TRF2 fails to accumulate at the laser-induced DSBs, but accumulate at a higher laser dose of 2.65 e^{+11} W/cm^2. Despite the lack of recruitment of TRF2 at DSBs, phosphorylated TRF2 at threonine 188 does accumulate at the lower laser dose in damaged interphase and mitotic cells. Furthermore, inhibition of ATM did not prevent phosphorylation of TRF2 at threonine 188, but it delayed its kinetics. We also demonstrate that laser microirradiation as opposed to γ-irradiation, can be used to study damage foci formation and activation of the DNA damage response, whereas, the kinetics of DDR proteins are more difficult to asses. Additionally, our results support the involvement of TRF2 in the DNA damage response, which is recruited only to high irradiance lesions. Contrary to these results, we observed phosphorylation of TRF2 at DSBs produced by the lower dose irradiance.

Materials and Methods

Cell lines and cell culture

Potorous tridactylus (PtK2) kidney epithelial cells (American Type Culture Collection ATCC, CCL 56), YPF-TRF2, and eGFP-53BP1 stably expressing PtK2 cells, were grown as previously described [17]. HeLa 1.2.11 cells (kindly donated by Eros Lazzerini Denchi's Lab) were grown in Gibco Advanced Minimum Essential Medium (MEM), supplemented with L-

Glutamine, 2% fetal bovine serum (FBS), and antibiotics. Cells were incubated at 37°C with 5% CO_2. For laser microirradiation and immunofluorescence experiments, cells were trypsinized (TrypLE^{TM} Express, Life Technologies) and plated on 35 mm gridded imaging dishes (MatTek) at approximately 20,000 cells per dish. YFP-TRF2 and eGFP-53BP1 stable expressing Ptk2 cells were grown on 35 mm imaging dishes. The media of the stable expressing YFP-TRF2 and eGFP-53BP1 was replaced before laser microirradiation with Hanks' Balanced Salt Solution (HBSS, 1X) to prevent the absorption of the laser light by the phenol red. Cells were monitored via-fluorescence microscopy with a Zeiss inverted microscope (Axiovert 200M) equipped with a Hamamatsu Orca cooled CCD Camera.

Laser microirradiation

Laser microirradiation was performed as previously described (18). Briefly, the RoboLase ablation software (19), was used to control a 200 femtosecond pulsed Ti:Sapphire 800 nm near-infrared (NIR) laser (Coherent Inc., Santa Clara, CA) coupled to a motorized inverted Zeiss microscope (Axiovert 200 M). The laser was focused to a diffraction-limited 0.7 μm spot with a Zeiss 63X /1.4 NA phase contrast oil objective. To determine the irradiance at the focal spot, the transmission of the objective at 800 nm was measured using the double-objective method [ref]. The objective transmission was 0.50% as previously shown (17, 20) and the corresponding laser irradiance at the focal spot was $2.43e^{+11}W/cm^2$ (17).

Laser-fluorescence microscopy

To study the recruitment of 53BP1 and TRF2, stable expressing PtK2 cells were microirradiated with the microscope-laser ablation system described above. Damaged cells were

monitored (minutes) via fluorescence microscopy. To study the kinetics of phospho-TRF2, PtK2 cells grown on 35 mm gridded dishes were microirradiated at a single focal spot on a chromosome, monitored by fluorescence after laser microirradiation (minutes), and fixed for subsequent antibody staining.

Gamma irradiation

Gamma irradiation was performed using a cesium-137 source irradiator as previously described (21). Briefly, a 24-well plate containing HeLa 1.2.11 cells grown on coated poly-lysine cover slips, was place on a rotator inside the irradiator. Irradiation was performed at a dose rate of 5 Gy/min.

ATM inhibitor (ATMi) treatment

HeLa 1.2.11 cells grown on poly-lysine cover slips on a 24-well plate one day before irradiation. Cells were incubated 1 hr prior to irradiation with ATM inhibitor (ATMi, ku-55993) at 10μM. After irradiation cells were incubated at 37°C with 5% CO_2 and fixed with 3.0% formaldehyde at specific time points (minutes-hour).

Immunofluorescence and imaging

Cells grown on gridded culture dishes and on polysine-coated cover slips were fixed with 3.0% formaldehyde-tris-buffer saline (TBS) for 10 min at room temperature (RT) and placed on ice. Antibody staining was performed as previously described [17]. The following primary antibodies were used: anti-γ-H2AX (07-164; Millipore), anti-phospho-TRF2 (Thr188) (07-737,

Millipore), anti-TRF2 (img-124A, IMGENEX), anti-actin (A4700, Sigma). After the incubation, cells were washed twice in PBS/0.05% Tween-20 for 5 min at RT, and incubated with secondary antibodies (Invitrogen; 1:1,000) for 1hr at RT. DNA was stained with 4, 6-diamidino-2-phenylindole (1:1000 in PBS) for 5 min at RT. Samples were imaged using a Hamamatsu Orca cooled CCD Camera. Images were analyzed using ImageJ software (NIH, Bethesda, MD).

Immunoblotting

HeLa 1.2.11 cells were grown on a 24-well plate were lysed with 100 μl hot 2X Laemmli buffer (Bio-Rad) supplemented with 6% SDS, followed by sonication. Proteins were resolved by 5% - 10% SDS-PAGE and transferred to a PVDF membrane. The membrane was blocked with 5% BSA for 1 h and incubated overnight with primary antibodies (1:1000), and secondary HRP using a 1:1000 mix (Promega).

Data Analysis

The statistical analysis was performed using a unpaired Students *t*-test to determine the significance of DDR protein recruitment on treated and untreated damaged cells ($p < 0.05$). Tests were obtained using GraphPad Prism version 6.00 (GraphPad Software Inc.).

Results

Double-stand breaks (DSBs) can be induced with a short pulse NIR femtosecond laser

The focal point of an 800 nm femtosecond laser is known to produce submicron lesions in interphase and mitotic cells (17, 22, 23). To investigate whether the 800 nm femtosecond laser is

capable of producing DNA breaks, specifically DSBs, we investigate the recruitment of 53BP1 (p53-binding protein 1), a well-known repair protein required for DSB repair and checkpoint regulation (24). Stably expressing eGFP-53BP1 PtK2 cells were subjected to different irradiations doses to find the adequate dose for DSB induction. After microirradiation, cells were monitored via fluorescence microscopy for the recruitment of 53BP1. Our results show that at an irradiance of 2.43 e^{+11} W/cm^2, 53BP1 is rapidly accumulated (Figure 4.1A). On the other hand, at an irradiance of 2.65 e^{+11} W/cm^2 or higher, 53BP1 fails to accumulate at laser-induced DNA breaks (Figure 4.1B). This result suggests, that DSBs are laser-dose specific.

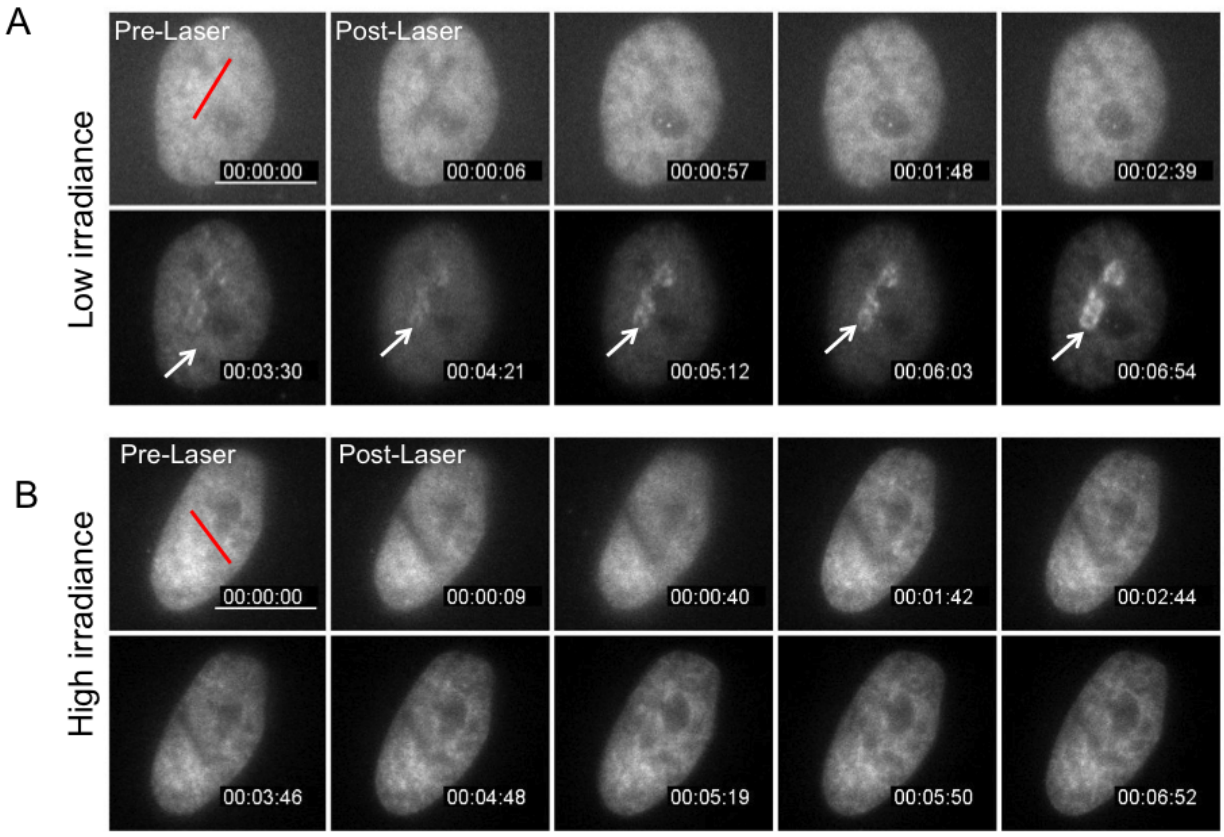

Figure 4.1 The near infrared femtosecond laser induces the recruitment of 53BP1 to DNA lesions. (A) Stably expressing eGFP-53BP1 cells before were subjected to laser microirradiation (pre-laser). Red line indicates the area where the laser strip is introduced (0 sec). DNA lesions were introduced at a low irradiance of 2.43 e^{+11} W/cm^2 (post-laser). White arrow shows recruitment of

53BP1 at laser induced damage site. (B) Stably expressing cell before laser microirradiation (pre-laser). DNA lesions were introduced at a high irradiance of 2.65 e^{+11} W/cm^2 (post-laser). Recruitment of 53BP1 is absent at this irradiance. Cells were followed 6 min post-laser. Scar bar 10 μm. N = 3 of independent experiments.

TRF2 does not accumulate at laser-induced DSBs

It is well known that TRF2 plays a role in DSB repair (11). To investigate the possible association of TRF2 with laser induced DSBs, we monitored the recruitment of YPF-TRF2 stably expressing cells via fluorescence microscopy after laser-induced DSBs at a low irradiance (2.43 e^{+11} W/cm^2). We observed that at this irradiance, TRF2 fails to accumulate (Figure 4.2A) as previously observed (13). However, when DNA breaks were introduced using a higher irradiance of 2.65 e^{+11} W/cm^2 (where 53BP1 fails to accumulate), a rapid accumulation of TRF2 is observed (Figure 4.2B). Furthermore, inhibition of ATM does not prevent TRF2 accumulation at this high irradiance (Figure 4.3). These results suggest that TRF2 accumulates to higher laser induced DNA lesions distinct from 53BP1.

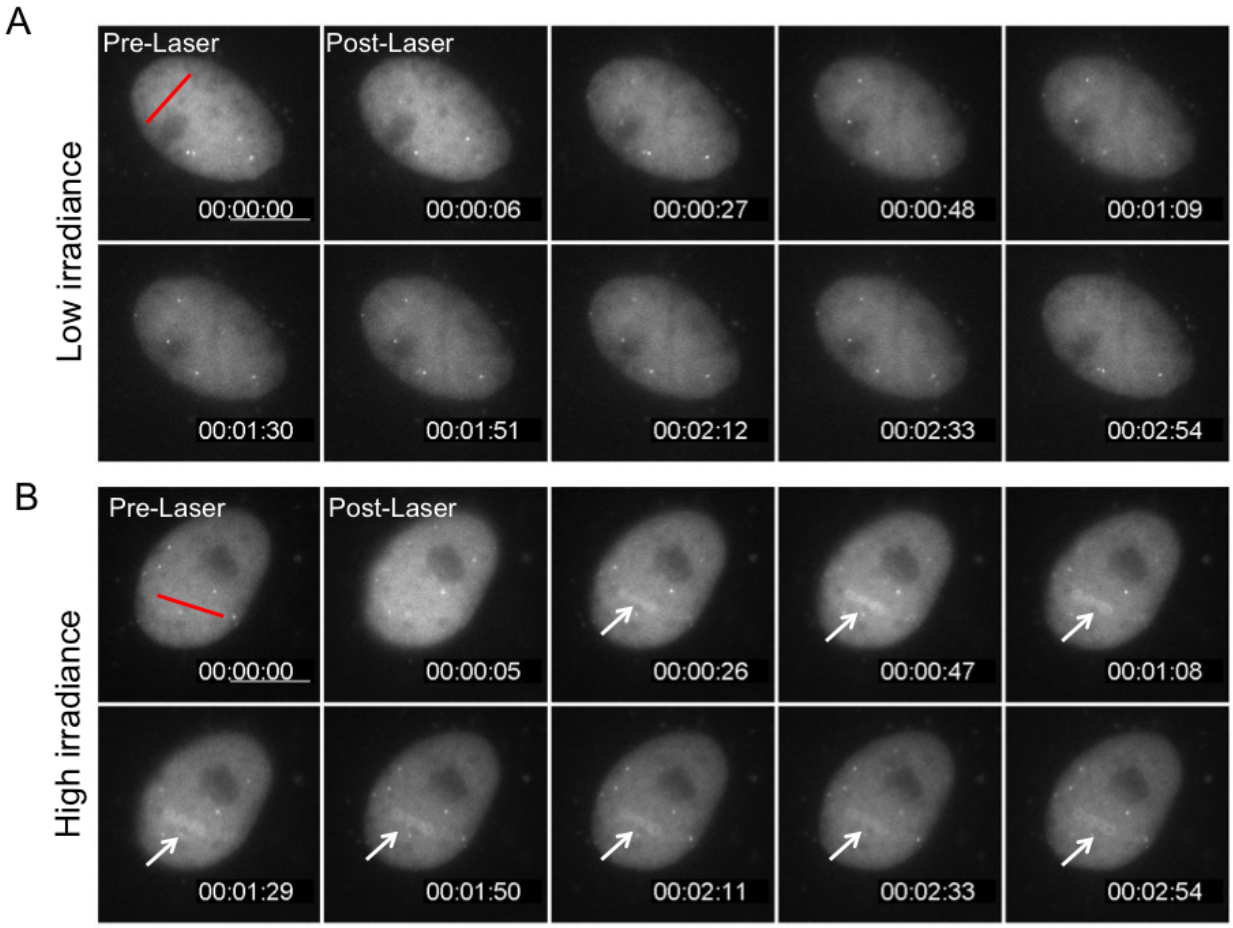

Figure 4.2 TRF2 recruits to high irradiance DNA lesions.
(A) Stably expressing YFP-TRF2 cells before laser microirradiation (pre-laser). DNA lesions were introduced at a low irradiance of of 2.43 e^{+11} W/cm^2 (post-laser). (B) Stably expressing cell before laser microirradiation (pre-laser). DNA lesions were introduced at a high irradiance of 2.65 e^{+11} W/cm^2 (post-laser). White arrow shows the recruitment of TRF2 at high irradiance lesions. Cells were monitored for 3 min post-laser. Scar bar 10 μm. N = 3 of cells were analyzed.

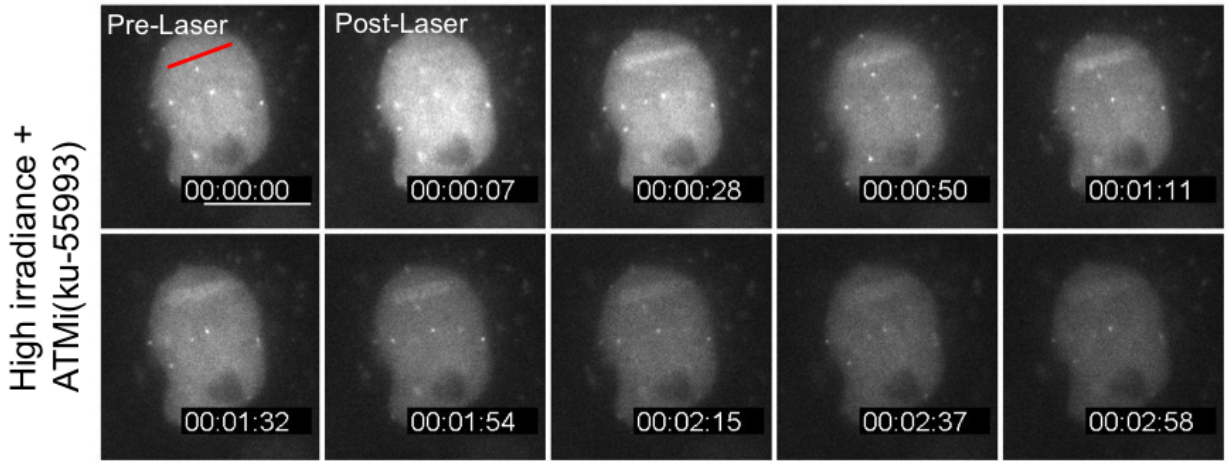

Figure 4.3 Recruitment of TRF2 is independent of ATM.
Stably expressing YFP-TRF2 cells were incubated 1 hr prior laser microirradiation with 10 m of ATM inhibitor (ATMi, ku-55993). Cell was monitored before laser microirradiation (pre-laser), and after damage using a high irradiance of 2.65 e^{+11} W/cm^2 (post-laser). Cell was monitored for 3 min. Scar bar 10 μm. N = 3 of cells tested.

Having established that TRF2 fails to accumulate at DSBs produced with a low irradiance (2.43 e^{+11} W/cm^2), we wanted to investigate whether phosphorylated TRF2 at threonine 188 had a similar response. We found the presence of phosphorylated TRF2 on threonine 188 at laser-induced DSBs at both low and high irradiance in PtK2 and HeLa 1.2.11 cells (Figure 4.4A, 4.4B, low). However, based upon the spread of phosphorylated H2AX (γH2AX) into the nucleus of PtK2 and HeLa1.2.11 cells, it appeared that the damage was more severe at the higher irradiance (2.65 e^{+11} W/cm^2) (Figure 4.4A, 4.4B, high).

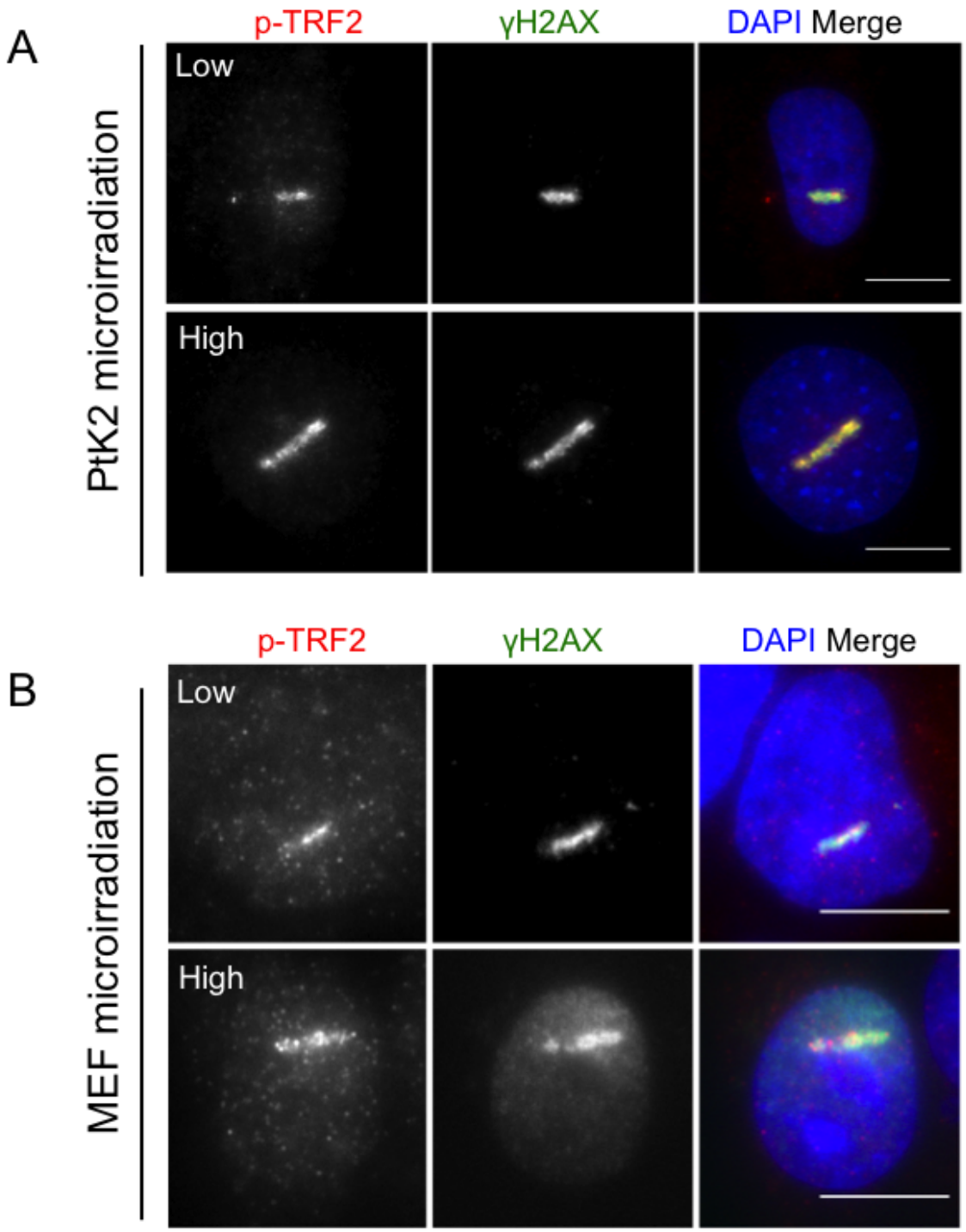

Figure 4.4 Low and high laser-induced breaks results in the phosphorylation of TRF2 on threonine 188.
(A) Ptk2 cells were irradiated at 2.43 e^{+11} W/cm^2 (low), and 2.65 e^{+11} W/cm^2 (high). (B) HeLa 1.2.11 cells were irradiated at 2.43 e^{+11} W/cm^2 (low), and 2.65 e^{+11} W/cm^2 (high). PtK2 and HeLa 1.2.11 cells were monitored for 10 min and fixed stained with phosphophorylated TRF2 on threonine 188 (p-TRF2) and DNA break marker anti-γH2AX and co-stained with DAPI. Scar bar 10 μm. N = 3 of cells tested.

ATM delays the kinetics of phosphorylated TRF2 at laser-induced DSBs

Recent evidence suggests that phosphorylation of TRF2 is dependent on ATM (15). To verify whether phosphorylated TRF2 is dependent on ATM when laser induced DSBs are produced by low irradiance, we used a small molecule inhibitor specific to ATM (ATMi). Cells were treated with ATMi 1 hr prior to laser microirradiation, and assessed by immunofluorescence. Immediate phosphorylation of TRF2 was observed at DSBs along with γH2AX in interphase cells (Figure 4.5A, 5 min, Figure 4.5C, N = 15). In contrast, Inhibition of ATM did not prevent TRF2 phosphorylation, but rather, delayed its kinetics (Figure 4.5B, 10 min, Figure 4.5C, N = 15) at laser induced DSBs. To investigate whether phosphorylation of TRF2 (Thr188) also is present at DSBs of mitotic cells in the presence or absence of ATM inhibition, unsynchronized mitotic PtK2 cells were damaged at one single chromosome site. The cells were fixed at specific time points (Figure 4.6A, 4.6B). Our results confirm the presence of phosphorylated TRF2 damaged chromosomes in mitotic cells had similar kinetics to treated (ATMi) and untreated interphase cells (Figure 4.6C). These results confirm that ATM inhibition suppresses the phosphorylation of TRF2 at laser-induced DSBs.

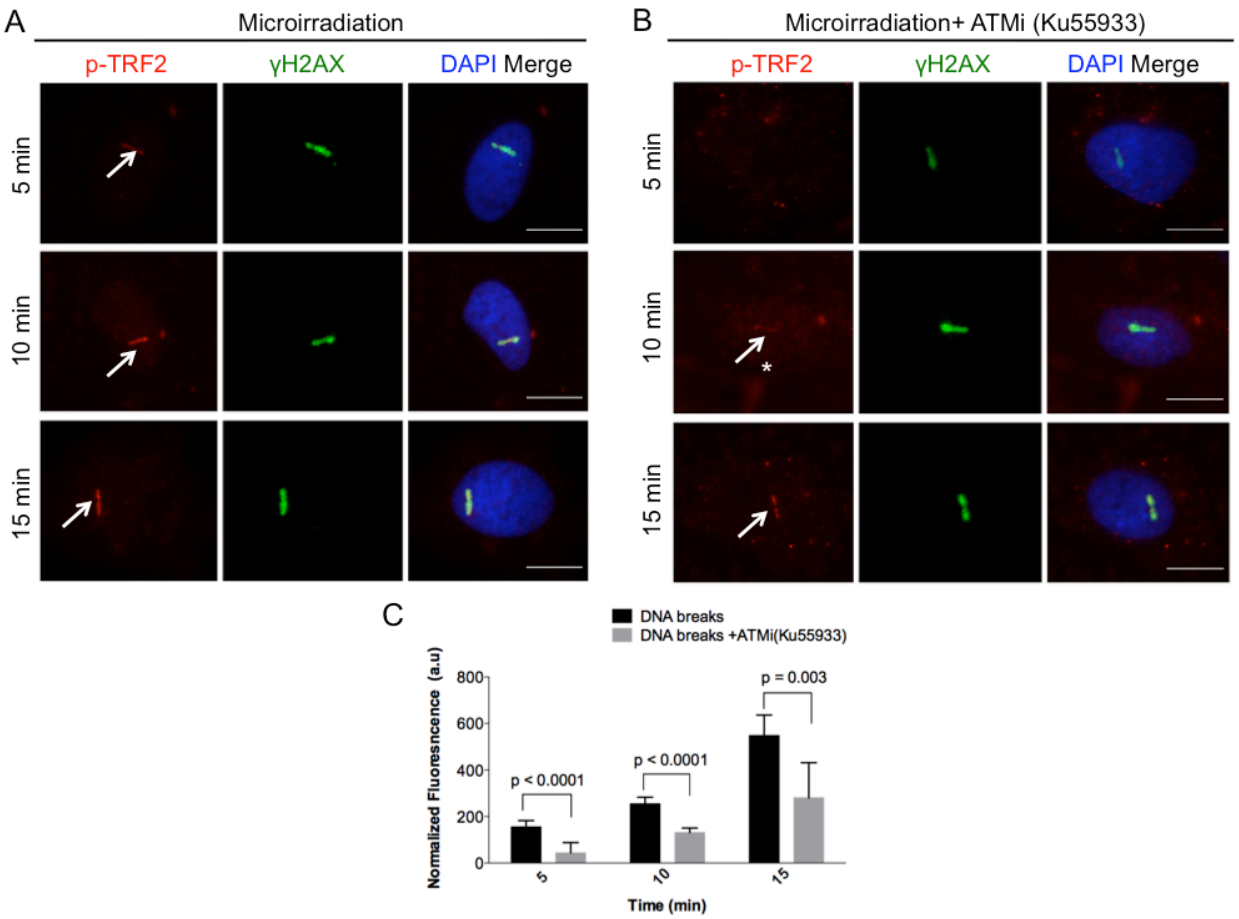

Figure 4.5 Inhibition of ATM delays phosphorylation of TRF2 on threonine 188 at low irradiance breaks on interphase cells.
(A) Interphase PtK2 cells were microirradiated, monitored at different time points (minutes) and fixed. (B) PtK2 cells were incubated 1 hr prior microirradiation with ATM inhibitor (ATMi, ku-55993). Samples were stained with phosphorylated TRF2 (p-TRF2) and DSB break marker anti-γH2AX and co-stained with DAPI. Scar bar 10 μm. N = 3 of cells tested. (C) Kinetics of normalized fluorescence of phosphoryated TRF2 (p-TRF2) of irradiated interphase cells treated and untreated with ATMi. Cells were microirradiated and microscopically monitored at specific time points and fixed (minutes).

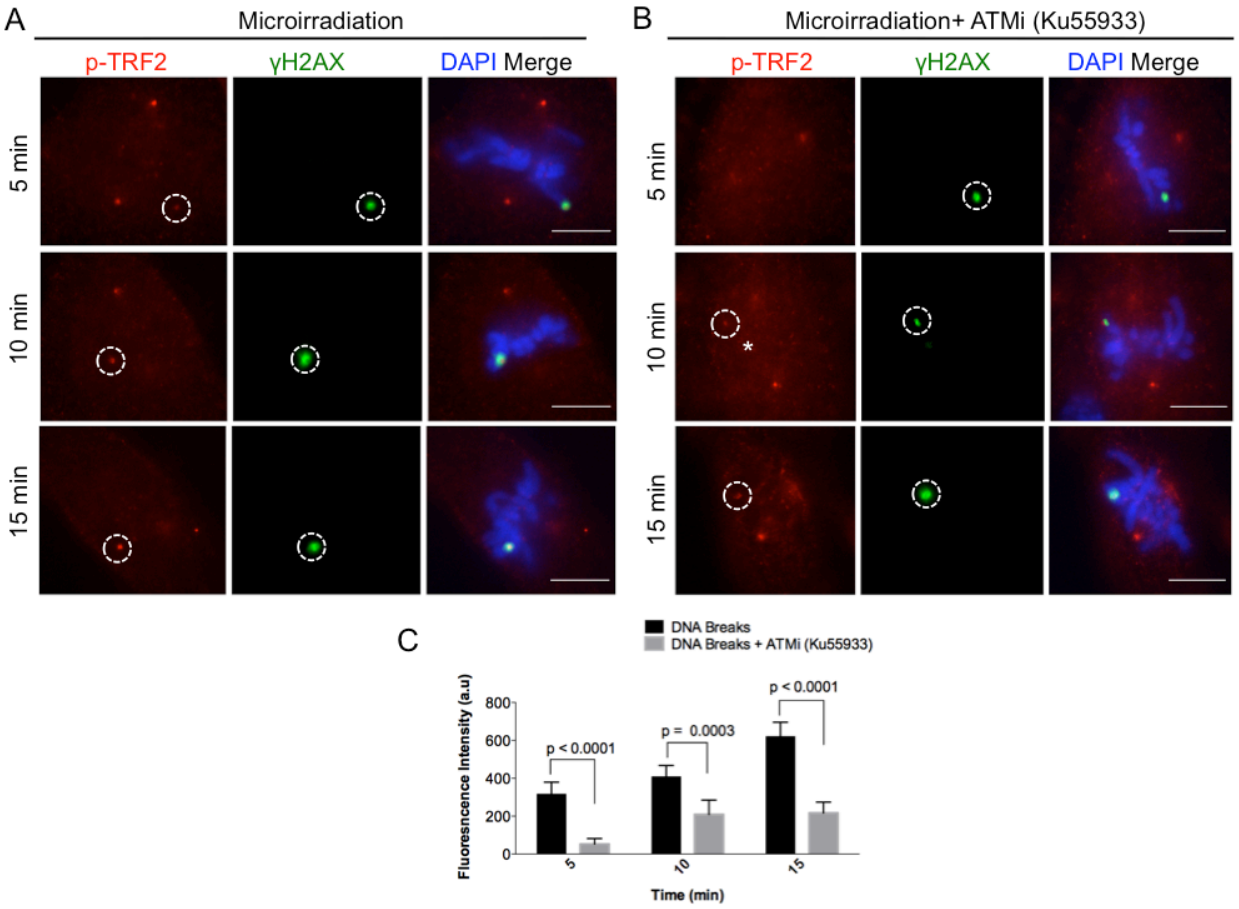

Figure 4.6 Inhibition of ATM delays phosphorylation of TRF2 on threonine 188 at low irradiance breaks of mitotic cells.
(A) Mitotic PtK2 cells were microirradiated, monitored at different time points (minutes) and fixed. (B) PtK2 cells were incubated 1 hr prior microirradiation with ATM inhibitor (ATMi, ku-55993). Samples were stained with phosphorylated TRF2 (p-TRF2) and DSB break marker anti-γH2AX and co-stained with DAPI. Scar bar 10 μm. N = 3 of cells tested. (C) Kinetics of normalized fluorescence of phosphoryated TRF2 (p-TRF2) of irradiated mitotic cells treated and untreated with ATMi. Cells were microirradiated and microscopically monitored at specific time points and fixed (minutes).

Kinetics of phosphorylated TRF2 is difficult to assess with γ-irradiation

Having established that phosphorylation of TRF2 forms damage foci at laser-induced DNA lesions of interphase and mitotic cells, we next examined whether γ-irradiation can be used as an alternative method to assess the kinetics of TRF2 of interphase and mitotic damaged cells.

Ionizing irradiation (IR) is known to produce DSBs, however, the kinetics of DDR factors are more difficult to assess compared to the kinetics following laser microirradiation. To assess TRF2 phosphorylation of interphase and mitotic cells, cells were irradiated, fixed and stained. We observed at irradiated interphase and mitotic cells that γH2AX increased with time (Figure 4.7A, 4.8A). However, phosphophorylation of TRF2 was minimal and did not co-localized with γH2AX. We also determined that γH2AX was delayed when ATM was inhibited. Although phsohpo-TRF2 did not form damage foci, either in interphase, or mitotic irradiated cells, our results revealed a delayed of endogenous accumulation of phospho-TRF2-Thr188 when ATM was inhibited (Figure 4.7B, 4.8B). Collectively, these results demonstrate the presence of phosphorylated TRF2 on threonine 188 at laser-induced DNA lesions in interphase and mitotic cells, but not at IR-induced lesions.

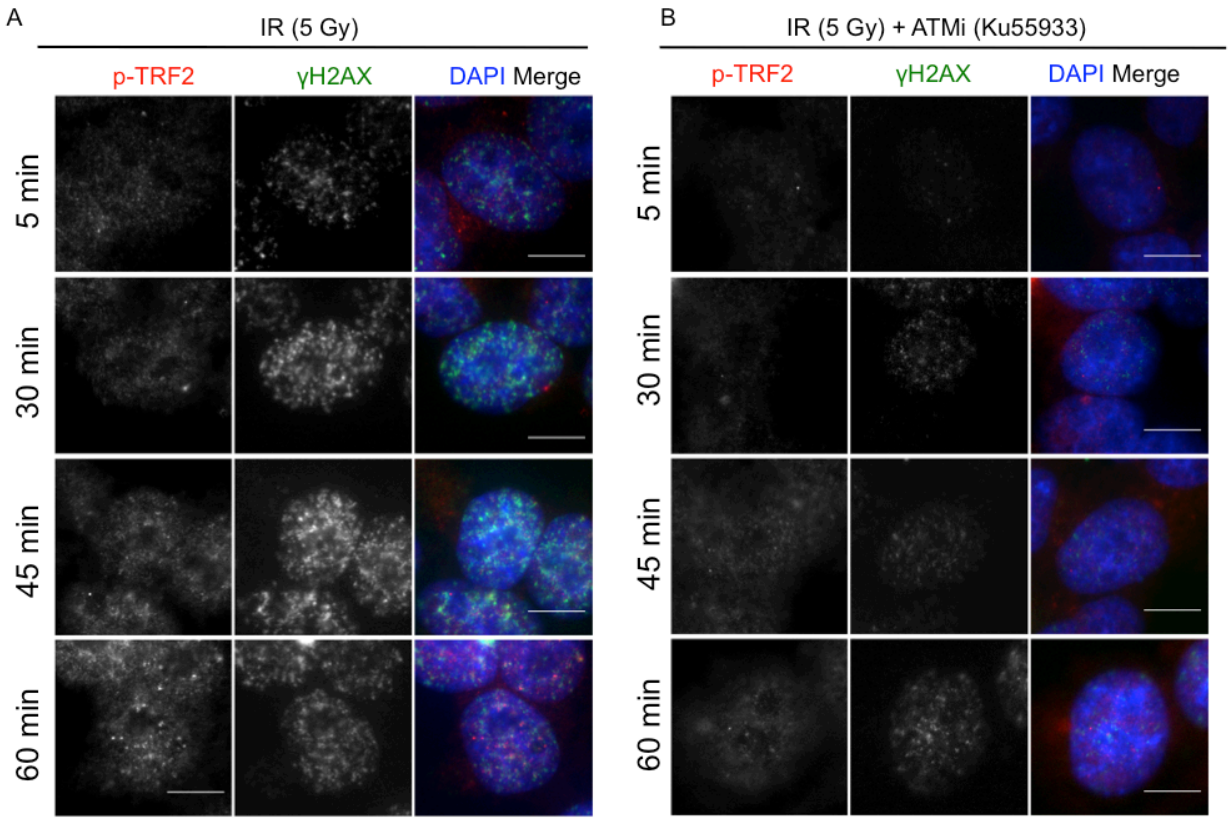

Figure 4.7 Gamma-irradiation of interphase cells does not result in phosphorylation of TRF2 at laser-induced DNA lesions.
(A) Interphase HeLa 1.2.11 cells were irradiated, monitored at different time points (minutes) and fixed. (B) HeLa 1.2.11 cells were incubated 1 hr prior microirradiation with ATM inhibitor (ATMi, ku-55993). Damage cells were microscopically monitored at specific time points and fixed (minutes). Samples were stained with TRF2 (Thr188) and DSB break marker anti-γH2AX and co-stained with DAPI. Scar bar 10 μm. N = 3 of cells tested.

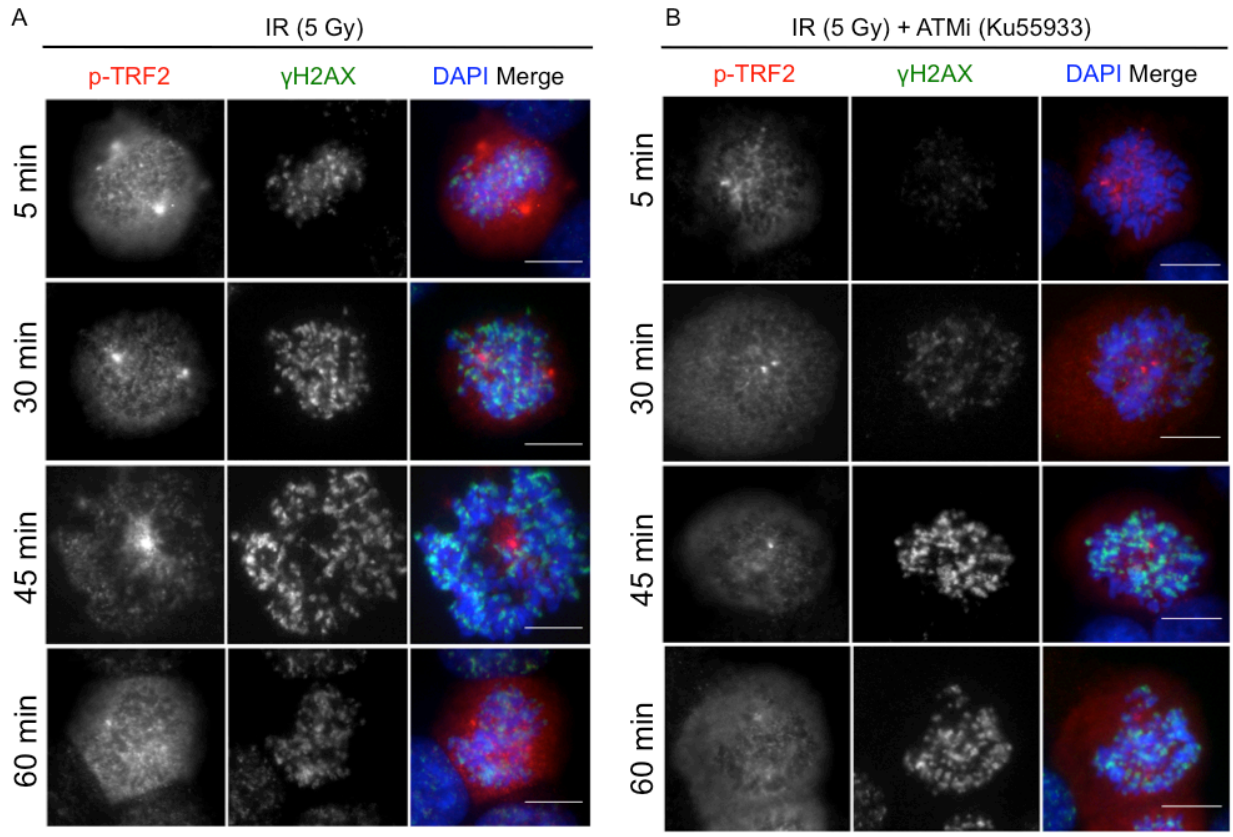

Figure 4.8 Gamma-irradiation of mitotic cells does not induce prominent phosphorylated TRF2 foci.
(A) Interphase HeLa 1.2.11 cells were irradiated, monitored at different time points (minutes) and fixed. (B) HeLa 1.2.11 cells were incubated 1 hr prior microirradiation with ATM inhibitor (ATMi, ku-55993). Damage cells were microscopically monitored at specific time points and fixed (minutes). Samples were stained with phosphorylated TRF2 (p-TRF2) and DSB break marker anti-γH2AX and co-stained with DAPI. Scar bar 10 μm. N = 3 of cells tested.

Discussion

Recently, it has been shown that the NIR femtosecond laser can be used as a tool to generate DNA lesions such as single strand breaks (SSBs) and double strand breaks (DSBs) in a submicron focal spot of mitotic chromosomes (17, 22). This approach permits live monitoring of

DDR and repair factors without damaging the cell membrane. However, in many studies using the laser microbeam approach to study DDR, whether using the short-pulsed NIR laser, or other lasers, specific parameter details are not provided, such a pulse duration, irradiance (W/cm2), and diameter of the focal spot (25).

Despite these deficiencies, laser microirradiation is an effective alternative tool to study DNA repair mechanisms in cells. One protein shown to accumulate to non-telomeric DNA in response to laser damage and which plays a role in DDR besides protecting chromosome ends is TRF2. However, the specificity for the type of DNA damage that TRF2 is recruited to, is still in question. Nonetheless, TRF2 is phosphorylated in response to DSB damage at threonine 188 and its phosphorylation is dependent on the activity of ATM.

In the present study, we used a NIR femtosecond laser combined with immunofluorescence to introduce DNA lesions and to monitor the recruitment of eGFP-53BP1 and YFP-TRF2. We found that using the NIR femtosecond laser at a dose of 2.43 e^{+11} W/cm^2 is sufficient to introduce DSBs in PtK2 cells. On the other hand, when using a laser dose of 2.65 e^{+11} W/cm^2 or higher, 53BP1 fails to accumulate. Furthermore, our studies show that the higher laser dose (2.65 e^{+11} W/cm^2) is necessary for TRF2 to accumulate at laser-induced DNA lesions. This result supports a TRF2's role in DDR as well as its role in telomere protection. Furthermore, the results suggest that TRF2 is not only recruited to DSBs, but also to multiple types of DNA lesions. Even at a higher irradiance it has being shown that the mechanism of damage is via a nonlinear multiphoton process at the focal spot (23, 26). It is well known that at higher laser irradiances, the types of lesions produced in cells are DSB, SSBs and base modifications (23, 25). This could explain why TRF2 only accumulates at high irradiance laser breaks, suggesting that it

requires multiple types of lesion. Further study is necessary to specifically test what type of DNA damage activates TRF2 recruitment.

Despite the lack of accumulation of TRF2 at the lower irradiance, our results suggest that low and high irradiances result in the phosphorylation of TRF2 (Thr188) in damaged interphase and mitotic cells. We also found that ATM inhibition does not prevent phosphorylation ofTRF2 (Thr188), but delays its recruitment to laser-induced DNA lesions. On the other hand, IR fails to form damaged foci of phosphorylated TRF2 (Thr188) in mitotic and interphase cells. This suggests that in order for phosphorylated TRF2 (Thr188) to form damaged foci, a high volume of breaks is required in a confined focal spot. The results reported here suggest that by titrating the laser dose of the femtosecond NIR laser, specific DNA lesions can be created such as DSBs and SSBs. Factors such as TRF2 require a larger amount of damage in the focal spot in order to be recruited. The results of this study further suggest that laser microirradiation can be used as a tool to study the kinetics of DDR associated proteins that are difficult to assess using conventional ionizing irradiation.

Conclusion

A microscope-focused 800-nm fs NIR laser can be used to introduce multiple types of DNA lesions by varying the irradiation dose. A low NIR irradiance of 2.43 e^{+11} W/cm^2 is sufficient to induce DSBs as assayed by the recruitment of 53BP1. However, TRF2 fails to accumulate at DNA lesions at this dose, but is recruited when a higher NIR irradiance of 2.65 e^{+11} W/cm^2 is used. Furthermore, phospho-TRF2-Thr188 forms damaged foci at high and low irradiance in interphase and mitotic cells, however, phospho-TRF2-Thr188 does not form damage foci when ionizing radiation is used as an alternative method to induce DNA damage. These results

demonstrate that laser microirradiation can be used to study the kinetics of DDR and repair factors, however careful attention must be given to laser dose.

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Chapter 5 Summary and Conclusions

There is considerable research on the DNA damage response (DDR) of interphase cells, but little is known about the DDR during mitosis, especially at telomeres. It is well known that uncapped mitotic telomeres are able to activate the upstream components of the DDR, resulting in the phosphorylation of H2AX and the recruitment of MDC1 (1). In addition, it was recently found that interphase telomeres lack a repair mechanism, since they show persistent accumulation of γH2AX damage foci (2). However, future studies need to confirm the lack of repair of mitotic uncapped telomeres by using an alternative assay that will allow the detection of newly synthesized DNA, such as bromodeoxyuridine (Brdu) incorporation assay. In addition, there is limited information on whether damaged telomeres are able to recruit downstream components of the DDR, and repair factors, while the cell is in mitosis.

In this thesis, I have investigated the signaling and molecular mechanisms associated with DSBs, and the effect of their damage at telomere-containing chromosome ends in mitotic marsupial PtK2 (*Protorous tridactylus*) cells. A focused femtosecond NIR (800 nm) laser microirradiation system was used to produce submicron DNA lesions on a single chromosome of a dividing cell. The recruitment of DDR factors and repair proteins were assessed by using immunofluorescence or standard fluorescence microscopy. The results show that DNA lesions (DSBs and SSBs) can be introduced into specific chromosomal domains while the cells are in anaphase. Additional results show the presence of factors involved in cell cycle arrest such as Chk1, Chk2 and phospho-p53 at the chromosome ends. This was not observed by other researchers (3-5) while the cells are in anaphase. However, we also found that phosphorylated p53 is undetected at DNA lesions of internal chromosome regions (chromosome arms) during

anaphase. This result indicates that specific chromosomal domains activate the DDR differently, despite the production of the same amount of DNA damage. It also suggests that the DDR may be not fully activated at specific chromosomal domains, and provides further insight about the integrity of the chromosome ends and how they may respond differently to damage during the anaphase stage of mitosis. We believe that the difference in response may be due to the location of the DNA lesions—at the chromosome end as opposed an internal chromosome arm. This finding may relate to the fact that evidence suggests that telomeres are prone to damage which results in genomic instability [need reference here]. In conclusion, introducing DNA breaks to the telomere-containing chromosome end in anaphase cells, results in a unique response, which is different from chromosome arms.

Experiments were also performed where a single chromosome end containing a telomere (TIP) was microirradiated, and compared to microirradiated internal regions of the chromosome (Non-TIP), in mitosis. The goals of these experiments were to assess the activation of the DDR. In addition, we wanted to study the effect of DNA lesions, while the chromosomes were aligned at the metaphase plate. The results of these experiments demonstrate that damage to single chromosome ends (TIPs) as opposed to an internal chromosomal arm (Non-TIPs) results in the recruitment of specific signaling factors such as ATM, MDC1, FANCD2, and WRN. In addition, DNA lesions at TIPs result in a delay in mitotic progression that is dependent on the activity of ATM, Chk1 and the spindle assembly checkpoint (SAC) kinase, Mps1. It was further found, that cells with damaged TIPs transition into G1, but with unresolved damage that is often segregated into a single micronuclei. Cells with damaged Non-TIPs do not activate a mitotic checkpoint, and do not form micronuclei. These results suggest that specific chromosomal domains respond differently to damage. Our findings suggest a direct connection between telomere damage and

the activation of the SAC. How the SAC directly interacts with damaged telomeres is not well understood. However, there is evidence that suggest the direct connection between the DDR factors such as Chk1, which participate in the activation of the SAC protein components such as Burb1. This study investigated whether the activation of the DDR at chromosome ends containing telomeres plays a role in the activation of the SAC. The results show that inhibiting the main protein kinase ATM, Chk1 and one of the SAC components Mps1, can restore the anaphase delay caused by the selective damage of telomeres. This result connects the SAC in response to telomere damage. However, future studies need to address the direct activation of the DDR at damaged telomeres and the SAC. Also, future studies should investigate the proteins that interact and are responsible for linking the two signaling mechanism.

This research demonstrates the role of telomeres in mitosis—to protect the rest of the chromosome. If the telomere containing region of the chromosome is damaged it will result in the activation of the DDR leading to the missegregation of DNA, which may be the next step to aneuploidy and chromosome instability. Based on these results it is important to investigate whether specific chromosomal domains in non-marsupial mammalian cells respond similarly.

In summary, we found a specific recruitment of factors when cells where damaged during anaphase compared to those damaged in metaphase. Damaged anaphase chromosome ends showed phosphorylated p53, while chromosome arms did not. However, in metaphase, DNA lesions at both ends and arms resulted in phosphorylation of p53. This result suggests that specific stages of mitosis (prometaphase, methaphase, telophase) may have unique DDR at specific chromosomal domains.

Next, the kinetics of TRF2 was examined when the cells are microirradiated with the NIR laser. The results show that a higher laser dose results in the recruitment of TRF2, while a lower

dose recruits 53BP1. Furthermore, previous evidence suggests that TRF2 is phosphorylated at threonine 188 (phsopho-TRF2) in response to DSB damage (6). Using the femotosecond NIR laser we were able to study the kinetics of phosphophorylated TRF2 at mitotic chromosomes and in interphase cells. In addition, we found that inhibition of ATM does not abrogate phosphorylation of TRF2, but delays its accumulation at the damage sites. In addition, I have shown that laser microirradiation, as opposed as γ-irradiation, can be used as a method to study the kinetics of DDR and repair proteins using fluorescence microscopy either by antibody staining or by fusion protein expression. These experiments provide insights into the functionality of the NIR at difference laser doses, which can be use to study the activation of DDR and repair pathways. However, future studies should address the specificity of the pathways activated by the NIR.

In addition, future studies should pursue that mechanism of micronuclei formation in response to telomere damage by inhibiting DDR factors using shRNA or small molecule inhibitors. This will identify potential DDR factors or repair proteins involved in the process of micronuclei formation. Furthermore, it will be important to determine whether there is a direct signaling between the kinetochors and telomeres, which may also lead to micronuclei formation when the DDR is activated. These studies may involve the use of shRNA specific for kinetochore proteins, and will assess whether damaged telomere-containing chromosome ends have the ability to form micronuclei in the absence of kinetochore proteins.

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