The inhibition of checkpoint activation by telomeres does not involve exclusion of dimethylation of histone H4 lysine 20 (H4K20me2) [version 1; referees: awaiting peer review]

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Abstract
DNA double-strand (DSBs) breaks activate the DNA damage checkpoint machinery to pause or halt the cell cycle. Telomeres, the specific DNA-protein complexes at linear eukaryotic chromosome ends, are capped DSBs that do not activate DNA damage checkpoints. This “checkpoint privileged” status of telomeres was previously investigated in the yeast *Schizosaccharomyces pombe* lacking the major double-stranded telomere DNA binding protein Taz1. Telomeric DNA repeats in cells lacking Taz1 are 10 times longer than normal and contain single-stranded DNA regions. DNA damage checkpoint proteins associate with these damaged telomeres, but the DNA damage checkpoint is not activated. This severing of the DNA damage checkpoint signaling pathway was reported to stem from exclusion of histone H4 lysine 20 dimethylation (H4K20me2) from telomeric nucleosomes in both wild type cells and cells lacking Taz1. However, experiments to identify the mechanism of this exclusion failed, prompting our re-evaluation of H4K20me2 levels at telomeric chromatin. In this short report, we used an extensive series of controls to identify an antibody specific for the H4K20me2 modification and show that the level of this modification is the same at telomeres and internal loci in both wild type cells and cells lacking Taz1. Consequently, telomeres must block activation of the DNA Damage Response by another mechanism that remains to be determined.

Keywords
Fission yeast, H4K20me2, histone, methylation, DNA damage, checkpoint, telomere
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Author roles: Audry J: Data Curation, Formal Analysis, Investigation, Methodology, Validation, Visualization, Writing – Review & Editing; Wang J: Conceptualization, Data Curation, Investigation, Methodology, Validation, Visualization, Writing – Original Draft Preparation, Writing – Review & Editing; Eisenstatt JR: Investigation; Berkner KL: Conceptualization, Funding Acquisition, Methodology, Validation; Runge KW: Conceptualization, Funding Acquisition, Methodology, Project Administration, Resources, Supervision, Validation, Visualization, Writing – Original Draft Preparation, Writing – Review & Editing

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Introduction
Genome instability is a potentially lethal event for an eukaryotic cell, and a mutational force for genetic diseases such as cancer. DNA double-strand breaks (DSBs) can drive genome instability and are sensed by the DNA damage checkpoint, a defined set of evolutionarily-conserved proteins that bind the DSB to signal a pause or arrest of the cell cycle and recruit proteins to repair the DNA lesion. Telomeres, the physical ends of linear eukaryotic chromosomes, are specialized DSBs that suppress DNA damage checkpoint activation by an unknown mechanism(s), even though telomeres are bound by many of the DNA damage checkpoint proteins that signal cell cycle arrest. Carneiro et al. (Nature 467: 228–232) addressed this question using Schizosaccharomyces pombe cells that lack Taz1, the protein that binds to double-stranded telomere repeats. Telomeres in tas1Δ cells have single-stranded DNA regions that are bound by checkpoint and DNA repair proteins, but cells do not arrest. Immunoﬂuorescence co-localization results from Carneiro et al. indicated that the ortholog of the human DNA damage checkpoint protein 53BP1 (Crb2) found at DSBs was not recruited to telomeres. Crb2 can bind to dimethylated lysine 20 of histone H4 (H4K20me2) in nucleosomes. Carneiro et al. presented data that H4K20me2 was depleted near telomeres in wild type and tas1Δ cells, suggesting a mechanism for checkpoint suppression. Efforts to pursue this exciting result by ourselves and others failed. We therefore carefully re-evaluated the presence of H4K20me2 at different chromosomal loci, and found that H4K20me2 is not depleted near telomeres, indicating that checkpoint suppression occurs by some other mechanism(s).

Methods
Construction of the H4K20R strains
Wild type (yJRE20-1) and histone H4 lysine 20 mutant (yJRE21-1) strains were previously described and were constructed as follows: The 5’ flanking region, the H4 gene, and the 3’ flanking region of each histone H4 gene were separately cloned into a pFA vector 5’ of the selection marker (hhf1 into pFA6a arg3MX6; hhf2 into pFA6a his3MX6; hhf3 into pFA6a ura4MX6). Approximately 500 base pairs 3’ to the initial fragment was cloned 3’ of the selection marker to the appropriate vector. The inclusive distance between the last A for the histone H4 TAA stop codon and the first G in the Asc I site from the pFA6a marker was 441 bp from H4.1 to arg3’, 707 bp from H4.2 to his3’ and 464 bp from H4.3 to ura4’ (plasmid maps are included in Supplementary File 1). Each construct was verified by restriction enzyme digestion and DNA sequencing of the fragments. Site-directed mutagenesis was used to mutate lysine 20 to arginine (H4K20R) at each gene copy; for wild type strains, the site was left unmutated. The resulting mutant constructs were verified by enzyme digestion and capillary dye terminator dideoxyDNA sequencing at ACGT (ACGT, Inc., Germantown, MD) to confirm the codon change corresponding to H4K20 (examples of aligned sequences are available in Supplementary File 2). Linearized fragments containing the 5’ fragment, selectable marker, and 3’ fragment were separately transformed into FY1645 (hhf1, h+) or FY1646 (hhf2 and hhf3, h+). Confirmation of integration was done by restriction digestion and DNA sequencing of the PCR product of the H4 gene. The strains with hhf1 (h+) and hhf3 (h+) marked and/or mutated were crossed to generate a strain in which hhf2 is the only unmarked gene copy. The resulting h+ strain was then crossed with the hhf2 (h+) marked strain to generate a strain in which all three loci of the histone H4 gene contain a selectable marker and are either wild type or mutated to arginine at lysine 20. Confirmation via digestion and DNA sequencing was performed after each cross. The H4K20R strain has been previously shown to be sensitive to DNA damaging agents. The strains and primers used during strain construction are available upon request.

ChiP assay
The strains used are described in Table 1. For the control strains lacking H4K20 methylation created by transformation for these experiments, two (yJRE141) or three (JA008) independent transformants were independently assayed in parallel. Cells were grown at 32°C in 300 ml in EMMG + AHRULK (yJRE141-3 and yJRF141-6) or EMMG + AHRULK + G418 (All other cells. EMMG is described in Moreno et al. and AHRULK + G418 contains 225 mg/l adenine, histidine, arginine, uracil, leucine, lysine and 200 mg/l G418 sulfate). Mid-log cells (9-12 × 10^6/ml or 0.8-1.2 OD_600) were cross-linked with 1% formaldehyde for 15 min at room temperature and washed twice with cold HBS buffer (50 mM HEPES-NaOH pH 7.5, 140 mM NaCl). Cell pellets were stored at -80°C. All subsequent steps were performed at 4°C. Cell pellets were resuspended in ChiP-lysis buffer and lysed using mechanical disruption by bead-beater (Mini-Beadbeater-16, Bio Spec, Bartlesville, OK, USA) with 0.5 mm glass beads (11079105, Biospec) using 4 cycles of 45 sec followed by 60 sec on ice. The lysate was sonicated for 10 cycles on maximum power (30 sec ON and 59 sec OFF) in a Diagenode Bioruptor XL with sample tubes soaked in an ice water bath. Solubilized chromatin protein (2-4 mg) was used for each ChiP while 5 µl was saved as Input. Antibodies (2 µg) against H4K20me2 (GeneTex GT282 [RRID: AB_296888] Lot #GR133660-1) were mixed with chromatin and incubated at 4°C while rocking for 4 h. Dynabeads Protein G (50 µl, Cat. No. REF 10004D; Life Technologies Carlsbad, CA, USA) was then added and rocked overnight at 4°C. Beads were washed with ChiP lysis buffer, ChiP lysis buffer with 500 mM NaCl, Wash buffer and TE buffer (10 mM Tris, 1 mM EDTA pH 7.5) successively. Beads were then resuspended in 145 µl of TES (1X TE with 1% SDS). Supernatant (120 µl) was resuspended in TES buffer (115 µl) was added and incubated in the Thermo mixer at 65°C, 1000 rpm (rotation per min) overnight to reverse cross-linking. For Input samples, TES buffer (115 µl) was added and incubated in the Thermomixer with the ChiP samples. Samples were treated with RNase A (2 µl of 10mg/ml added to each sample) for 30 min at 55°C, and purified by QIAagen PCR purification column (Cat.No. 28106; Hilden, Germany). All samples from the same assay were processed for ChiP assay at the same time.
qPCR analysis for ChIP

Input samples were diluted to 1/100 with ddH₂O while beadsonly-ChIP, H4-ChIP and H4K20me2-ChIP samples were diluted to 1/10. Template DNAs (4 µl) were added to 5 µl of Roche LightCycler 480 SYBR Green I Master (2X) and primers (final concentration 0.6 µM) for a 10 µl total reaction volume. Each sample was run in triplicate on the same 384-well PCR plate (Roche LightCycler 480 Multiwell Plate 384, clear) in a Roche LightCycler 480. H4K20me2 immunoprecipitation levels were normalized to the total H4 immunoprecipitation levels at each locus (14-16). The primers used are shown in Table 2, all primers were custom syntheses purchased from Integrated DNA Technologies (Skokie, IL, USA). Each locus was assayed using two or three primer pairs in the same qPCR assay for each ChIP, and the results for all primer pairs for a specific locus were averaged to obtain the final ChIP signal. The level of H4K20me2 at each locus was calculated as a ratio of H4K20me2 ChIP level to H4 ChIP level, where each ChIP level is expressed as a percent of input chromatin in the immunoprecipitated DNA (i.e. amount of DNA in H4K20me2 ChIP H4K20me2/amount DNA in the input chromatin divided by amount of DNA in H4 ChIP/amount DNA in the input chromatin).

Cell extract preparation

Cells of 5 OD (5 × 10⁷ cells) were collected and resuspended in 200 µl SDS loading buffer without dye and reducing agent (50 mM Tris, 2% SDS, 10% glycerol). Cells were lysed using mechanical disruption by FastPrep 120 (Thermo Savant; Thermo-Fisher Scientific, Waltham, MA, USA) with 0.5 mm glass beads, in cold room, using 2 cycles of 40 sec of disruption followed by 60 sec on ice. Cell lysis efficiency, monitored under microscope, always reached a minimum of 90%. The lysate was collected by poking holes on the bottom of the tubes and spinning into new tubes at 1000 rpm for 1 min at 4°C. The protein concentration was measured by BCA assay (23225 Pierce, Thermo-Fisher Scientific, Waltham, MA, USA) on 96-well plate. After adding 4X SDS loading buffer, lysate of 10 µg was heated at 95°C for 5 min, spun down, and loaded into each lane on SDS-PAGE gel. The rest of the lysates were kept at -20°C.

Recombinant histone H4 preparation

Recombinant histone H4 (MLA-modified) H4K20me1 or H4K20me2 or H4K20me3 (31224, 31225, 31226; Active Motif®, Carlsbad, CA, USA) and unmodified recombinant histone H4 (Active Motif® 31223) were resuspended in PBS buffer.

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**Table 1. Yeast strains used in this stud.**

<table>
<thead>
<tr>
<th>Name</th>
<th>Genotype</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>yJR201-1</td>
<td>hr H4.1::arg3' H4.2::his3' H4.3::ura4' ade6-210 arg3A-4 his3A-1 leu1-32 urea-D18</td>
<td>This lab®, used for western as WT</td>
</tr>
<tr>
<td>yJR21-1</td>
<td>hr H4.1-K20R::arg3' H4.2-K20R::his3' H4.3-K20R::ura4' ade6-210 arg3A-4 his3A-1 leu1-32 urea-D18</td>
<td>This lab®, used for western as H4K20R</td>
</tr>
<tr>
<td>ySL298</td>
<td>hr set9::CYC-terminator-kanMX (set9- strain)</td>
<td>Greeson et al.®, used for western as set9-kan-wt</td>
</tr>
<tr>
<td>ySL252</td>
<td>hr set9::CYC-terminator-kanMX (set9-deletion strain)</td>
<td>Greeson et al.®, used for western as set9A</td>
</tr>
<tr>
<td>yNTG41</td>
<td>hr set9-F178Y::CYC-terminator-kanMX</td>
<td>Greeson et al.®, used for ChIP as set9-F178Y</td>
</tr>
<tr>
<td>yNTG39</td>
<td>hr set9-F164Y::CYC-terminator-kanMX</td>
<td>Greeson et al.®, used for ChIP as set9-F164Y</td>
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<tr>
<td>yNTG43</td>
<td>hr set9-F195Y::CYC-terminator-kanMX</td>
<td>Greeson et al.®, used for western as set9-F195Y</td>
</tr>
<tr>
<td>yJR210-1</td>
<td>hr ade6-210 arg3-D4 his3-D1 leu1-32::pFA-LEU2-I-SceI ura4-D18 gal1-3'::ura4-48bp TelORpt-I-SceI-hph*</td>
<td>This lab®, used for ChIP as wild type</td>
</tr>
<tr>
<td>JA002-3</td>
<td>taz1Δ::kanMX introduced into yJR210-1 by transformation</td>
<td>This work, used for ChIP as taz1Δ</td>
</tr>
<tr>
<td>JA008-1</td>
<td>set9Δ::kanMX introduced into yJR210-1 by transformation</td>
<td>This work, used for ChIP as set9A</td>
</tr>
<tr>
<td>JA008-2</td>
<td>set9Δ::kanMX in yJR210-1, independent transformant from JA008-1</td>
<td>This work, used for ChIP as set9A</td>
</tr>
<tr>
<td>JA008-3</td>
<td>set9Δ::kanMX in yJR210-1, independent transformant from JA008-1 and JA008-2</td>
<td>This work, used for ChIP as set9A</td>
</tr>
<tr>
<td>yJR141-3</td>
<td>hr ade6-210 arg3-D4 his3-D1 leu1-32::pFA-LEU2-TETp-I-SceI ura4-D18 hhh1K20R::arg3'- hhh2K20R::his3'- hhh3K20R::nat'- leu1-32::pFA-LEU2-TETp-I-SceI gal1-3'::ura4-48 bp TelORpt-I-SceI-hph*</td>
<td>This lab®, used for ChIP as H4K20R</td>
</tr>
<tr>
<td>yJR141-6</td>
<td>Independent isolate of yJR141-3</td>
<td>This lab®, used for ChIP as H4K20R</td>
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</table>
(in HPLC grade water) and used at the working concentration of 50 ng/μl except for H4K20me3 which was at 2.5 ng/μl. After adding 4X SDS loading buffer, 200 ng of recombinant histone H4 was heated at 95°C for 5 min, spun down, and loaded into each lane on SDS-PAGE gel. The rest of the proteins were stored at -20°C.

**Western analysis**

SDS-PAGE gels were prepared with a 15% resolving gel and a 4% stacking gel using 40% Acrylamide/Bis solution (BioRad 161-0146), Tris buffer and SDS. The gel was run in 1x SDS-Glycine-Tris running buffer with Odyssey One-Color Molecular Weight Protein Marker (Li-Cor 928-40000). The gel was run in 1x SDS-Glycine-Tris running buffer with Odyssey One-Color Molecular Weight Protein Marker (Li-Cor 928-40000). The proteins were transferred onto nitrocellulose membrane (Li-Cor 926–31092) using Genie transfer system for 1 h with 1X transfer buffer with 20% methanol and 0.05% SDS. The membrane was stained with Ponceau S and the blot above the 25 kDa marker band was removed. The cut membrane was then rocked with Odyssey blocking buffer (Li-Cor 927-40000) at room temperature for 1 h, followed by incubation with anti-H4K20me2 antibodies (GeneTex GT282 [RRID: AB_2728656] Lot #41582) diluted 1:2000 in Odyssey blocking buffer with 0.2% Tween-20 at 4°C overnight. Anti-H4 antibody (Abcam ab10158 [RRID: AB_296888] Lot #GR133660-1) was diluted at 1:10000 in Odyssey blocking buffer with 0.2% Tween-20. The anti-H4K20me2 blot was treated with the secondary antibody 680RD Goat anti-Mouse IgG (Li-Cor 926-68070 [RRID: AB_10956588]) in Odyssey blocking buffer with 0.2% Tween-20 at room temperature and rocked for 1 h and kept away from light during the incubation. For anti-H4 blots, the secondary antibody was Goat anti-Rabbit antibody IgG (800CW Li-Cor 926-32211 [RRID: AB_621843]). The blots were scanned by the Odyssey® CLx Imaging system to acquire Western blot signal and analyzed with the Image Studio™ software (v. 4.0).

### Results and discussion

If the DNA damage checkpoint at telomeres is severed by excluding H4K20me2 from telomeric chromatin, the presence of H4K20me2 in telomeric nucleosomes would activate the DNA damage response in \textit{taz1\Delta} cells, causing slower growth or cell cycle arrest. H4K20me2 constitutes ~25% of total H4 in \textit{S. pombe} 19, implicating a telomere-associated demethylase to deplete H4K20me2 at telomeres in \textit{taz1\Delta} cells.
to prevent checkpoint-mediated arrest. However, both a genome-wide screen of gene deletion mutants (D. Durocher, pers. comm.) and our screen of demethylase mutants failed to identify a mutant that caused taz1Δ cells to arrest, to grow poorly or to recruit more Crb2. We therefore re-evaluated H4K20me2 levels by chromatin immunoprecipitation (ChIP). We first identified commercial antibodies specific for H4K20me2 by western analysis using 11 different samples. Positive controls were extracts from wild type cells, cells where the single S. pombe H4K20 methylase gene set9 is marked and functional (set9-kan-wt) and recombinant H4 where the only modification is a chemical mimetic for K20me2. Negative controls included recombinant H4 where the only modifications were mimetics of 0, 1 or 3 methyl groups on lysine 20, and extracts from cells where all three copies of the H4 gene have lysine 20 mutated to arginine (H4K20R). A series of set9 mutants that methylate H4K20 to contain 0 (set9Δ), 1 (set9-F164Y, set9-F178Y), or 1 and 2 methyl groups (set9-F195Y) were also assayed. The specific antibody identified (Figure 1A) was used in ChIP to monitor H4K20me2 at the telomeric loci assayed in Carneiro et al. and two internal chromosomal loci in wild type and taz1Δ cells, and in mutants that lack H4K20 methylation, set9Δ and H4K20R. Total H4 levels at these loci were monitored with an antibody that recognizes all H4 forms.

We found that H4K20me2 levels are similar at all three loci in wild type and taz1Δ cells, and clearly distinguishable from the set9Δ and H4K20R negative controls (Figure 1B). These data argue that while the damaged telomeres in taz1Δ cells block checkpoint activation, the mechanism is unlikely to be the suppression of H4K20me2 in telomeric chromatin. These results and conclusion are consistent with the genetic screen results that did not identify a demethylase required to sever the checkpoint in taz1Δ cells and suggest that searches for combinations of demethylase mutants that activate the checkpoint in taz1Δ cells will not be fruitful. Rather, broader approaches to investigate the differences between telomeres and DSBs may be required, including much more extensive characterization of the post-translation modifications of proteins at or near telomeres. While H4K20me2 levels are not reduced at telomeres, it is worth noting that checkpoint activation is the sum of multiple protein interactions and modifications, e.g. phosphorylation of histone H2A and modification of several checkpoint proteins. Reducing the...

Figure 1. H4K20me2 is not excluded from the telomere repeat-adjacent nucleosomes in wild type or taz1Δ cells. An antibody that specifically recognizes H4K20me2 was identified (A) and used in ChIP to measure levels of H4K20me2 in chromatin at two standard internal loci and loci adjacent to the telomere repeats (B). H4K20me2 levels are expressed as the ratio of the H4K20me2 ChIP levels (% of DNA in anti-H4K20me2 IP compared to input chromatin) over H4 ChIP levels (% of DNA in anti-histone H4 IP compared to input chromatin). Wild type and taz1Δ cells have the same levels at all loci and are clearly distinguishable from the negative controls (P values compared to wild type levels; all taz1Δ strains > 0.18; all set9Δ and H4K20R strains < 0.023, individual values are presented in Table 3). Each western blot in panel A used a separate, identically run gel to analyze the samples shown. M stands for molecular weight markers.
efficiency of some of these interactions may be sufficient to impair checkpoint signaling at \textit{taz1}∆ cell telomeres. Results from such studies may provide an understanding of the anti-checkpoint activity of telomeres so that it may be modulated to treat telomere-related diseases such as cellular aging and cancer\textsuperscript{23}.

**bioRxiv**
A previous version of this article is available from bioRxiv - [https://doi.org/10.1101/251389\textsuperscript{24}]

**Data availability**
Dataset 1: unedited blot images [10.5256/f1000research.15166.d209374\textsuperscript{25}]

Dataset 2: Excel workbook containing the Ct values from the PCRs and the location of the primers within the genes and telomere repeat adjacent DNA. [10.5256/f1000research.15166.d209375\textsuperscript{26}]

**Competing interests**
No competing interests were declared

**Grant information**
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The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

**Table 3. P values for H4K20me2 levels compared to wild type in Figure 1.** Assays were performed as described in Materials and Methods with two or more independent ChIP experiments. Each ChIP experiment was analyzed in triplicate. \(P\) values are from t-tests comparing each locus in a mutant strain to the same locus in the wild type strain, where values <0.05 are considered significant.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Locus</th>
<th>(P)</th>
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<tbody>
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<td>\textit{taz1}∆</td>
<td>Internal locus \textit{act1}</td>
<td>0.8913</td>
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<tr>
<td>\textit{taz1}∆</td>
<td>Internal locus \textit{hip3}</td>
<td>0.3377</td>
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<td>\textit{taz1}∆</td>
<td>Telomere: adjacent to telomere repeats</td>
<td>0.1842</td>
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<tr>
<td>\textit{set9}∆</td>
<td>Internal locus \textit{act1}</td>
<td>0.0082</td>
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<tr>
<td>\textit{set9}∆</td>
<td>Internal locus \textit{hip3}</td>
<td>0.0137</td>
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<tr>
<td>\textit{set9}∆</td>
<td>Telomere: adjacent to telomere repeats</td>
<td>0.0213</td>
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<tr>
<td>\textit{H4K20R}</td>
<td>Internal locus \textit{act1}</td>
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<td>Internal locus \textit{hip3}</td>
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<tr>
<td>\textit{H4K20R}</td>
<td>Telomere: adjacent to telomere repeats</td>
<td>0.0220</td>
</tr>
</tbody>
</table>

**Supplementary material**
Click here to access the data.

Supplementary File 2: Examples of plasmid sequences used to validate mutation
Click here to access the data.
References


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