RESEARCH NOTE

REVISED The inhibition of checkpoint activation by telomeres does not involve exclusion of dimethylation of histone H4 lysine 20 (H4K20me2) [version 2; peer review: 2 approved, 1 not approved]

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Abstract

DNA double-strand breaks (DSBs) 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 those 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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REVISED **Amendments from Version 1**

This version was revised to include data that rebut the objections of Ferreira and Nakamura, the two senior authors of the Carneiro *et al.* 2010 paper that claimed that H4K20me2 is excluded from *Schizosaccharomyces pombe* telomeres and we show is not the case. Due to an editorial error the link to dataset 2 in the version 1 article was not made available. Thus, we include the original Dataset 2 again in this version 2, for readers to see. Additionally, we provide Dataset 3 which contains the original dataset 2 along with two new spreadsheets which show that normalizing H4 ChIP data to input chromatin produces an artifact of low H4 levels for telomeric chromatin fragments. We also add a Supplementary Figure 1 showing that the available lot of Abcam ab9052 anti-H4K20me2 antibody has issues that make it a poor choice for ChIP compared to the Gentex GT282 antibody that we characterized and used.

See referee reports

Editorial note:

The link to dataset 2 in the version 1 paper was not provided due to an editorial error. Thus, we now include below the original dataset 2 for readers to see (labelled Dataset 2), alongside the revised dataset 2 provided for the version 2 article (labelled Dataset 3).

Introduction

Genome instability is a potentially lethal event for a 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[1] and recruit proteins to repair the DNA lesion[2,3]. 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[4]. 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[5]. Telomeres in *taz1Δ* cells have single-stranded DNA regions that are bound by checkpoint and DNA repair proteins, but cells do not arrest[5,6]. 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[5]. Crb2 can bind to dimethylated lysine 20 of histone H4 (H4K20me2) in nucleosomes[7]. Carneiro *et al.* presented data that H4K20me2 was depleted near telomeres in wild type and *taz1Δ* cells, suggesting a mechanism for checkpoint suppression[5]. 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[8] 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*$^-$)[9]. 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[8]. 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.*[10] 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[11] and lysed using mechanical disruption by bead-beater (Bio Spec Mini-Beadbeater-16) with 0.5 mm glass beads (Biospec 11079105) 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_2728656] Lot #41582) or total histone H4

Table 1. Yeast strains used in this study.

Name	Genotype	Source
yJRE20-1	*h⁻ H4.1::arg3⁺ H4.2::his3⁺ H4.3::ura4⁺ ade6-210 arg3Δ-4 his3Δ-1 leu1-32 ura4-D18*	This lab[8], used for western as *WT*
yJRE21-1	*h⁻ H4.1-K20R::arg3⁺ H4.2-K20R::his3⁺ H4.3-K20R::ura4⁺ ade6-210 arg3Δ-4 his3Δ-1 leu1-32 ura4-D18*	This lab[8], used for western as *H4K20R*
ySLS298	*h⁻ set9::CYC-terminator-kanMX* (*set9⁺* strain)	Greeson *et al.*[12], used for western as *set9-kan-wt*
ySLS252	*h⁻ set9Δ::CYC-terminator -kanMX* (*set9*-deletion strain)	Greeson *et al.*[12], used for western as *set9Δ*
yNTG41	*h⁻ set9-F178Y::CYC-terminator -kanMX*	Greeson *et al.*[12], used for western as *set9-F178Y*
yNTG39	*h⁻ set9-F164Y::CYC-terminator -kanMX*	Greeson *et al.*[12], used for western as *set9-F164Y*
yNTG43	*h⁻ set9-F195Y::CYC-terminator -kanMX*	Greeson *et al.*[12], used for western as *set9-F195Y*
yJRE210-1	*h⁺ ade6-210 arg3-D4 his3-D1 leu1-32::pFA-LEU2-I-SceI ura4-D18 gal1-3'::ura4⁺-48bp TeloRpt-I-SceI-hph⁺*	This lab[13], used for ChIP as wild type
JA002-3	*taz1Δ::kanMX* introduced into yJRE210-1 by transformation	This work, used for ChIP as *taz1Δ*
JA008-1	*set9Δ::kanMX* introduced into yJRE210-1 by transformation	This work, used for ChIP as *set9Δ*
JA008-2	*set9Δ::kanMX* in yJRE210-1, independent transformant from JA008-1	This work, used for ChIP as *set9Δ*
JA008-3	*set9Δ::kanMX* in yJRE210-1, independent transformant from JA008-1 and JA008-2	This work, used for ChIP as *set9Δ*
yJRE141-3	*h⁻ ade6-210 arg3-D4 his3-D1 leu1-32 ura4-D18 hhf1K20R::arg3⁺ hhf2K20R::his3⁺ hhf3K20R::nat^R leu1-32::pFA-LEU2-TETp-I-SceI gal1-3'::ura4⁺-48 bp TeloRpt-I-SceI-hph⁺*	This lab[8], used for ChIP as H4K20R
yJRE141-6	Independent isolate of yJRE141-3	This lab[8], used for ChIP as H4K20R

(Abcam ab10158 [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, Life Technologies, Cat. No. REF 10004D) 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[11]. Beads were then resuspended in 145 µl of TES (1X TE with 1% SDS). Supernatant (120 µl) was recovered and incubated in a Thermomixer 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)(Roche 10109142001) for 15 min at 37°C and Proteinase K (2 µl of 20mg/ml added to each sample)(Roche 03115879001) for 30 min at 55°C, and purified by QIAgen PCR purification column (Cat.No. 28106)[14]. 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 ddH_2O while beads-only-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 immunoprecipitate levels were normalized to the total H4 immunoprecipitate levels at each locus[15–17]. 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 (5×10^7 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

Table 2. Primers used for qPCRs for ChIP. All primers were custom syntheses purchased from IDTdna.com.

Name	Sequence	Reference
79 act1 1-1Fw	TGC CGA TCG TAT GCA AAA GG	Oya *et al.*, 2013[18]
80 act1-1Rev	CCG CTC TCA TAC TCT TG	Oya *et al.*, 2013[18]
139 act1-2Fw	GCA AGC GTG GTA TTT TGA CC	This study
140 act1 2Rev	TCA GTC AAC AAG CAA GGG TG	This study
141 act1-3Fw	TAC CAC TGG TAT CGT CTT GG	This study
142 act1-3Rev	TAG TCA GTC AAG TCA CGA CC	This study
143 hip3-1Fw	AGC CAA ATT TGA CGG TGT TC	This study
144 hip3-1Rev	AGA CCT GGA CGG CAT TTT TA	This study
145 hip3-2Fw	GGT GCC AAG ATT GTT TAT CCA	This study
146 hip3-2Rev	ACG ACG TAT CCG ACA TCC TC	This study
147 hip3-3Fw	ACG ATG CCG AGT AGT TCA GC	This study
148 hip3-3Rev	TTC GTT GTT GTG TGC CTT TC	This study
135 Telo-1Fw	CAG TAG TGC AGT GTA TTA TGA TAA AAA TGG	Carneiro *et al.*, 2010[5]
136 Telo-1Rev	CAG TAG TGC AGT GTA TTA TGA TAA TTA AAA TGG	Carneiro *et al.*, 2010[5]
121 Telo-2Fw	TAT TTC TTT ATT CAA CTT ACC GCA CTT C	Kanoh *et al.*, 2005[19]
122 Telo-2Rev	CAG TAG TGC AGT GTA TTA TGA TAA TTA AAA TGG	Kanoh *et al.*, 2005[19]

mechanical disruption by FastPrep 120 (Thermo Savant) 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 (Pierce 23225) 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 (Active Motif® 31224, 31225, 31226) and unmodified recombinant histone H4 (Active Motif® 31223) were resuspended in PBS buffer (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 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. In some experiments, GT282 was replaced with Abcam ab9052 ([RRID:AB_1951942] lot #GR99672-1). 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).

Dataset 1. unedited blot images

http://dx.doi.org/10.5256/f1000research.15166.d209374

Dataset 2. Original excel workbook containing the Ct values from the PCRs and the location of the primers within the genes and telomere repeat adjacent DNA

https://dx.doi.org/10.5256/f1000research.15166.d220802

Dataset 3. Revised excel workbook containing the Ct values from the PCRs, the location of the primers within the genes and telomere repeat adjacent DNA and the ChIP values of total H4 normalized to input chromatin

https://dx.doi.org/10.5256/f1000research.15166.d219736

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 *taz1Δ* cells, causing slower growth or cell cycle arrest. H4K20me2 constitutes ~25% of total H4 in *S. pombe*[20], implicating a telomere-associated demethylase to deplete H4K20me2 at telomeres in *taz1Δ* 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[21]. 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)[8]. 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[12]. 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. We also tested the antibody Carneiro *et al.* reported using to detect H4K20me2, Abcam ab9052. We found that this lot of ab9052 did not recapitulate the reactivity of the original antibody reported by Greeson[12] for different *set9* mutants and showed reduced reactivity to H4K20me2 (Supplementary Figure 1) compared to the GT282 antibody (Figure 1A), so this antibody was not used. Total H4 levels at these loci were monitored with an antibody that recognizes all H4 forms.

We found that H4K20me2 levels are similar at telomeres and the two internal loci in wild type and *taz1Δ* cells, and clearly distinguishable from the *set9Δ* and H4K20R negative controls (Figure 1B). These results were obtained by normalizing H4K20me2 signals to total H4 signals, which allows the direct comparison of this H4 modification at loci which contain nucleosomes. Carneiro *et al.* normalized to their ChIP signals to total input chromatin[5]. However, because telomere repeats are

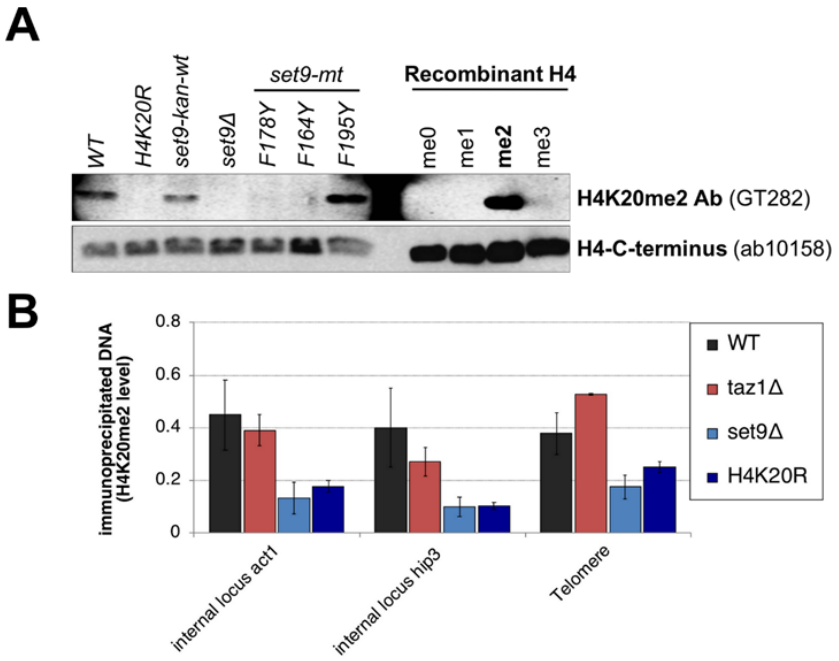

Figure 1. *H4K20me2* is not excluded from the telomere repeat-adjacent nucleosomes in wild type or *taz1*Δ cells. An antibody that specifically recognizes H4K20me2 was identified (**A**) and used in ChIP to measure levels of H4K20me2 in chromatin at two standard internal loci and loci adjacent to the 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.

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.

Strain	Locus	*P*
taz1Δ	Internal locus *act1*	0.8913
taz1Δ	Internal locus *hip3*	0.3377
taz1Δ	Telomere: adjacent to telomere repeats	0.1842
set9Δ	Internal locus *act1*	0.0082
set9Δ	Internal locus *hip3*	0.0137
set9Δ	Telomere: adjacent to telomere repeats	0.0213
H4K20R	Internal locus *act1*	0.0230
H4K20R	Internal locus *hip3*	0.0161
H4K20R	Telomere: adjacent to telomere repeats	0.0220

bound by non-nucleosomal proteins[22], this normalization gives a much lower ChIP signal for total H4 and, thus, a lower signal for all H4 modifications. An example of this lower H4 signal is shown in the third spreadsheet of Dataset 3, where the level of H4 at wild type telomeres is 1/5 to 1/9 that of internal loci. Therefore, normalization of H4K20me2 ChIP signals to total H4 is necessary to monitor the fraction of modified H4 at telomeres.

The results in Figure 1B 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[23,24]. Reducing the efficiency of some of these interactions may be sufficient to impair checkpoint signaling at *taz1Δ* 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[25].

bioRxiv

A previous version of this article is available from bioRxiv - https://doi.org/10.1101/251389[26]

Data availability

Dataset 1: unedited blot images 10.5256/f1000research.15166.d209374[27]

Dataset 2: Original 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.d220802[28]

Dataset 3: Revised excel workbook containing the Ct values from the PCRs, the location of the primers within the genes and telomere repeat adjacent DNA and the ChIP values of total H4 normalized to input chromatin. 10.5256/f1000research.15166.d219736[29]

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Supplementary material

Supplementary File 1: Plasmid maps. Files are in Snapgene Reader format, a free software package available here: http://www.snapgene.com/products/snapgene_viewer/

Click here to access the data.

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

Supplemental Figure 1. Western blot results with the ab9052 antibody.
Click here to access the data.

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http://www.doi.org/10.5256/f1000research.15166.d219736

Open Peer Review

Current Peer Review Status: ✗ ✓ ✓

Version 2

Reviewer Report 15 October 2018

https://doi.org/10.5256/f1000research.17976.r39273

✓ **Jeffrey S. Thompson**
Department of Biology, Denison University, Granville, OH, USA

Audry et al. examine a previously published observation (Carneiro et al., 2010[1]) that checkpoint activation is inhibited at telomeres as a result of exclusion of histone H4K20me2 at these loci. The prior observation was done via ChIP, using a polyclonal antibody from Abcam. As addressed in the present manuscript, the inability to identify a demethylase that is associated with this effect raised questions as to the certainty of the claim. The investigators of the current study have identified what appears to be a superior antibody to evaluate this modification. The antibody from GeneTex shows improved specificity for the dimethylated state relative to that observed for the previously utilized antibody from Abcam, and furthermore displays higher affinity for H3K20me2 relative to the currently available lot of the Abcam antibody.

The current study clearly demonstrates that H4K20me2 is detectable at telomeric loci, and that no differences are observed in taz1 mutant strains. The investigators took steps to normalize the ChIP data relative to total H4 levels to ensure that loci-specific variations in nucleosome levels were accounted for, which is completely appropriate for telomeric regions where histone levels are reduced relative to internal loci. Thus, the findings presented here raise further doubts about the role of H4K20me2 in checkpoint inhibition at telomeres.

This study provides useful insights into the nature of DNA damage checkpoint activation, and the underlying mechanisms by which cells inhibit such pathways in response to native chromosomal features such as telomeres. While the original finding, implicating reduced H4K20me2 levels at telomeres as an inhibitor of checkpoint activation, was supported by the previously published observations, it is essential that such models adapt as new information is made available. The use of antibodies for gauging histone modification levels has been perpetually problematic for many years, as these reagents are routinely used to evaluate subtle molecular changes across large genomic landscapes, pushing the limits of the methodology. In particular, lot variation for polyclonal antibodies is a serious problem in this field. In the absence of more reliable methodologies, we must be willing to reconsider prior observations as new antibodies become available. The study presented here appropriately re-examines this prior observation with an improved reagent for detecting this modification, and the observations raise reasonable doubts regarding the original model. This study by no means settles the issue of the role (or lack thereof) of H4K20me2 in checkpoint inhibition, but the findings merit publication to ensure that other investigators

continue to re-examine this model.

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PubMed Abstract | Publisher Full Text

Is the work clearly and accurately presented and does it cite the current literature?
Yes

Is the study design appropriate and is the work technically sound?
Yes

Are sufficient details of methods and analysis provided to allow replication by others?
Yes

If applicable, is the statistical analysis and its interpretation appropriate?
Yes

Are all the source data underlying the results available to ensure full reproducibility?
Yes

Are the conclusions drawn adequately supported by the results?
Yes

Competing Interests: No competing interests were disclosed.

I have read this submission. I believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

Version 1

Reviewer Report 07 August 2018

https://doi.org/10.5256/f1000research.16521.r35871

Holger Richly
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The DNA damage response involves the setting of specific chromatin marks that are important to recruit or maintain DNA repair factors at the damage site. One such mark is H4K20me2, which has been linked to doublestrand break repair (DSB repair) (Fradet-Turcotte 2013[1]) and more recently to nucleotide

excision repair (NER) (Chitale et al 2018[2]). H4K20me2 is read by 53BP1 or Crb1, respectivley, which bridges the interaction with other DNA repair proteins thereby playing an essential role in chromatin signaling events at the damage site. Telomeres can be considered specialized DSBs in that they suppress the DNA damage checkpoint activation. One potential mechanism to explain this phenomenon was put forward by Carneiro and colleagues[3]. In their research paper it was reported that 53BP1 (Crb2) and H4K20me2 do not locate to telomeres, which would partially explain why the DNA damage checkpoint activation was suppressed. However, this exciting finding was never recapitulated by other research groups.

Here, Audry and colleagues carefully reassessed the occurence of H4K20me2 at telomeres. To this end H4K20 mutant *S. pombe*strains were generated and most of the comercially available antibodies specifically recognizing the H4K20me2 mark were tested. Importantly, the authors found an antibody that shows the desired specificity when probing it against recombinant proteins and protein extracts from mutant strains. Employing this highly specific antibody in ChIP experiments they can unequivocally show that telomeres are decorated with H4K20me2, even in taz1 knockout cells, which is in stark contrast to the previous findings by Carneiro and colleagues. Thus, the findings presented here refute the idea that the DNA damage checkpoint is suppressed by exclusion of H4K20me2. This important finding will create a profound repercussion for the research field of genome stability as it shows that the mechanisms for checkpoint suppression at telomers have yet to be uncovered.

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Is the work clearly and accurately presented and does it cite the current literature?
Yes

Is the study design appropriate and is the work technically sound?
Yes

Are sufficient details of methods and analysis provided to allow replication by others?
Yes

If applicable, is the statistical analysis and its interpretation appropriate?
Yes

Are all the source data underlying the results available to ensure full reproducibility?
Yes

Are the conclusions drawn adequately supported by the results?

Yes

Competing Interests: No competing interests were disclosed.

I have read this submission. I believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

Reviewer Report 17 July 2018

https://doi.org/10.5256/f1000research.16521.r36001

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Carneiro et al. 2010 described a mechanism whereby telomeres can prevent cell cycle arrest even when Rad3-Rad26 (ATR-ATRIP) and Rad9-Rad1-Hus1 (9-1-1) checkpoint sensor complexes are massively accumulated at telomeres and activated, due to the elimination of telomere protein Taz1. We showed that telomeres in taz1Δ cells block Chk1 phosphorylation by limiting accumulation of Crb2 (53BP1 ortholog) at telomeres, and found that forced targeting of Crb2 to telomeres leads to Chk1 phosphorylation and cell cycle arrest in taz1Δ cells, but not in wild-type cells. In order to explain this phenomenon, we investigated status of the known recruiters of Crb2, and found that 1- Rad4/Cut5 (TopBP1 ortholog) is present and activated, 2- phosphorylated H2A (γ-H2A) is also present, but 3- H4K20me2 is surprisingly missing at telomeres. Therefore, we proposed that shortage of H4K20me2 could contribute to telomere's ability to prevent Crb2-dependent cell cycle arrest. Inaddition, we determined that other subunits of fission yeast shelterin complexes, Pot1 and Ccq1, play critical roles in preventing Crb2 accumulation and Chk1 activation. Furthermore, we found that elimination of Set9, a sole enzyme responsible for generating H4K20me, me2 and me3, was able to partially rescue accumulation of Crb2 and strong cell cycle arrest phenotype found in taz1Δ ccq1Δ cells (Fig. S5D). Taken together, these observations suggested that the fission yeast shelterin complex contribute in attenuation of Chk1 activation via histone-dependent and -independent regulation of Crb2. It is also important to note that elimination of set9Δ or γ-H2A only partially abrogate checkpoint activation in response to DNA damages in fission yeast, due to the existence of CDK-dependent modulation of Cut5-Crb2 interaction that allow recruitment and activation of Crb2 at DNA damages even in the absence histone modifications.

We view with great interest the data presented by Audry and colleagues. Their work alerts the community that the basis of the checkpoint inhibition mechanism at telomeres is far from being fully understood. We do not view, however, that this constitutes a matter of incompatible results.
In fact, our results are experimentally different from the current study:

1. We did not normalize H4K20me2 levels over H4 levels as in their Fig. 1B, instead we measured different forms of H4K20 methylation as a ratio of total input. While normalization could be useful in

certain circumstances, it could also introduce unintended artefact. Thus, without evaluating their original raw data, it is nearly impossible to determine if their newly identified H4K20me2 antibody behave substantially different from antibody utilized in our previous study (Abcam ab9052). Normalization may simply reflect fewer H4 levels at telomeres and, consequently, provide an explanation to our previous finding that lessH4K20me2 and Crb2 accumulate at telomeres.

2. We would also like to point out that the H4K20me2 antibody utilized in our study was originally validated by SL Sanders (Sanders et al. 2004, Fig. 1), and widely used (more than 20 publications to date). Using the same antibody as our study, others have since corroborated our results. ChIPseq experiments initially performed by the group of S Grewal, NIH alerted us to lower levels of this mark near telomeres (personal communication). More recently, Svensson et al. 2015, looking at genome-wide distribution of H4K20me2 (Fig S4B), independently observed reduced levels of this mark at pericentromere and subtelomere regions. Therefore, we are somewhat surprised and disappointed that this manuscript does not directly compare their newly identified antibody against the previously established H4K20me2 antibody.

In any case, a potential explanation to our seemingly contradictory results may reside on different affinities ofthe antibodies used. It may be that this new reagent is able to pick up low amounts of H4K20me2 that we were previously unable to detect. If so, this would sustain a quantitative model whereby very low levels of this chromatin modification may be unable to provide sufficient enough Crb2 to trigger a sustained checkpoint response. Nevertheless, since experimental evidences strongly suggests that Set9-dependent H4K20 methylation alone cannot provide a full explanation to the refractory nature of telomeres to Crb2 as we outlined above, we do believe that it is very important to further investigate how telomeres prevent checkpoint activation in collaboration or in parallel to regulation of chromatin status, in particular, using independent techniques that would support each other.
We openly divulge our identity:
Miguel Godinho Ferreira and Toru Nakamura

Is the work clearly and accurately presented and does it cite the current literature?
Partly

Is the study design appropriate and is the work technically sound?
No

Are sufficient details of methods and analysis provided to allow replication by others?
Partly

If applicable, is the statistical analysis and its interpretation appropriate?
Partly

Are all the source data underlying the results available to ensure full reproducibility?
No

Are the conclusions drawn adequately supported by the results?
No

Competing Interests: We are authors in the paper Carneiro et al. 2010.

We have read this submission. We believe that we have an appropriate level of expertise to state that we do not consider it to be of an acceptable scientific standard, for reasons outlined above.

Author Response 26 Sep 2018

Kurt Runge, Case Western Reserve University, Cleveland, USA

Rebuttal to Ferreira and Nakamura:

The inclusion of Drs. Ferreira and Nakamura as reviewers was a major failure of reviewer selection system for the F1000Research Preclinical Reproducibility and Robustness channel. We are directly disputing results from a paper where Drs. Ferreira and Nakamura were the senior authors. While the editorial team tries to remove reviewers with a positive bias,they relied on software to select reviewers that included authors of the disputed publication and their former collaborators. Under this open review system, the editors and authors are supposed to agree on a set of reviewers, but reviewers were selected before we could respond. The editorial staff did not vet these reviewers and thus allowed the inclusion of the most negatively biased reviewers possible. As we show below, their comments distort the facts about our paper and are without merit.

The point of our paper is that H4K20me2 is as clearly detectable in nucleosomes adjacent to telomere repeats as it is at internal loci, requiring re-evaluation of this mechanism for checkpoint suppression proposed in Carneiro et al. The review by Ferreira and Nakamura follows our discussions with them for more than one year in which we provide H4K20me2 localization and they turn the discussion to Pot1, Ccq1 and Crb2. Our paper is only about H4K20me2 localization.

H4K20me2 exclusion from nucleosomes near telomere repeats was a fundamental mechanistic conclusion of Carneiro et al. 2010. This paper frequently states H4K20me2 is excluded from telomeres and that this exclusion is the mechanism for severing the checkpoint response. For example, their results contain the statement:
“In contrast, H4K20me2 was undetectable at telomere in earlier wild-type or *taz1*Δcells.”
Their final model in Figure 4g diagrams this exclusion and the legend states:

“Pot1 and Ccq1, together with the Taz1 complex, define a chromatin-privileged region that excludes H4K20me2 and prevents stable Crb2^{53BP1}association” (spelling error corrected)

Their penultimate paragraph in the text constituting the discussion begins with:

“The inability of telomeres to stably recruit Crb2^{53BP1}is probably due to the lack of H4K20me2 epigenetic marks at chromosome ends.” This paragraph ends with:

“In contrast, the absence of H4K20me2 marks regions where DNA perceived as damaged, such as chromosome ends, would not interfere with genome stability thus precluding a full checkpoint response.”

It is worth noting that Ferreira and Nakamura also cite data in their rebuttal that are not in their paper (there is no figure S5D), and overstate the claims of some of the data shown (Figure S5A shows immunofluorescence data of overexpressed Crb2 in very sick cells with multiple mutations and global changes in chromatin as a bargraph without error bars, so these data are, at best, very indirect for changes in H4K20me2 levels). They also avoid mentioning their own contradictory data that Crb2 can be detected at telomeres by ChIP with another epitope tag (presented by Dr. Nakamura at the 2013 Cold Spring Harbor Telomeres and Telomerase meeting), so the overall picture is more complex than they present. The focus of their summary on Pot1, Ccq1, Crb2 and phosphorylated H2A is irrelevant to our paper and distracts from the main point: H4K20me2 is present in nucleosomes adjacent to the telomere repeats at levels comparable to internal loci.

We show below that the two main points raised by Ferreira and Nakamura are simply not true, misrepresent our data and contradict their own previous discussions with us.

Objection 1. (A) Normalization to H4 levels versus total input chromatin in ChIP and (B) the absence of the original data.

1. A. The normalization to input chromatin in Carneiro et al. biases the results to lower levels of nucleosomes and H4 adjacent to telomeres, and leads to the erroneous conclusion that H4K20me2 is reduced near telomeres. Telomere repeats are bound by specific binding proteins, so chromatin fragments that include telomere repeat chromatin will have fewer histones in total. For example, the ~300 bp of telomere repeats in wild type cells are bound by non-nucleosomal telomere-specific binding proteins (i.e. Taz1 and Pot1). A 500 bp chromatin fragment containing the telosome will have fewer histones than an internal fragment of the same size. Normalization to input chromatin will thus yield a reduced signal for H4 and will give an artifactual decrease in all H4 modifications. Our data supporting this point are presented in the new Dataset 3 described in 1. B below.

In contrast to normalization by input chromatin, normalization to total histone H4 at a locus allows one to assay the level of modification in the nucleosomes present, regardless of the adjacent non-nucleosomal chromatin. It allows the direct assay of the major claims in Carnerio et al. 2010 quoted above.

1. B. We submitted the original data in a spreadsheet that was linked to our paper as Dataset 2, which had an associated doi in the pdf version of the paper that we proofed, but that link was not yet live active. This doi entry linked to an error webpage and was absent in the HTML version due to a processing mistake. We are surprised that Ferreira and Nakamura state that the data were not provided when the defective hyperlink was obvious. The editorial staff would have provided the data if these reviewers asked for it.

Our revised version contains the original Dataset 2 and has used these data to show that normalization to input chromatin gives reduced levels of histone H4 at telomeres (e.g. 1/5 to 1/9 the level of internal loci) and presents this analysis in a new third worksheet of Dataset 3. H4K20me2 signals near telomeres will therefore be low due to the artifact of normalizing non-nucleosomal chromatin fragments to input chromatin that is primarily nucleosomal.

Objection 2. The H4K20me2-specific antibody we used.

We find the wording of this objection extremely odd as Dr. Toru Nakamura told us in 2012 that while the original SL Sanders antibody sold by Abcam (as ab9052) was specific and good for ChIP, this original antibody was no longer available and subsequent lots sold under the same catalog number did not work well for ChIP. We therefore find this reversal of opinion to criticize our work puzzling, as repeating the experiment with the working anti-H4K20me2 antibody from Abcam is impossible as it no longer exists.

We did test the Abcam antibody sold as ab9052 in 2014 in case a newer lot showed better specificity. However, our tests confirmed Dr. Nakamura's statement that the newer antibody showed less reactivity with H4K20me2, and showed a different reactivity to the histones from the yeast mutants originally tested in by the Sanders lab in Greeson et al. (2008). The reactivity for the histones in the *crb2-F178Y* mutant appears to be different with the new lot of antibody and the reactivity with the pure H4K20me2 mimic is reduced (Supplemental Figure 1). We do not know what else this antibody recognizes in the *crb2-F178Y* mutant. In contrast, the GT282 antibody we used showed stronger selectivity and reactivity with H4K20me2 without the issues that accompany ab9052.

We cannot comment on the Svenson et al. 2015 paper as we did not address their findings in our paper and do not know what lot of antibody they used for their experiments. We only test whether the nucleosomes adjacent to the telomere repeats are depleted in the H4K20me2 modification, and find that they are at the same level as two different internal loci. Therefore, checkpoint suppression must occur by a mechanism distinct from H4K20me2 exclusion.

Ferreira and Nakamura include another puzzling statement after these two points.

"It may be that this new reagent is able to pick up low amounts of H4K20me2". Our data show that the levels at internal loci and telomeres are the same, so any quantitative models regarding Crb2 accumulation at telomere would apply to internal loci and would not explain telomere-specific checkpoint suppression.

In conclusion, the nucleosomes adjacent to telomere repeats have the same levels of H4K20me2 as the internal genes act1 and hip3, so some other mechanism than H4K20me2 exclusion prevents checkpoint activation at telomeres.

Kurt Runge
This rebuttal was reviewed and accepted by all authors of this paper by Audry et al.

Competing Interests: We are the authors of the Audry et al. paper.

Reader Comment 10 Oct 2018

F1000 Research, UK

The authors raise an important concern about the peer review of their article. F1000Research operates an open peer review, which takes place after publication. The peer-review process is led by the authors, who suggest referees, following defined referee criteria; the peer review is administered through the F1000 team. Occasionally the F1000 team provides further support by identifying qualified experts to peer review; authors are usually given an opportunity to approve such referee suggestions within a certain time frame.
We apologise that in the case of this article, a referee was invited without awaiting the authors' approval.
The referee's competing interest in the context of this article is stated as part of their report.

Competing Interests: No competing interests were disclosed.