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Long G2 accumulates recombination intermediates and disturbs chromosome segregation at dysfunction telomere in *Schizosaccharomyces pombe*

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ABSTRACT

Protection of telomere (Pot1) is a single-stranded telomere binding protein which is essential for chromosome ends protection. Fission yeast Rqh1 is a member of RecQ helicases family which has essential roles in the maintenance of genomic stability and regulation of homologous recombination. Double mutant between fission yeast *pot1Δ* and *rqh1* helicase dead (*rqh1-hd*) maintains telomere by homologous recombination. In *pot1Δ rqh1-hd* double mutant, recombination intermediates accumulate near telomere which disturb chromosome segregation and make cells sensitive to microtubule inhibitors thiabendazole (TBZ). Deletion of *chk1*$^{+}$ or mutation of its kinase domain shortens the G2 of *pot1Δ rqh1-hd* double mutant and suppresses both the accumulation of recombination intermediates and the TBZ sensitivity of that double mutant. In this study, we asked whether the long G2 is the reason for the TBZ sensitivity of *pot1Δ rqh1-hd* double mutant. We found that shortening the G2 of *pot1Δ rqh1-hd* double mutant by additional mutations of *wee1* and *mik1* or gain of function mutation of Cdc2 suppresses both the accumulation of recombination intermediates and the TBZ sensitivity of *pot1Δ rqh1-hd* double mutant. Our results suggest that long G2 of *pot1Δ rqh1-hd* double mutant may allow time for the accumulation of recombination intermediates which disturb chromosome segregation and make cells sensitive to TBZ.

1. Introduction

The major role of DNA damage checkpoints is to delay cell cycle transition in response to DNA damage which allocates time for this damage to be repaired [1,2]. In higher eukaryotes and in fission yeast, entry to mitosis is tightly regulated by protein kinases Wee1 and protein phosphatase Cdc25 [3–5]. In response to DNA damage, Chk1, a protein kinase that is essential for DNA damage checkpoint arrest, phosphorylates both Wee1 and Mik1. Active Wee1 and Mik1 consequently phosphorylate Cdc2 at Tyr 15 and arrest cell cycle at G2/M boundary [6,7]. Mutation of *wee1* and *mik1* abrogates cell cycle arrest at G2/M boundary and accelerates entry to mitosis at cell size smaller than wild type [8,9].

Eukaryotic chromosome ends are protected by DNA-protein complex called telomere. In fission yeast, Pot1 is a single stranded telomere binding protein that plays important roles in chromosome end protection and telomere length regulation [10]. Deletion of *pot1*$^{+}$ results in rapid telomere loss, chromosome circularization and activation of DNA damage response [10]. Fission yeast RecQ helicase Rqh1 has an anti-recombination function and suppresses the accumulation of aberrant recombination intermediates [11,12]. The double mutant between *pot1Δ* and *rqh1* helicase dead *(rqh1-hd)* point mutant, in which lysine 547 is mutated to alanine, maintains chromosome ends by homologous recombination (HR) [13]. This recombination intermediates accumulate near telomeres even in M phase and cells enter mitosis with that accumulated intermediates which would disturb chromosome segregations and make cells sensitive to microtubules inhibitor thiabendazole (TBZ)

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[13]. In fission yeast and vertebrates, POT1 inactivation elicits DNA damage response, aberrant HR and chromosome segregation defects [10,15–17]. Although DNA damage activation acts as a surveillance mechanism which is critical for genome stability and cell viability, interestingly, in *pot1Δ rqh1-hd* double mutant, activation of DNA damage checkpoint adversely affects the cell's viability in presence of TBZ. In that double mutant, activation of DNA damage checkpoint arrests cell cycle at G2/M boundary and gives that mutant its characteristic long G2 phenotype [13]. This long G2 may allow time for recombination intermediates to be accumulated at chromosome ends and may be the reason for the TBZ sensitivity of *pot1Δ rqh1-hd* double mutant. It was previously reported that deletion of *chk1*$^+$ or mutation of its kinase domain suppresses both the accumulation of recombination intermediates and the TBZ sensitivity of *pot1Δ rqh1-hd* double mutant [14]. As deletion of *chk1*$^+$ overrides the G2/M transition delay of *pot1Δ rqh1-hd* double mutant, in this study, we addressed a question whether the long G2 is the reason for the TBZ sensitivity of *pot1Δ rqh1-hd* double mutant. We found that shortening the G2 phase of *pot1Δ rqh1-hd* double mutant by mutation of Chk1 downstream kinases, *wee1* and *mik1* also suppresses both the accumulation of recombination intermediates and the TBZ sensitivity of *pot1Δ rqh1-hd* double mutant. Moreover, shortening the G2 of *pot1Δ rqh1-hd* double mutant in cells harboring Cdc25-independent gain of function mutant allele of *cdc2* (*cdc2–3w*) also suppresses both the accumulation of recombination intermediates and the TBZ sensitivity of *pot1Δ rqh1-hd* double mutant. Based on these data, we proposed that long G2 phase of *pot1Δ rqh1-hd* double mutant would allow time for recombination intermediates to be accumulated near telomere which would disturb chromosome segregation and would be the reason for the TBZ sensitivity of that double mutant.

2. Material and methods

2.1. Strain construction and growth media

The strains used in this study are listed in Table 1. The *pot1Δ rqh1-hd wee1–50 mik1Δ* mutant *(pot1::KanMX rqh1-K547A wee1–50 mik1::ura4)* expressing Pot1 from a plasmid (pPC27-*pot1*$^+$-hemagglutinin [HA], containing *leu1* gene) was created by mating *h*$^-$ *wee1–50 mik1:ura4* double mutant with *h*$^+$ *pot1 rqh1-hd* double mutant (*pot1::KanMX rqh1-K547A(pPC27-leu1-pot1*$^+$*-HA)). pot1Δ rqh1-hd wee1–50 mik1Δ* mutants that do not have pPC27-*pot1*$^+$-HA were selected on yeast extract agar (YEA) plates containing 100 μM 5-fluorodeoxyuridine (FUDR). The *pot1Δ rqh1-hd cdc2–3w* triple mutant (*pot1::KanMX rqh1-K547A cdc2–3w*) expressing Pot1 from a plasmid (pPC27-*pot1*$^+$ [HA], containing *ura4* gene) was created by mating *h*$^+$ *pot1Δ rqh1-hd* double mutant (*pot1::KanMXrqh1-K547A*) expressing Pot1 from a plasmid (pPC27-*pot1*$^+$ [HA], containing *ura4* gene) with *h*$^-$*cdc2–3w. pot1Δ rqh1-hd cdc2–3w* triple mutants that do not have pPC27-*pot1*$^+$-HA were selected on yeast extract agar (YEA) plates containing 2 g/liter 5-fluoroorotic acid (FOA). Cells were grown in either YEA medium (0.5% yeast extract, 3% glucose, and 40 μg/ml adenine) or Edinburgh Minimal Media EMM with the required supplements at indicated temperature.

2.2. Measurement of telomere length

Telomere length was measured using southern hybridization as a previously described procedure [18] with an AlkPhos Direct Kit (GE healthcare) plus telomere fragments derived from pNSU70 [19] were used as a probe.

2.3. Pulsed-field gel electrophoresis

PFGE was performed as previously described by Baumann et al. [20]. For the detection of NotI-digested chromosomes, *Schizosaccharomyces pombe* NotI-digested chromosomal DNA was fractionated in 1% agarose gel with 0.5 × TBE (50 mM Tris–HCl, 5 mM boric acid, and 1 mM EDTA [pH 8.0]) buffer using the CHEF Mapper PFGE system at 6 V/cm (200 V) and a pulse time of 60–120 s for 24 h. DNA was visualized by staining with ethidium bromide (1 μg/ml) for 30 min.

2.4. Microscopy

Microscope images of living cells were obtained using an AxioCam digital camera (Zeiss) connected to an Axio Observer. Z1 microscope (Zeiss) with a plan-Apochromat 63 × objective lens (numerical aperture, 1.4). Pictures were captured and analyzed using AxioVision Rel. 4.8.2 software (Zeiss). A glass-bottom dish (Iwaki) was coated with 5 mg/ml lectin from *Bandeiraea simplicifolia* BS-I (Sigma).

3. Results

3.1. Deletion of wee1 and mik1 suppresses both the TBZ sensitivity and the accumulation of recombination intermediates at telomere of pot1Δ rqh1-hd double mutant

Chk1 is required to prevent entry to mitosis in response to DNA damage [21,22] and keeping cells at long G2. Deletion of *chk1*$^+$ or mutation of its kinase domain shortens the G2 of *pot1Δ rqh1-hd* double mutant (Fig. 1A and B) and suppresses both the TBZ sensitivity and the accumulation of recombination intermediates near telomere in that double mutant [14]. This result highly suggests that there is relevance between the long G2 phenotype of *pot1Δ rqh1-hd* double mutant and the TBZ sensitivity and the accumulation of recombination intermediates. To address evidence that

Table 1
Schizosaccharomyces pombe strains used in this study.

Strain name	Genotype	Source
GT000	*h*$^+$ *leu1–32 ura4-D18 ade6-M210 pot1::kanMX6 rqh1-K547A (pPC27-ura4-pot1*$^+$*-HA)*	Takahashi et al.
GT002	*h*$^-$ *leu1–32 ura4-D18 ade6-M210 pot1::kanMX6 rqh1-K547A*	Takahashi et al.
FY16194	*h*$^-$ *cdc2–3w ade6-M216 ura4-D18*	NBRP
AH010	*h*$^-$ *leu1–32 ura4-D18 ade6-M210 pot1::kanMX6 rqh1-K547A cdc2–3w (pPC27-ura4- pot1*$^+$*-HA)*	This study
AH009	*h*$^-$ *leu1–32 ura4-D18 ade6-M210 pot1::kanMX6 rqh1- K547A cdc2–3w*	This study
MY444	*h*$^-$ *leu1–32 ura4-D18 wee1–50 mik1::ura4*	NBRP
KM032	*h*$^+$ *ura4-D18 leu1–32 pot1::kanMX6 rqh1-K547A wee1–50 mik1::ura4 (pPC27-leu1- pot1*$^+$*-HA)*	This study
KM031	*h*$^+$ *ura4-D18 leu1–32 pot1::kanMX6 rqh1-K547A wee1–50 mik1::ura4*	This study
TH025	*h*$^+$ *leu1–32 ura4-D18 ade6-M210 pot1::kanMX6 rqh1-K547A (pPC27-leu1- pot1*$^+$*-HA)*	Nakano et al.
YI002	*h*$^-$ *leu1–32 ura4-D18 ade6 pot1::kanMX6 (pPC27-ura4- pot1*$^+$*-HA)*	Nanbu et al.
SIH60	*h*$^-$ *leu1–32 ura4-D18 ade6 pot1::kanMX6 rav1::hphMX6*	This study

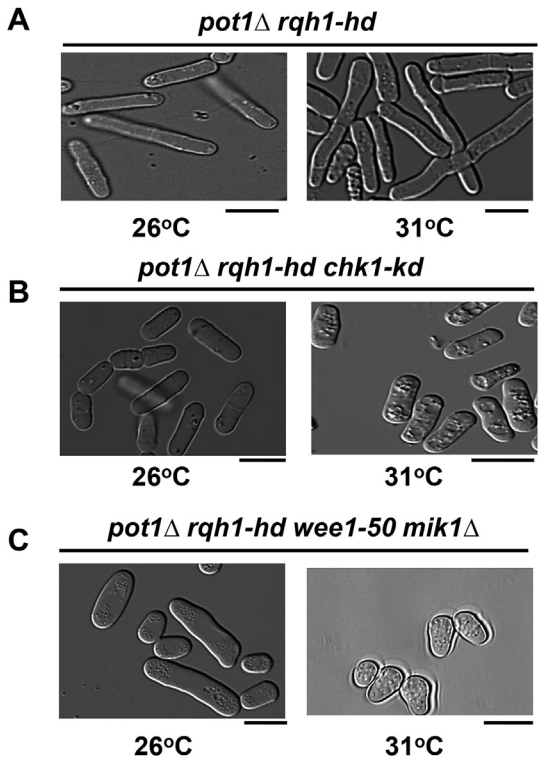

Fig. 1. The cell length of vegetative growing *pot1Δ rqh1-hd* cells, *pot1Δ rqh1-hd chk1-kd* cells and *pot1Δ rqh1-hd wee1–50 mik1Δ* cells at indicated temperatures. Bar = 10 μm.

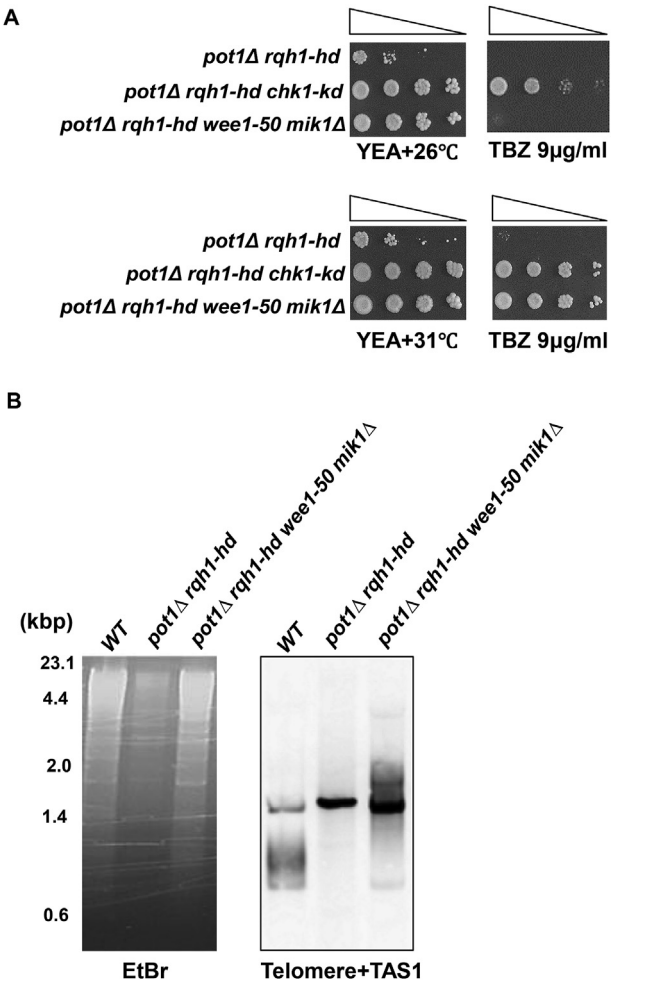

Fig. 2. Mutation of *wee1* and *mik1* suppresses the TBZ sensitivity of *pot1Δ rqh1-hd* double mutant. (A and B) Spotting assay of 10-fold serial dilutions of log phase cells. The *pot1Δ rqh1-hd*, *pot1Δ rqh1-hd chk1-kd*, *pot1Δ rqh1-hd wee1–50 mik1Δ* cells expressing Pot1 from plasmid, and *pot1Δ rqh1-hd wee1–50 mik1Δ* mutant cells were spotted onto YEA plate in the absence and in the presence of 9 μg/ml TBZ at indicated temperature. (C) The telomere lengths of wild-type, *pot1Δ rqh1-hd*, and *pot1Δ rqh1-hd wee1–50 mik1Δ* cells were analyzed using southern hybridization. Genomic DNA was digested using EcoRI and separated by 1.5% agarose gel electrophoresis and hybridized to 1 kb-DNA fragment containing telomere fragment of 300 bp plus telomere-associated sequence (TAS1) of 700 bp. To estimate the total amount of DNA, the gel was stained with ethidium bromide (EtBr) before blotting onto the membrane.

supports this relevance, we tried to find another mutant that shortens the G2 phase of *pot1Δ rqh1-hd* double mutant. Activation of Chk1 results in activation of downstream kinases Wee1 and Mik1 that consequently inhibitory phosphorylate Cdc2 at Tyr 15 and hold cells at long G2 [6,7]. Mutation of *wee1, wee1–50*, which is a temperature sensitive loss of function allele of *wee1* and *mik1* null mutation results in premature mitotic entry at smaller size than wild type [23] and shortens the G2 of *pot1Δ rqh1-hd* double mutant at semi-restrictive temperature 31 °C (Fig. 1C).

We tested the TBZ sensitivity of *pot1Δ rqh1-hd* cells harboring *wee1* temperature sensitive mutant, *wee1–50*, and *mik1* null mutant. Interestingly, we found that mutation of both *wee1* and *mik1* suppressed that TBZ sensitivity of *pot1Δ rqh1-hd* double mutant at semi-restrictive temperature 31 °C (Fig. 2A and B). This supports our suggestion that long G2 phase may be the reason for the TBZ sensitivity of *pot1Δ rqh1-hd* double mutant.

Telomeres in *pot1Δ rqh1-hd* double mutant are maintained by homologous recombination and the chromosome end fragments at EcoR1 sites are highly amplified [13]. The same band pattern was observed in *pot1Δ rqh1-hd wee1–50 mik1Δ* (Fig. 2C) which suggests that the telomeres are also maintained by homologous recombination as in *pot1Δ rqh1-hd* double mutant.

3.2. Mutation of wee1 and mik1 suppresses the accumulation of recombination intermediates near telomere in pot1Δ rqh1-hd double mutant

Previous analysis of the chromosome end fragments of *pot1Δ rqh1-hd* by pulsed-field gel electrophoresis (PFGE) revealed that NotI-digested fragments M, L, I and C that located at the ends of chromosome I and II were almost absent [13]. This result indicates that the chromosome ends of *pot1Δ rqh1-hd* double mutant may be entangled and were unable to enter the gel which may be attributed to the accumulation of recombination intermediates at chromosome ends of that mutant. We analyzed the chromosome end fragments of *pot1Δ rqh1-hd wee1–50 mik1Δ* mutant cells using PFGE to test if short G2 phenotype of *pot1Δ rqh1-hd wee1–50 mik1Δ* will suppress the accumulation of recombination intermediates. Interestingly, the NotI-digested fragments M, L, I and C were detected in *pot1Δ rqh1-hd wee1–50 mik1Δ* mutant cells (Fig. 3A and B). This result suggests that long G2 may be the reason for the accumulation of recombination intermediates at chromosome ends of *pot1Δ rqh1-hd* double mutant.

3.3. Gain of function mutation of Cdc2 suppresses the TBZ sensitivity of pot1Δ rqh1-hd double mutant

To provide further evidence that supports the relevance between long G2 and the TBZ sensitivity of *pot1Δ rqh1-hd* double mutant. We searched for another mutant which has short G2

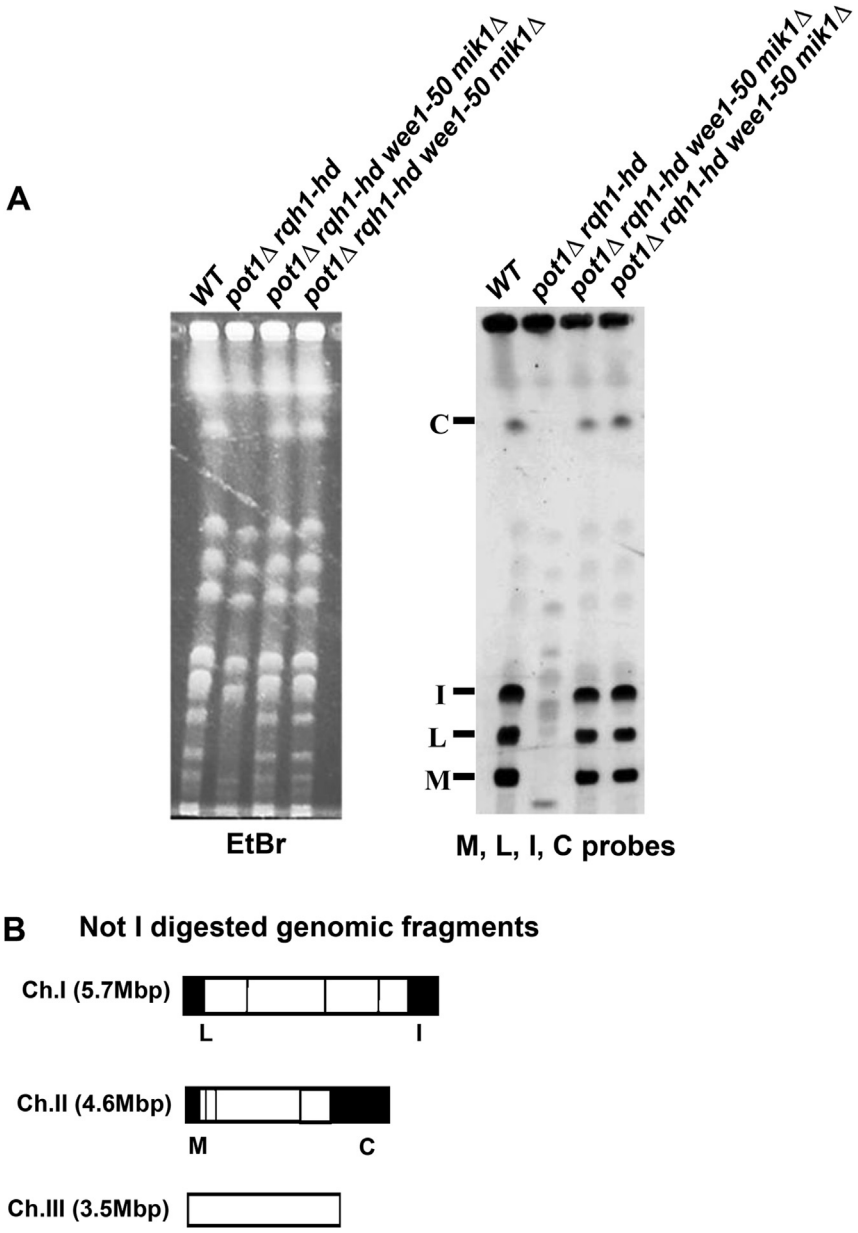

Fig. 3. The chromosome end fragments of *pot1Δ rqh1-hd wee1–50 mik1Δ* cells are linear. (A) Chromosomal DNA from wild-type, and two independent *pot1Δ rqh1-hd wee1–50 mik1Δ* cells incubated at 31 °C was digested with NotI and analyzed using PFGE. Probes specific to NotI fragments (M, L, I, and C) were used. (B) NotI restriction site map of *S. pombe* chromosomes. Chromosome I, II, and III are shown.

phenotype in *pot1Δ rqh1-hd* double mutation background. Cdc2 and its regulatory subunit cyclin B is the master regulator for entry to mitosis [9,24,25]. *cdc2–3w*, which is Cdc25-independent gain of function allele of Cdc2, has short G2 and pre-mature enters to mitosis at smaller size than wild type [26]. *cdc2–3w* mutation also reduces the G2 length of *pot1Δ rqh1-hd* double mutant (Fig. 4A). By testing the TBZ sensitivity of *pot1Δ rqh1-hd cdc2–3w* triple mutant, interestingly, we find that *cdc2–3w* mutation suppresses the TBZ sensitivity of *pot1Δ rqh1-hd* double mutant (Fig. 4B) which further supports our hypothesis regarding the relevance between the long G2 of *pot1Δ rqh1-hd* double mutant and its TBZ sensitivity.

Moreover, by looking at the telomeres in *pot1Δ rqh1-hd cdc2–3w*, we detected the same band pattern as it was observed in *pot1Δ rqh1-hd* double mutant which suggests that telomeres in *pot1Δ rqh1-hd cdc2–3w* triple mutant are also maintained by homologous recombination as in *pot1Δ rqh1-hd* double mutant (Fig. 4C).

3.4. *cdc2–3w mutation suppresses the accumulation of recombination intermediates at chromosome end fragments of pot1Δ rqh1-hd double mutant*

To further test the relevance between the long G2 and the accumulation of recombination intermediates, the chromosome end fragments of *pot1Δ rqh1-hd cdc2–3w* triple mutant cells were analyzed by PFGE. We found that the NotI-digested fragments M, L, I and C were detected in *pot1Δ rqh1-hd cdc2–3w* cells and the chromosome end fragments could enter the gel (Fig. 4D) which also suggests that shortening the G2 phase of *pot1Δ rqh1-hd* double

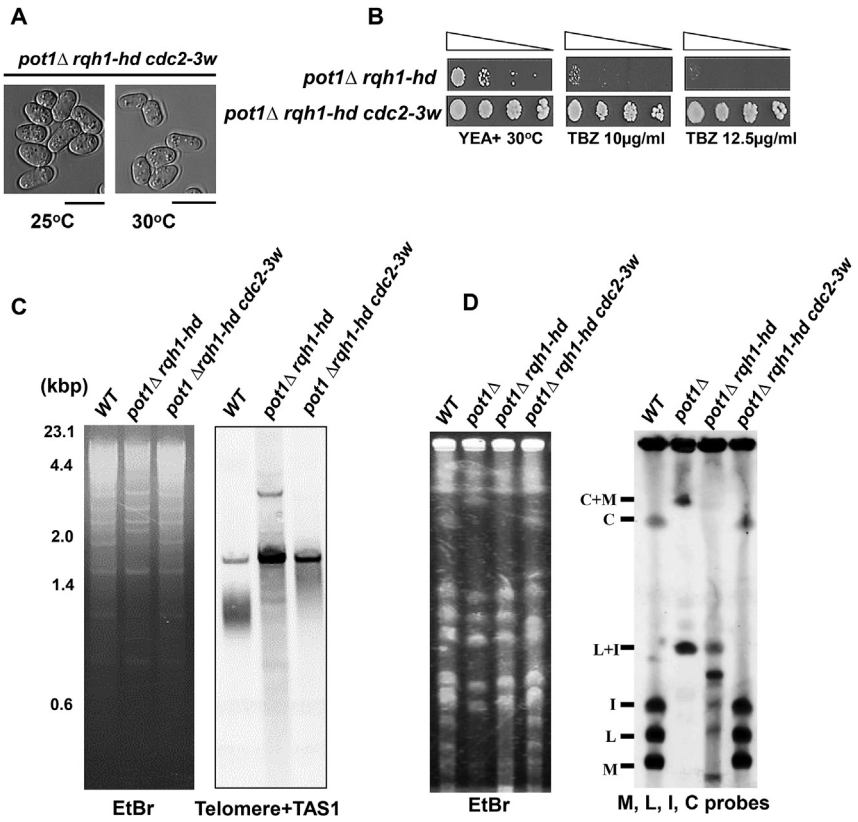

Fig. 4. Gain of function mutation of Cdc2 suppresses the TBZ sensitivity of *pot1Δ rqh1-hd* double mutant. (A) The cell length of vegetative growing *pot1Δ rqh1-hd cdc2–3w* cells at indicated temperature. Bar = 10 μm. (B) Spotting assay of 10-fold serial dilutions log phase cells were spotted on YEA and YEA containing TBZ (10, 12.5 μg/ml) at indicated temperature. (C) The telomere lengths of wild-type, *pot1Δ rqh1-hd*, and *pot1Δ rqh1-hd cdc2–3w* mutant cells were analyzed using southern hybridization. (D) NotI-digested *S. pombe* chromosomal DNA from *pot1Δ rav1Δ*, wild-type, and *pot1Δ rqh1-hd cdc2–3w* isolate were analyzed using PFGE.

mutant suppresses the accumulation of recombination intermediates.

4. Discussion

pot1Δ rqh1-hd double mutant maintains telomere by HR [13]. In human, telomere uncapping results in activation of DNA damage response and restrains mitotic entry [27–29]. In chicken cells, depletion of POT1 results in an acute DNA damage response at telomeres and G2 arrest [30]. Disruption of the two POT1 genes in mouse embryonic fibroblasts causes compromised proliferation, a severe telomere DNA damage response and elicits aberrant HR at telomeres [31–33]. These facts suggest that deletion of *pot1*$^+$ also activates DNA damage and elicits inappropriate recombination in *pot1Δ rqh1-hd* double mutant. *rqh1-hd* mutant cells show hyper-recombination phenotype and accumulation of recombination intermediates near telomere in *pot1Δ rqh1-hd* double mutant was reported [13]. The accumulation of recombination intermediates at chromosome end fragments results in defect in chromosome segregation and makes cells sensitive to TBZ [13]. The PFGE analysis of the NotI-digested genomic DNA also revealed that the chromosome end fragments of *pot1Δ rqh1-hd* double mutant were not able to enter the gel [13] possibly due to the accumulation of recombination intermediates at chromosome end fragments. Moreover, *pot1Δ rqh1-hd* double mutant displays long G2 phenotype (Fig. 1A) which would be attributed to the activation of DNA damage checkpoint as deletion of *chk1*$^+$ or mutation of its kinase domain overrides the G2/M arrest and shortens the G2 of that double mutant (Fig. 1B). In addition, deletion of *chk1*$^+$ or mutation of its kinase domain suppresses both the accumulation of recombination intermediates near telomere and the TBZ sensitivity of *pot1Δ rqh1-hd* double mutant [14]. Therefore, in this study we tested the possibility whether long G2 caused by Chk1-dependent DNA damage checkpoint activation pathway is the reason for the accumulation of recombination intermediates near telomere and the TBZ sensitivity of *pot1Δ rqh1-hd* double mutant. Long G2 would allow time for the accumulation of recombination intermediates at chromosome end fragments of *pot1Δ rqh1-hd* double mutant which disturbs chromosome segregation and makes cells sensitive to TBZ.

To study this relevance between long G2 phenotype of *pot1Δ rqh1-hd* double mutant and its TBZ sensitivity, we searched for other mutants that shorten the G2 of *pot1Δ rqh1-hd* double mutant. If long G2 is the reason for the accumulation of recombination intermediates and the TBZ sensitivity of that double mutant, mutations that reduce the G2 of *pot1Δ rqh1-hd* double should suppress both the accumulation of recombination intermediates and the TBZ sensitivity. Mutations that are able to abrogate the inhibitory phosphorylation of Cdc2 result in dramatic reduction in cell length and rapid entry to mitosis at very small size [8,9,26]. Both Chk1 downstream kinases, Wee1 and Mik1, act together in the inhibitory phosphorylation of Cdc2 at Tyr 15 and consequent cell cycle arrest [7]. The loss of function of both Wee1 and Mik1 results in premature mitotic entry at smaller size than wild type cells [6,7]. Moreover, *S. pombe* Cdc2 which is a homolog to *S. cerevisiae* CDC28 plays a key role in regulation of the cell cycle and entry to mitosis [9,24,25]. *cdc2–3w* which is Cdc25-independent gain of function allele of Cdc2 bypasses checkpoint control and rapidly enters to mitosis at reduced cell sizes [26]. Both *pot1Δ rqh1-hd* cells

harboring *wee1–50 mik1Δ* double mutation, which express loss of function temperature sensitive allele of Wee1 and lack Mik1, and *pot1Δ rqh1-hd cdc2–3w* mutant cells have short G2 (Figs. 1C and 4A) and undergo rapid mitotic entry. By testing the TBZ sensitivity of *pot1Δ rqh1-hd wee1–50 mik1Δ* and *pot1Δ rqh1-hd cdc2–3w* mutant cells, we find that *wee1–50 mik1Δ* double mutation and *cdc-3w* single mutation suppress the TBZ sensitivity of *pot1Δ rqh1-hd* double mutant (Figs. 2A and B and 4B). These results substantiate our hypothesis regarding the relevance between long G2 of *pot1Δ rqh1-hd* double mutant and its TBZ sensitivity. In addition, we analyzed the chromosome end fragments of both *pot1Δ rqh1-hd wee1–50 mik1Δ* and *pot1Δ rqh1-hd cdc2–3w* mutant cells by PFGE to test if short G2 phenotype of these mutants will suppress the accumulation of recombination intermediates at telomere of *pot1Δ rqh1-hd* double mutant. Interestingly, the chromosome end fragments of both *pot1Δ rqh1-hd wee1–50 mik1Δ* and *pot1Δ rqh1-hd cdc2–3w* cells could enter the gel as the NotI-digested fragments M, L, I, and C, which are located at the ends of chromosomes I and II could be detected (Figs. 3A and 4D). This result suggests that long G2 would contribute to the accumulation of recombination intermediates at the chromosome end fragments of *pot1Δ rqh1-hd* double mutant.

Our findings provide a case in which cell cycle arrest induced by activation of DNA damage checkpoint pathway worsens the cells' viability by contributing to accumulation of toxic recombination intermediates at telomere. In *S. pombe srs2Δ rqh1Δ* double mutant, deletion of DNA damage checkpoints *rad9*$^+$ or *chk1*$^+$ also shortens the G2 and rescues the slow growth phenotype of that double mutant. Moreover, the slow growth phenotype of *srs2Δ rqh1Δ* is seems to be recombination dependent as deletion of recombination genes *rad51*$^+$ or *rad57*$^+$ increases cells' viability. Therefore, in *srs2Δ rqh1Δ* double mutant, it is also highly possible that long G2 caused by activation of DNA damage checkpoint exaggerates the hyper-recombination phenotype of *srs2Δ rqh1Δ* by contributing to more accumulation of recombination intermediates which adversely affects cell growth [34].

In conclusion, our results strongly demonstrate strong relevance between the accumulation of recombination intermediates near telomere and the TBZ sensitivity of *pot1Δ rqh1-hd* double mutant in the way that long G2 would allow time for the accumulation of recombination intermediates which disturbs the chromosome segregation and would be the reason for the TBZ sensitivity of that double mutant.

Conflict of interest

None.

Acknowledgment

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Transparency document

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