博 士 論 文
（Doctoral Thesis）

Characterizing the factors that are required for the maintenance of circular chromosomes using fission yeast

（分裂酵母を用いた環状染色体の維持に必要な因子の理解）

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2018 年 9 月
（September 2018）

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主　論　文
（Main Thesis）

Abstract

A telomere is a region of repetitive nucleotide sequences at each end of a eukaryotic chromosome. Telomere usually protects cells from genomic instability and cellular senescence. DNA double-strand break generates sticky end which fuses with end to end and creates circular chromosome. In addition, loss of telomere also creates chromosome circularization. Circular chromosomes are found in many eukaryotes. Circular chromosomes in human have been linked to some genetic diseases and cancer. My research emphasizes to develop therapies to kill cancer cells carrying circular chromosomes. But genes that are involved in the maintenance of circular chromosome are not known in human. In addition, there has no advanced therapy yet discovered that can selectively kill this kind of cancer cells. In this research, I have used *Schizosaccharomyces pombe*. *S. pombe* is genetically tractable. Most of the genes related to chromosome maintenance are conserved in mammalian counterparts. Specifically, I have used *pot1* deletion strain which has circular chromosome.

The purpose of this research is to find out the genes required for the maintenance of circular chromosome. These target genes could be used for a possible therapy of cancer where most of the cells carrying circular chromosomes. To identify genes that are involved in the maintenance of circular chromosome in *S. pombe*, I tried to find out a gene that will be synthetically lethal with *pot1* deletion strain. The cell cycle checkpoint protein Rad9-Hus1-Rad1 (9-1-1) complex plays an important role to maintain the genomic integrity. However, the role of the 9-1-1 complex on the maintenance of circular chromosomes is not known. I constructed *pot1*Δ and the 9-1-1 complex double mutant cell which exhibits the feature of the circular chromosome. I

analyzed the sensitivity of *pot1*Δ and the 9-1-1 complex double mutant strains with agent causing replication stress. Here, I have investigated the effect of 5-fluorodeoxyuridine (Fudr) which is an anticancer drug. I found that the checkpoint-defective 9-1-1 complex single mutant strains (*rad9*Δ*, rad1*Δ*, hus1*Δ*)* were sensitive to Fudr, although fission yeast does not express thymidine kinase (*tk*). I also found that *pot1*Δ and the 9-1-1 complex double mutant strains (*pot1*Δ *hus1*Δ*, pot1*Δ *rad1*Δ *and pot1*Δ *rad9*Δ) showed greater sensitivity to Fudr than that shown by each single mutant (*pot1*Δ*, hus1*Δ*, rad1*Δ and *rad9*Δ). I found that Fudr causes cell cycle arrest in S phase. In addition, Fudr treatment of *pot1*Δ and the 9-1-1 complex double mutant strains exhibited more DNA damage than that shown by each single mutant. Thus, the 9-1-1 complex is required for the maintenance of circular chromosome when DNA replication is arrested.

The *S. pombe* phosphatidylinositol 4-kinase, Pik1p, is homologous to human PI 4-kinase IIIβ. Pik1p is found to associate with Golgi. Pik1 is involved in sorting proteins in Golgi. However, the role of $pik1^{+}$ at the telomere or the maintenance of circular chromosomes are not reported. To obtain the double mutant strain that will be synthetically lethal with *pot1Δ*, I used mutagenic alkylating agent, EMS to mutagenize the *pot1Δ* cells carrying $pot1^{+}$ expressing plasmid. I obtained a candidate, which had an unknown mutation in *pot1Δ* carrying $pot1^{+}$ expressing plasmid. I found that *pot1Δ* is synthetically lethal with unknown EMS-induced mutation in this candidate strain. I used a browser-accessible tool, mutation discovery and identified that this unknown mutation is *pik1* mutation. I called this mutation as *pik1-1*. There may be two possible reasons behind the lethality between *pot1Δ* and *pik1-1* double mutant. Pik1 is either required for

the formation of chromosome circularization or the maintenance of chromosome circularization. I found that *pik1* is not required for the formation of the circular chromosome. Therefore, it supports the second possibility where *pik1* could be required for the maintenance of circular chromosome.

In this research, I found that the 9-1-1 complex is required for the maintenance of circular chromosome when DNA replication is arrested. I also found that the $pik1^{+}$ could be required for the maintenance of circular chromosome. These kinds of finding will directly contribute to selectively kill cancer cells that have circular chromosomes in human.

Contents

Chapter 1. Introduction

1.1. Fission Yeast *Schizosaccharomyces pombe*

Fission yeast *Schizosaccharomyces pombe* was first discovered by Paul Linder from a millet beer in 1893 in a Brewery Association Laboratory in Germany. The word schizo means to split or fission and pombe means beer. The *S. pombe* was isolated from East African beer where pombe means "beer" in Swahili. *S. pombe* is used in traditional brewing and recognized as a model organism in molecular and cellular biology. *S. pombe* is first developed for the experimental model in the 1950s by Urs Leupold for analyzing genetics and by Murdoch Mitchison for studying the cell cycle [1,2]. Sir Paul Maxime Nurse, a renowned fission yeast researcher was awarded 2001 Nobel Prize in Physiology or Medicine for the discovery of protein molecules that regulate the division of cells in the cell cycle together with Lee Hartwell and Tim Hunt. *S. pombe* is a unicellular eukaryote, whose cells are rod-shaped and maintain their shape using cell tips. *S. pombe* cells are divided by medial fission to produce 2 daughter cells of equal in size that are used as the powerful tool in cell cycle analysis. Each cell has to pass a cycle including Gap1 phase (G1) where cell increases in size and the G1 checkpoint control mechanism ensures that the cell is ready or not for going to DNA synthesis. The DNA replication occurs in the synthesis phase (S). G2 phase (Gap2 phase) is the Gap between DNA synthesis and mitosis where the cell continues to grow. In mitosis phase, cell growth stops and cellular energy is focused on the orderly division into two daughter cells. Metaphase checkpoint which is in the middle of mitosis ensures that the cell is ready to complete the cell duplication [3]. To ensure the proper cell division this cycle consists of some control mechanisms known as cell cycle checkpoints. Active

checkpoints detect problems and activate proteins to halt the cell cycle for repairing damaged DNA. On the other hand, loss of control mechanism of the cell cycle allows to divide cells. Among the four cell cycle phases, S-phase ensures to duplicate whole genome (Fig. 1.1. B). The fission yeast cell divides rapidly with a generation time of 2 to 4 hours. The cell is usually 3 to 4 micrometers in diameter and about 7 to 14 micrometers in length. *S. pombe* genome is (about 12 Mb) divided in just three chromosomes, chromosome I (5.7 Mb), chromosome II (4.6 Mb) and chromosome III (3.5 Mb), and contains 4,970 protein-coding genes and 450 non-coding RNAs. The 3 chromosomes are linear but the formation of circular chromosomes is also observed in fission yeast mutant. If the *S. pobme* loses the simple repeats at the terminal ends of the chromosome as well as much of the subtelomeric region, it can fuse end to end and form a circular chromosome. The three chromosomes I, II and III are containing 35, 65, and 110 kb long centromeres, respectively [4,5]. The features of *S. pombe* chromosomes are similar to those of higher eukaryotes, including large centromeres comprising repetitive sequences and conserved heterochromatin proteins [6]. On the other hand, budding yeast consists of slightly larger genome divided amongst 16 chromosomes. Budding yeast contains simple point centromeres, non-conserved silencing proteins, small origins and a limited set of telomere proteins. Thus, fission yeast has become a model of choice for analyzing fundamental mechanisms of transcription, translation, DNA replication, DNA damage and repair, cell cycle controls and signal transduction [3]. There are fifty homologs in *S. pombe* that are involved in human diseases including cystic fibrosis, hereditary deafness, diabetes and the major group of human disease-related genes those are implicated in cancer. There are 23 genes that are associated to DNA damage and repair, checkpoint controls, and the cell cycle, and all

these processes are important to maintain genomic instability of the cells. These events play an important role in finding out the more about the evolution of unicellular and multicellular eukaryotic organisms compared to other organisms such as bacteria which do not contain nucleated cells [6].

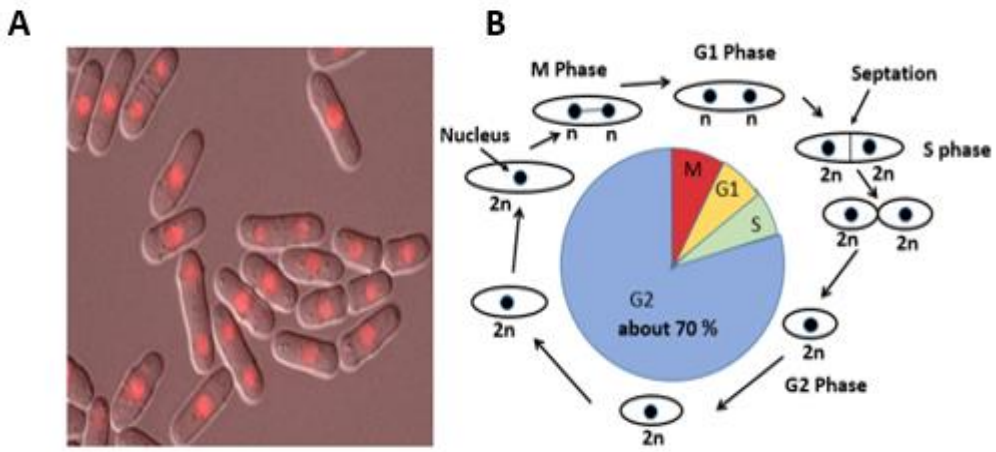

Fig. 1.1. (A) Picture of *S. pombe* cells. (B) Cell cycle of *S. pombe* cells where denoted G2 phase, M phase, G1 phase and S phase with their genomic contents, n and 2n in different phase. About 70% during the total time of cell cycle in *S. pombe* spends to G2 phase and other 30% time spends to M, G1 and S phase.

1.2. Telomere, the end protector of eukaryotic chromosome

A telomere is a region of repetitive nucleotide sequences at each end of a eukaryotic chromosome. It acts as a cap at the end of each strand of DNA that should protect chromosomes, like the plastic tips at the end of our shoelaces. Without the coating (tips), shoelaces are destroyed. Similarly, without telomeres, DNA strands are damaged. Telomere usually protects cells from genomic instability, cellular senescence and DNA mutations. It is also necessary for maintaining genomic integrity and cellular replication. Barbara McClintock studied on corn and Herman Muller studied on fruit

flies in the 1930s where they collaboratively found that chromosomes have specialized ends to protect fusion from end to end. McClintock performed X-ray treatment on corn that was leaded to the formation of a circular chromosome. From the circularized chromosome that have lost their protective end, she named this protective end as telomere [7]. Elizabeth Blackburn found *Tetrahymena* telomere carrying hundreds of base pair repeats of the sequence TTGGGG. Carol Greider who worked in Elizabeth Blackburn laboratory discovered telomerase, an enzyme that is responsible for the synthesis of the telomeric repeats in *Tetrahymena*. In 2009, Elizabeth Blackburn, Carol Greider, and Jack Szostak were together awarded Nobel Prize in Physiology or Medicine for their outstanding research on the discovery of the enzyme telomerase [8]. Including mammal's eukaryotes, telomeric DNA consists of single-stranded TTAGGG repeats and a single-stranded G-rich overhang where the shelterin complex binds. These binding induces to form t-loop, a cap structure which protects the DNA-damage-sensing machinery from mistakenly repairing telomeres. Loss or mutation of shelterin complex causes telomere uncapping and thereby activates damage-signaling pathways. The activation of damage-signaling pathways may lead to non-homologous end joining (NHEJ), homologous recombination (HR), senescence and apoptosis. When telomere shortage is occurred to an unhealthy point then the DNA-damage checkpoint recognizes the chromosome ends as a broken. Therefore, the DNA-damage checkpoint proteins halt the cell division. Pot1 which is an important component of shelterin complex, is conserved from yeast to human. Pot1 is essential for telomere protection. In *S. pombe*, deletion of *pot1* causes immediate telomere loss and chromosome circularization. Then the survival of cell depends on chromosome circularization [10].

1.3. Loss of telomere leads to cell senescence and cancer

The DNA replication is semi-conservative that the two strands of DNA (mother strand) are separated and each strand is used as a template for a new strand of DNA (daughter strand). New strands of DNA (leading and lagging strand) are synthesized as the complementary of mother strands. DNA polymerase extends DNA in 5'-3' direction by the help of a primer that provides 3'-OH. Both leading and lagging strands need a primer as a support for the initial synthesizes of DNA and this primer is removed after completion of DNA synthesis. When the piece of RNA primer at the terminal 3' end is removed then the DNA cannot be extended. Then it causes end replication problem and thus telomere becomes short in each replication [11]. Normally in somatic cells, telomerase gene expression remains silent or sleeping that cannot synthesize any telomere. However, cancer cell/tumor is found to have the high level of telomerase gene expression (telomerase switch on). When the level of telomerase concentration becomes very high it can easily bind to the telomere through the barrier of shelterin complex. Simply, when telomere becomes very short cell becomes senescence (no longer divide the cells). If this shortage of telomere is elongated by telomerase or other alternative telomere lengthening pathways, then this deprotected telomere causes malignant tumor (Fig. 1.2) [9].

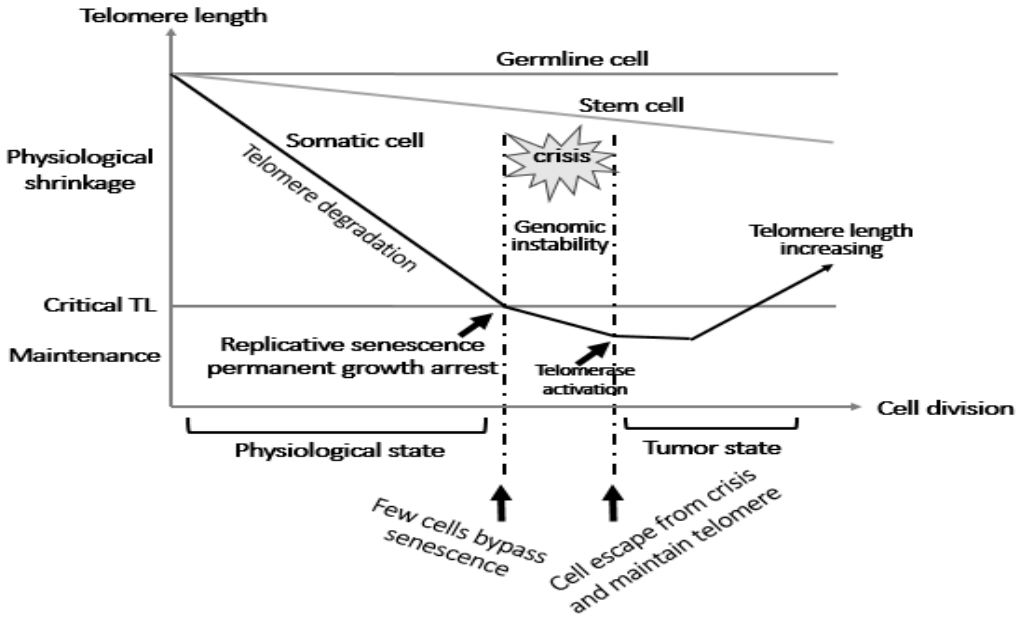

Fig. 1.2. Germline and stem cells usually do not lose their telomeric DNA after each replication but somatic cells lose their telomeric DNA. When cells lose their telomeric DNA in an excessive level then cell cycle checkpoint proteins recognize it as a damaged DNA. Then cell cycle delays for repairing damaged DNA or stops cell cycle to perform programmed cells death due to excessive damage. The damaged cells which cross the critical point then result in genomic instability. After this, if cells lose more telomeric DNA and cross the crisis level then cells become tumor in nature [9].

1.4. Circular chromosome-related disease in human

Circular chromosomes are unstable in eukaryotic cells that will induce genomic instability. This instability induces to chromosome loss or rearrangement which can result in other genomic instability of the cells. When DNA double-strand break occurs at both arms of the chromosome, these sticky ends induce to fuse with one another and form circular chromosomes. In addition, telomere dysfunction results in circular chromosome due to loss of telomere. Circular chromosomes are found in many

eukaryotes. Circular chromosomes in human have been linked to some genetic disease and some types of cancer [12]. Ring chromosome 20 causes epilepsy which is a neurological disease. It hampers the supply of electrical activity in the brain [13]. Dermatofibrosarcoma protuberans is a malignant tumor where cells contain about 70% circular chromosome. In addition, atypical lipomatous cells also contain about 85% circular chromosome. There are some other types of cancer cell that also carry circular chromosome such as, liposarcoma (21.1%), malignant mesenchymal neoplasia (10%), acute lymphoblastic leukemia (3.4%), malignant fibrous histiocytoma (11.5%), gallbladder (21.1%), pancreas (11.5%), breast (5.7%), lung (8.8%) and others [14]. There are some diseases that are related to the incidence of the circular chromosome in human. For instance, ring chromosomes 1, 2, 14,18 and 21 cause short stature, growth and intellectual developmental disorder. Ring chromosomes 2, 14, 18 and 21 cause microcephaly (head is usually smaller than normal size). Ring chromosome 14 causes distinct facial features, scoliosis and ocular anomalies such as abnormal retinal pigmentation. Ring chromosome 21 causes seizures, learning disabilities, heart defect and cleft lip and plate [15,16]. Thus, it is important for the researchers to find out the possible therapy which can reduce the risk of genomic instability of circular chromosome or kill the cancer cells that most of them contain circular chromosomes.

1.5. Clinical management of Circular chromosome

There is no possible therapeutic strategy to kill cancer cells that carry circular chromosomes. The conventional chemotherapy is barely used for the treatment of dermatofibrosarcoma protuberans (DFSP). Radiation therapy or imatinib mesylate have been used for treating DFSP. However, case reports did not show any significant values for the conventional therapy in the treatment of DFSP [17]. Excision of the tumor is

strongly proposed. Recently some studies are reported that the chromosome therapy is the best for treating circular chromosome associated disease. Understanding of the mechanism behind the survival of circular chromosome in eukaryotic cell will be helpful to develop the drugs to kill cancer in human.

1.6. The aim of this thesis

Telomere dysfunction induces genomic instability of the cells and cancer. Excessive loss of telomere leads to chromosome circularization which is a feature of some types of cancer and genetic diseases in human. Yet researchers did not know the mechanism of the survival of cells with circular chromosome in eukaryotes. In addition, there is no therapy proposed that can kill cancer cell carrying circular chromosome. The ultimate goal of my research is to kill cancer cells carrying circular chromosome. To acquire this goal, I want to identify the genes that are required for the survival of cells with circular chromosomes in *S. pombe*. In *S. pombe* most of the genes that are related to chromosome maintenance are conserved in mammalian counterparts. Therefore, it is expected that the human homologues of *S. pombe* genes that are required for the maintenance of circular chromosome will be required for the maintenance of circular chromosome in human. Identification of the maintenance of circular chromosome genes in *S. pombe* will be helpful to selectively kill human cancer cells that carry circular chromosomes. The inhibition of protein that is required for the maintenance of circular chromosome in human cancer cells carrying circular chromosome can kill cancer cells. Therefore, it is expected that the identification of *S. pombe* gene X (gene X is similar in *S. pombe* and human) that is required for the maintenance of circular chromosome will be helpful to selectively kill human cancer cells carrying circular chromosome by inhibiting of protein X produced from human homologue of *S. pombe* gene X.

Chapter 2. Fission yeast strains with circular chromosomes require the 9-1-1 checkpoint complex for the viability in response to the anti-cancer drug 5-fluorodeoxyuridine

2.1. Introduction

The cell cycle checkpoint signaling pathways are activated during the DNA damage. These pathways are important for maintaining the cellular integrity by repairing DNA, inducing apoptosis and arresting the cell cycle. In response to DNA damage, sensor proteins recognize the damage and block the cell cycle progression [18,19]. Likewise, cell cycle checkpoint protein complex, the 9-1-1 complex (Rad9-Hus1-Rad1) plays a role as a sensor of DNA damage [20]. The 9-1-1 complex is a heterotrimeric DNA clamp. The checkpoint proteins Rad1, Rad9 and Hus1 are similar in both human and *S. pombe* [21]. The 9-1-1 complex also denotes the structural similarity of the sliding clamp proliferating cell nuclear antigen (PCNA). The 9-1-1 complex is loaded onto DNA by Rad17-RFC complex [22]. DNA damage introduces to single stranded-DNA. The replication protein A (RPA) binds with this single-stranded DNA. RPA stimulates Rad17 to bind ssDNA, resulting in the loading of the 9-1-1 complex to this site [23]. Eukaryotic RFC complex consists of five subunits. The large subunit of RFC complex is replaced by cell cycle checkpoint protein Rad17 and forms Rad17-RFC2-5 complex [24]. Rad17-RFC2-5 complex binds to the 3′end of DNA and uses ATP to open the ring of the 9-1-1 complex. As a result, the 9-1-1 complex can encircle the DNA (Fig. 2.1) [21]. After that, the 9-1-1 complex activates DNA damage checkpoint proteins which cause cell cycle arrest and repair damaged DNA. The role of the 9-1-1 complex on the maintenance of circular chromosome is not known. *pot1*$^{+}$ is conserved from yeast to human and is essential for telomere maintenance. The deletion

of *pot1*$^{+}$ in *S. pombe* causes immediate degradation of telomere that results in chromosome circularization [25].

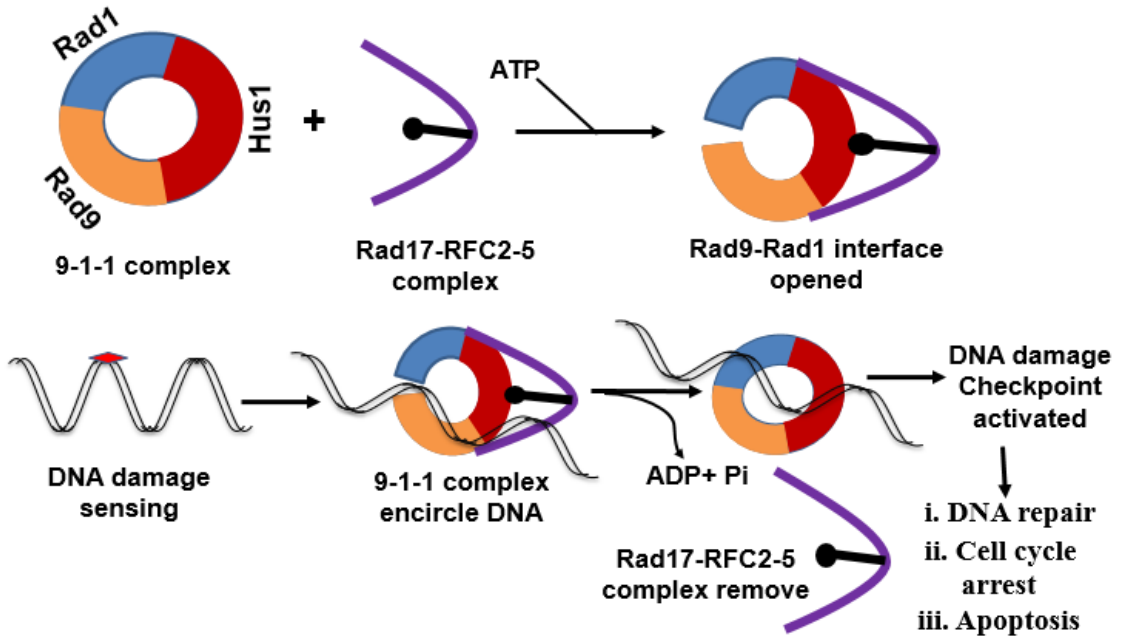

Fig. 2.1. After sensing DNA damage, the 9-1-1 complex opens their ring for encircling the DNA after binding with Rad17-RFC2-5 complex. After loading on to DNA, Rad17-RFC2-5 complex is released from the 9-1-1 complex and then 9-1-1 complex activates DNA damage checkpoint. Then cell cycle is arrested and damaged DNA is repaired.

DNA replication completely depends on the existence of available deoxyribonucleoside triphosphates. Deoxyuridine monophosphate (dUMP) is converted to deoxythiamine triphosphate (dTTP) in the presence of thymidylate synthase (TS) [26]. 5-fluorodeoxyuridine (Fudr) is used as a cancer chemotherapy. Fudr is phosphorylated to FdUMP by the activity of the thyamidine kinase (*tk* gene). FdUMP inhibits the thymidylate synthase that reduces or hampers the synthesis of dNTP [27]. The

imbalanced dNTP synthesis affects DNA replication that leads to DNA damage (Fig. 2.2) [28,29].

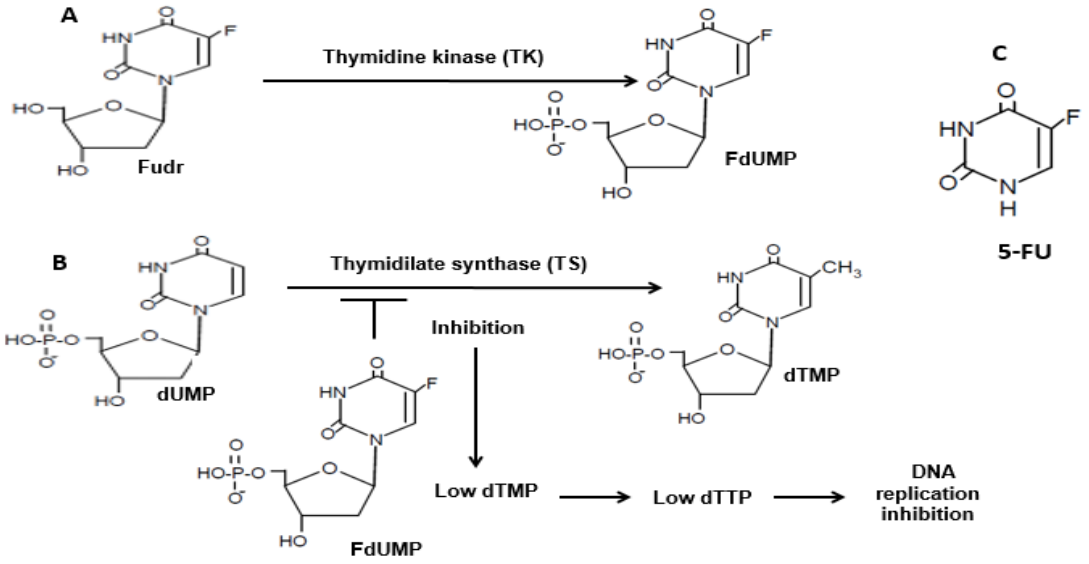

Fig. 2.2. (A) Fudr is converted to active (phosphorylation) FdUMP by the activity of thymidine kinase. (B) In cells, dUMP is converted to dTMP by the activity of thymidilate synthase which is required for the production of sufficient dNTP, necessary for the smooth DNA synthesis. Thymidilate synthase cannot work due to presence of active FdUMP that leads to stop or decrease the synthesis of dNTP and finally hampers the DNA synthesis. (C) Chemical structure of 5-FU.

Here, I investigated the effect of Fudr on fission yeast strains which have defect in DNA damage checkpoints and carry circular chromosomes. I found that the *hus1* single mutant is sensitive to Fudr. Notably, *pot1*Δ *hus1*Δ double mutant, which has circular chromosomes, exhibits greater sensitivity to Fudr than each single mutant (*pot1*Δ and *hus1*Δ*)*. My finding reveals that Fudr causes DNA replication arrest and induces DNA damage. I found that the 9-1-1 complex is required for viability in the absence of $pot1^{+}$ when DNA replication is arrested.

2.2. Materials and Methods

2.2.1. Strain construction and growth media

The strains used in this study are listed in Table 2.1. The *pot1*Δ *hus1*Δ double mutant which has the *pot1*$^{+}$ expressing plasmid (SH001), was created by deleting *hus1*$^{+}$ from YI002 by the lithium acetate transformation method using a *hus1::LEU2* DNA insert derived from SW794. The *pot1*Δ *rad1*Δ double mutant, which has the *pot1*$^{+}$ expressing plasmid (SH003), was constructed by excising *rad1*$^{+}$ from YI002 lithium acetate transformation method using a *rad1::LEU2* DNA fragment obtained from KT108. I used YEA plates containing 2mg/ml 5-Fluoroorotic acid (FOA) at 25^{o} C for the removal of *pPc27-ura4-pot1*$^{+}$*-HA* and finally obtained the *pot1*Δ *hus1*Δ and *pot1*Δ *rad1*Δ double mutants (SH002 and SH004). The *pot1*Δ *rad9*Δ double mutant, which contains the *pot1*$^{+}$ expressing plasmid (SH009), was generated by deleting from SH007 by the transformation method using a *rad9::ura4* DNA insert is taken from SH008. I used YEA plates containing 100µM Fudr, incubated at 36^{o} C for the removal of *pPC27-leu-pot1*$^{+}$ *-HA* to finally obtain the *pot1*Δ *rad9*Δ double mutant (SH010). To tag the Rad11 protein in *hus1*Δ (FY18394) and *pot1*Δ *hus1*Δ (SH002) cells with a monomeric red fluorescent protein (mRFP) at the C terminus, the pFA6a-mRFPnatMX6- rad11 plasmid was linearized with NspV and used to create SH005 and SH006 by transformation.

Table 2.1. *Schizosaccharomyces pombe* strains used in this study

Stain name	Genotype	Source
YI002	*h⁻ ade6 leu1-32 ura4-D18 pot1::kanMX6 pPC27-pot1⁺-HA*	[30]
SW794	*h⁻ ade6 leu1-32 ura4-D18 rqh1::kanMX6 hus1::LEU2 rad3::ura4⁺ his7*	Shao-Win Wang
SH001	*h⁻ ade6 leu1-32 ura4-D18 pot1::kanMX6 hus1::LEU2 pPC27-pot1⁺-HA*	This study
SH002	*h⁻ ade6 leu1-32 ura4-D18 pot1::kanMX6 hus1::LEU2*	This study
KT108	*h⁺ leu1-32 ura4-D18 ade6-M210 rad1::LEU2 tel1::ura4⁺*	Lab Stock
SH003	*h⁻ ade6 leu1-32 ura4-D18 pot1::kanMX6 rad1::LEU2 pPC27-pot1⁺-HA*	This study
SH004	*h⁻ ade6 leu1-32 ura4-D18 pot1::kanMX6 rad1::LEU2*	This study
1D	*h⁺ leu1-32 ura4-D18 his2-245 ade6-M216*	T. Toda
KTA037	*h⁻ leu1-32 ura4-d18 ade6 pot1::kanMX6*	[30]
FY18372	*h⁻ rad1::LEU2 leu1-32 ura4-D18*	NBRP
FY18394	*h⁻ hus1::LEU2 leu1-32 ura4-D18*	NBRP
TN004	*h⁺ rad11-mRFP::natMX6*	[30]
KTA038	*h⁻ leu-32 ura4-D18 ade6 pot1::kanMX6 rad11-mRFP::natMX6*	[30]
SH005	*h⁻ hus1::LEU2 leu1-32 ura4-D18 rad11-mRFP::natMX6*	This study
SH006	*h⁻ ade6 leu1-32 ura4-D18 pot1::kanMX6 hus1::LEU2 rad11-mRFP::natMX6*	This study
SH007	*h⁺ pot1::kanMX6 leu1-32 ura4-D18 ade6-M210 (pPC27-Leu-pot1⁺ -HA)*	Lab stock
SH008	*h⁻ leu1-32 ura4-D18 ade6 rad9::ura4⁺*	Lab stock
SH009	*h⁺pot1::kanMX6rad9::ura4⁺ -D18 ade6-M210 (pPC27-Leu-pot1⁺ -HA)*	This study
SH010	*h⁺pot1::kanMX6 rad9::ura4⁺*	This study

2.2.2. Fluorescence activated cell sorting (FACS)

S. pombe cells were cultured overnight in YEA liquid media to the concentration about $1x10^7$ cells/ml. Cultured 1 ml of cells are taken in microtube and centrifuged 2 min for 3000 rpm and discarded upper liquid portion. Then the cells were washed using sterile MiliQ and cells were fixed with addition of 300 µl 70% ethanol, and mixed using pipetting slightly. After that, centrifuged 15000 rpm for 3 min and discarded the liquid and added 1ml 50 mM sodium citrate and centrifuged again in the same condition and discarded liquid. Next, I added 200 µl sodium citrate and 10 µg/ml RNase and incubated about 2 h at 37°C. After that, 800 µl sodium citrate and 2.5 mg/ml propidium iodide (PI) are added for staining. Samples were then sonicated and analyzed using fluorescence-activated cell sorting (FACS) using a Becton Dickinson FACS Calibur.

2.2.3. Lactose gradient cell cycle analysis

S. pombe cells were grown overnight in YEA media of 100 ml to reach mid-log phase ($5x10^6$cells/ml) cells. Lactose gradients were prepared by using 10 ml aliquots of a 20% lactose solution in a clear 15 ml Falcon tube. The prepared sample was frozen at -80°C for 4 h and then thawed without causing any disturbance for 3 h at 30°C to generate a 10-30% gradient. Cells from the liquid culture were harvested by centrifugation at 3000 rpm for 3 min, and the cell pellets were re-suspended in 750 µl of sterile water. Then cells were slowly added to the top of the gradient using a cut off blue tip. Then the gradients were centrifuged at 1000 rpm for 8 min. The early G2 phase cells were brought by taking out around 0.1-0.4 ml from just down of side the top of a smear of cells using a cut off a blue tip. Collected cells were centrifuged at 13000 rpm

for 30 seconds in a microtube and resuspended in 500 µl media. After that, cells were incubated in YEA liquid media containing 300 µM Fudr at 30°C. The cell cycle of the sample was monitored using the sampling time of every 20 min from 0 to 300 min and staining with diamidino 2- phenylindole (DAPI) and counting the percentage of septated and mitotic cells under fluorescence.

2.2.4. Mating (Random spore analysis)

Same amount of freshly growing h^+ and h^- strains were taken in a microtube. Then 10 µl sterile water was taken and mixed properly by pipetting. Later on, a spot on the malt extract (ME (3% Bacto TM malt extract)) media to an area of about 1 cm was made and kept some times for drying it. After drying up the ME plate is incubated at 25°C about 4 days. Then 10 µl sterile water and some strain from the ME plate were taken on a slide. The strains were observed by a microscope to find out the formation of spore. After confirming the spore-forming, 100 µl sterile water was taken into a microtube and added 1 µl 0.5% glusulase, and mixed by slightly vortex. Then it was incubated around 8 h at 25°C. Usually, glusulase enzyme destroys the single cell membrane and then cell dies. On the other hand, spore-forming cell resists the glusulase enzyme and cell can survive. After incubating 8 h, cells were centrifuged 3000 rpm about 5 min at room temperature and discarded the liquid portion carefully. After an addition of 100 µl sterile water and mixing properly by pipetting, cells were spreading to the required media containing plate. The plate is then incubated at required temperature until the formation of colonies.

2.2.5. Lithium acetate transformation method

Ten ml of YEA or required liquid medium was taken to culture cells and

incubated overnight until reaching cells density around 1× 10^7 cells/ml. Cells were collected by centrifugation at 3000 rpm for 5 min and washed with sterile water (20 ml), and again performed centrifugation at 3000 rpm for 5 min to collect cells in a microtube. After that, cells were re-suspended with 0.1 M lithium acetate [pH 4.9] of 75 µl to reach a cell density 1×10^9 cells/ml. In another microtube, 290 µl polyethylene glycol was taken. Then two microtubes were incubated around 1h at 30°C. After incubation, 15 µl of desired PCR product or plasmid DNA (1500-2000 ng) was added to lithium acetate containing microtube. Then, the incubated 290 µl polyethylene glycol was also added into microtube containing the cells and mixed for a very short time by vortex. The mixture was incubated for 1h at 30° C. After that, cells were exposed to 42° C for 15-20 min and cooled down at room temperature for about 10 min. The cells were centrifuged at 3000 rpm for 4 min and the liquid was carefully discarded. Next, the cells were incubated with 10 ml ½ YEA liquid medium for 2 h. Then the cells were centrifuged 3000 rpm for 5 min and transferred to microtube and re-suspended in 100 µl sterile water to spread on the plate required medium, and incubated at the required temperature for 2-4 days and observed the formation of colonies.

2.2.6. Direct transformation method

Ten ml of YEA liquid medium containing cells were cultured and incubated overnight for getting cell density around 1× 10^7 cells/ml. One ml of cell culture was transferred into a microtube and centrifuged 4000-5000 rpm for about 2-3 min and was discarded the supernatant. The cells were re-suspended in 25 µl sterile water and mixed slightly by pipetting. Then, all cells (25 µl), 6 µl DNA PCR product, 90 ul transformation reagent and 4 µl carrier DNA (cDNA) were taken into a new microtube and mixed for a short time by vortex. The containing microtube was then incubated at

37° C for 2h. The cells were centrifuged at 5000 rpm for 3 min and the liquid was discarded. Then 1 ml of ½ YEA media was taken and the cells were transferred into a conical flask for shaking culture for 2 h at 30° C. After that, cells were collected using centrifugation and 100 µl sterile water was added for spreading cells on the plate containing required medium.

2.2.7. Measurement of telomere length

Telomere was identified using Southern hybridization. Ten ml of exponentially growing log-phase cells were collected using centrifugation at room temperature at 5000 rpm for 10 min. The solution was discarded and the cells were transferred into a microtube and re-suspended and the liquid was discarded again. One-hundred µl lysis buffer (2% Triton X100, 1% SDS, 10 mM Tris-HCl [pH 8.0], 10 mM EDTA, 5 Mm NaCl),100 µl volume of acid-washed glass beads and 100 µl of phenol/chloroform were added, and mixed by vortex at high speed for 20 min at 4°C. Taking out from the vortex, 100 µl lysis buffer, 100 µl phenol/chloroform and 200 µl TE buffer (10 mM Tris-HCl [pH 8.0], 1 mM EDTA) were added and then centrifuged at 15000 rpm for 10 min at room temperature. The aqueous layer was then transferred to a new microtube and 400 µl 2-propanol and 40 µl 3 M sodium acetate [pH 5.2] were added and mixed by hand up and down, and waited for around 10 min. The mixture was centrifuged at 15000 rpm for 10 min at room temperature (the DNA is precipitated at this stage). The DNA pellet was washed with about 500 µl 70% ethanol and centrifuged at 15000 rpm for 5 min at room temperature. The ethanol was discarded and the DNA was dried up and re-suspend in 100 µl TE buffer. Since, this extracted DNA contains both DNA and RNA, 1 µl of 1µg/ml RNase solution was added and incubated at 37°C about 4 h to degrade the RNA. After that, the degradation of RNA was confirmed using gel electrophoresis. After

confirmation of RNA degradation, 100 µl of phenol/chloroform was added and centrifuged at 15000 rpm for 10 min at room temperature. the upper portion containing DNA was transferred into a new micro tube and added 2.5× 100% ethanol and 0.1× 3M sodium acetate and kept at -20°C for ~ 30 min to allow the precipitation of DNA. Then the precipitation of DNA was collected by centrifugation at 15000 rpm for 10 min at 4°C, washed with 500 µl 70% ethanol and centrifuged at 15000 rpm for 5 min at 4°C. The ethanol was discarded and the DNA was dried up. The genomic DNA was then digested with *Eco*RI and separated using 1.5% agarose gel. After that, the vacuum blotting system (hybridization tank) was set and nitrocellulose membrane paper was placed on the middle. Then, the gel was placed on the membrane and mounted with denaturation buffer first (1.5 M NaCl,0.5 N NaOH) for 12 min and the denaturation buffer was discarded using vacuum pipe, then aspirated and mounted with neutralization buffer (1 M Tris-HCl [PH 5.0], 2 M NaCl) for 12 min and the buffer was discarded using vacuum pipe, then aspirated and mounted with 20x SSC (3 M NaCl, 0.3 M trisodium citrated dehydrate) for 1 h. Then the membrane was cross-linked by using UV cross-linker (1200 J/m3) and probed with DNA fragment containing telomere and telomeric-associated sequence (TAS1). The probe DNA was derived from a plasmid pSNU70. Then the probe DNA was amplified by PCR with pITNI as a template and purified using Gel Extraction Kit (QIAGEN). The probe hybridization was conducted according to the manufacturer protocol (AlkPhos DirectTM, GE Healthcare). The single-stranded telomeric DNA probe was labeled with (γ-^{32}P ATP) (GE Healthcare) by using T4 polynucleotide kinase. The membrane was then hybridized overnight with the labeled probe in hybridization buffer (Rapid-Hyb Buffer; GE Healthcare) with the rotary shaker at the 10 pmol probe at 55°C.

2.2.8. Pulsed-field gel electrophoresis (PFGE)

Fifty ml of exponential growing log-phase cells were harvested using centrifugation at 5000 rpm for 3 min. The liquid was discarded and the cells were mixed briefly in Zymolase enzyme (2 mg Zymolase dissolved in 1ml SP1 buffer, containing 1.2 M sorbitol, 50 Mm sodium citrate, 30 mM $Na_2HPO4. 12H_2O$, 40 mM EDTA pH [5.6]) where cell density of 5.5×10^8 cells/ml and incubated for 1h at 37° C. The cells were separated in four microtubes, then collected by centrifugation at 5000 rpm for 3 min. and re-suspended in 25 µl TSE buffer. Fifty mg low-melt preparative grade agarose (Bio-Rad, Richmond, CA) was dissolved in 5 ml TSE buffer (10 mM Tris HCl [pH 7.5], 0.9 M sorbitol, 45mM EDTA) in a small conical flask and kept it at 50° C for using. Sixty-five µl of agarose and 25 µl of TSE at 50° C were taken into microtube containing cells and mixed properly by pipetting and then this suspension was poured into the plug molds. Then it was kept at 4° C for 20 min for solidification. This solidified plugs were washed in PW1 (50 mM Tris-HCl [pH 7.5], 0.25 M EDTA, 1% SDS) for 2 h at 50° C and then it was transferred to PW2 (10 mM Tris-HCl [pH 9.0], 0.25 M EDTA, 1% wt./vol. N-lauroyl sarcosine, 1 mg/ml proteinase K) and incubated for 24 h at 50°C. The plug was again incubated at 50°C in fresh PW2 for 24 h, and then washed two times with 10 ml T10×E (10 mM Tris-HCl [pH 7.5], 10 mM EDTA), and incubated at 4°C for 30 min. After that, the plug was treated two times with 10 ml T10×E and 23 µl phenylmethylsulfonyl fluoride (PMSF) and incubated at 50°C for 30 min. The plug was washed twice 10 ml T10×E and incubated at room temperature for 30 min. The plug was washed twice in 1 ml sterile TE buffer and incubated at 4°C for 16 min. The plug was then transferred to 50 ml Falcon tube containing 10 ml 3 buffer (NEB) and 100 µl BSA (10 µg/ml) and incubated overnight at 37° C. Then the plug was

transferred into the new microtube that containing 300 µl 3 buffer, 3 µl BSA and 4 µl NotI and incubated at 37°C for 2 h. Next, additional 4 µl NotI was added and incubated overnight at 37° C. Then the plug was transferred into a new microtube added with 1 ml T10×E and incubated at 4° C for 30 min. After that, the plug was transferred to a new microtube and added 1ml 0.5×TBE buffer (50mM Tris-HCl, 5 mM boric acid, 1 mM EDTA, [pH 8.0] and incubated at 4°C for 30 min. NotI-digested chromosomal DNA was then used for fractionating in 1% agarose gel with 0.5% sterile TBE buffer at 14°C with CHEF Mapper PFGE system at 6 v/cm (200 V) and pulse time of 60-120s over 24 h. The DNA was then visualized by staining the gel with ethidium bromide (EtBr) (200 ml 0.5×TBE + 200 µl EtBr). After that, the DNA was blotted using Hybond-N+ nylon membrane and Southern hybridization was performed using probes that were amplified from pITNI by PCR and purified with Gel Extraction Kit to M, L, I, C probes [31,32].

2.2.9. Microscopy

Microscope images of living cells were acquired using an AxioCamdigital camera (Zeiss) connected to an AxioObserver.Z1microscope (Zeiss) with a Plan-Apochromat 63% objective lens (numerical aperture, 1.4). Pictures were taken and examined using AxioVision Rel.4.8.2 software (Zeiss). A glass-bottom dish (Iwaki) was coated with 5mg/ml lectin from *Bandeiraea simplicifolia* BS-I (Sigma).

2.2.10. Statistical analysis

Two independent experiments data were used to one-way analysis of variance (ANOVA) following by Duncan's multiple range tests. Data analysis were conducted using statistical applications and differences were maintained significance level at an alpha of 0.05. The statistical program was used Stat-View^{R} 5.0 (Mind Vision Software, Abacus, Concepts, Inc. Berkeley, CA, USA).

2.3. Results

2.3.1. Removing *pot1*$^+$ expressing plasmid from *pot1*Δ and the 9-1-1 complex double mutant strain

I created *pot1*Δ *rad1*Δ and *pot1*Δ *hus1*Δ double mutants carrying a plasmid expressing the *pot1*$^+$ and *ura4*$^+$ genes. Then I selected the loss of *pot1*$^+$ expressing plasmid on YEA containing FOA plate. The cell that carries *ura4*$^+$ accumulates a toxic intermediate in the presence of FOA. The cell that only had lost the *pot1*$^+$ expressing plasmid, which carries the *ura4*$^+$ gene, can survive. The colonies from the FOA plates were also streaked on plate containing EMM3+A to confirm the plasmid loss. Uracil was not added in the medium to confirm the plasmid loss because *pot1*$^+$ expressing plasmid carrying *ura4*$^+$ marker gene. If the strain carries plasmid it must be grown on plate containing EMM3+A. However, the strains could not grow on plate containing EMM3+A. This result suggests the loss of plasmid from the strain (Fig. 2.3). The colonies that did not grow on this selective media were selected to analyze the chromosome phenotype. If the 9-1-1 complex is required for the maintenance of circular chromosome, the double mutant will not survive. For this reason, I created *pot1*Δ and the 9-1-1 complex double mutant strain with linear chromosome using a plasmid expresses *pot1*$^+$. I successfully obtained the double mutant of *pot1*Δ and the 9-1-1 complex. This result suggests that *pot1*Δ and the 9-1-1 complex double mutant is not lethal.

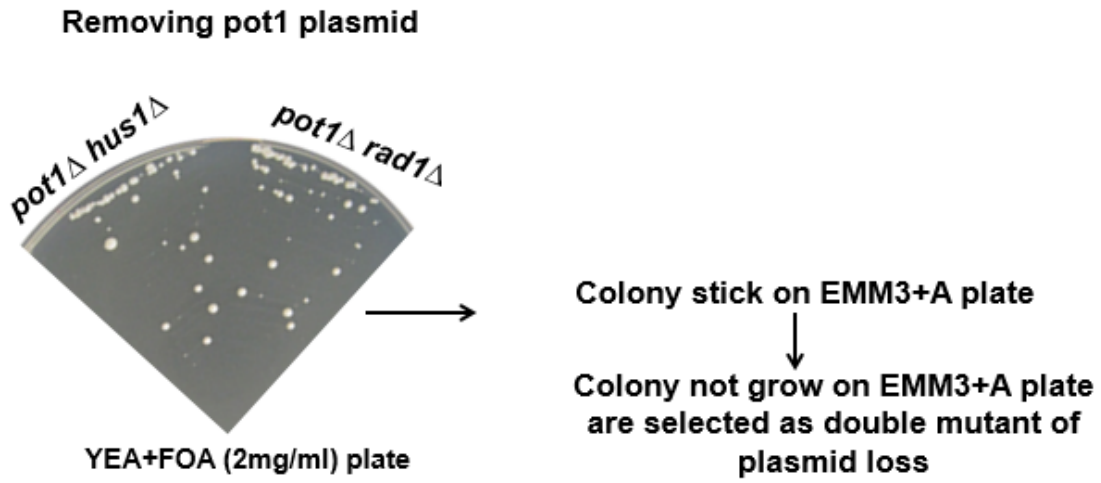

Fig. 2.3. *pot1*Δ *hus1*Δ and *pot1*Δ *rad1*Δ double mutant strains are streaked on YEA containing FOA plate to counter select the strain that lost *pot1*$^{+}$ expressing plasmid. The colonies then were streaked on plate containing EMM3+A to confirm about the plasmid lost and the colonies those did not grow on this selective plate were selected as plasmid loss.

2.3.2. *pot1*Δ *hus1*Δ and *pot1*Δ *rad1*Δ double mutants completely lost their telomeric DNA and had circular chromosomes

The *pot1* deletion leads to a complete loss of telomeric DNA. Then survival of cell relies on the circularization of chromosomes [33]. I wanted to know whether the *pot1*Δ and the 9-1-1 complex double mutant strains carry telomeric DNA or not. Then, I assessed telomere loss of these double mutant strains using Southern blotting. The loss of telomere indicates the chromosome circularization [33]. I used wild-type, *hus1*Δ *and* *rad1*Δ single mutant and *pot1*Δ *hus1*Δ and *pot1*Δ *rad1*Δ double mutant strains. I found that double mutant lost their telomeric signal but wild-type cells and the single mutant of the 9-1-1 complex (*hus1*Δ*, rad1*Δ*)* showed strong telomere signal (Fig. 2.4. A). These results demonstrate that both *pot1*Δ *hus1*Δ and *pot1*Δ *rad1*Δ double mutants carry

circular chromosomes.

Next, I monitored the chromosome structure by PFGE to confirm the chromosome circularization. The presence of NotI-digested chromosomal end fragments of M, L, I and C reveal linear chromosome. On the other hand, presence of C+M and L+I bands (chromosomal end fragments join together), and absence of chromosomal end fragment bands of M, L, I, and C reveal the circular chromosome. I detected chromosomal end fragment bands of M, L, I, and C only in wild-type cells. On the other hand, I detected only C+M and L+I bands both in *pot1*Δ *hus1*Δ and *pot1*Δ *rad1*Δ double mutant strains. This finding is similar in *pot1*Δ single mutant, which has circular chromosomes (Fig. 2.4. C). These results suggest that *pot1*Δ *hus1*Δ and *pot1*Δ *rad1*Δ double mutants have circular chromosome. I found L+I band in *pot1*Δ *rad1*Δ double mutants. On the other hand, I did not find C+M band in the expected position. I thought that the C+M band could be overlapped with L+I band in *pot1*Δ *rad1*Δ double mutant. To test this possibility, I performed probing using C, M and I+L separately. I found that C+M band overlapped with L+I band. This may be due to the chromosome rearrangement which occurred in breakage-fusion-bridge cycles after telomere loss. Overall, there results suggest that *pot1*Δ *hus1*Δ and *pot1*Δ *rad1*Δ double mutant have circular chromosomes.

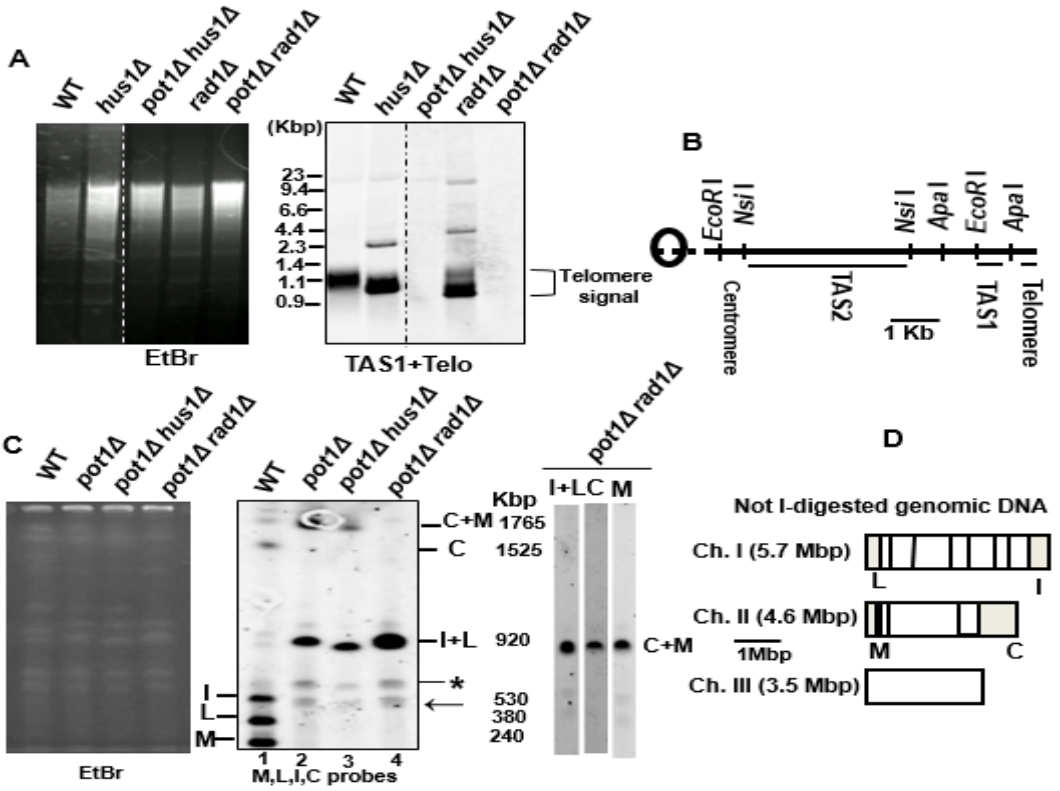

Fig. 2.4. (A) The wild-type, *hus1*Δ, *pot1*Δ *hus1*Δ, *rad1*Δ and *pot1*Δ *rad1*Δ cells were used to examine the telomere using Southern hybridization. *Eco*RI was used for genomic DNA digestion and DNA separated by 1.5% agarose gel electrophoresis. A telomere fragment (Telo) plus telomere-associated sequence (TAS1) was derived from pNSU70 [34] which was used as a probe. To estimate the total amount of DNA, the gel was stained with ethidium bromide (EtBr) prior to being blotted onto the membrane. The band with strong telomere band indicated as a telomere signal. The weak bands that were found in the above of telomere band may be non-specific bands or telomere bands that did not digest completely by *Eco*RI. The marker sizes are also mentioned **(B)** Restriction enzyme sites diagram around the telomere and telomere related sequences of TAS1 and TAS2 chromosome arm cloned in the plasmid pNSU70 [34]. **(C)** Chromosomal DNA in the wild-type, *pot1*Δ, *pot1*Δ*hus1*Δ, and *pot1*Δ *rad1*Δ cells were digested with NotI to examine the chromosome circularization using PFGE. The wild-type cell's linear chromosomes and the *pot1*Δ cell's circular chromosomes were used as a control in this experiment. In left EtBr stained PFGE agarose gel image was mentioned. (Middle) Specific probes for the telomeric NotI fragments (M, L, I, and C) were used. The asterisk in all lanes and arrow in lane 2,3 and 4 are indicated the non-specific band. The size of chromosome end fragments (I,L,M,C,C+M, I+L) were mentioned [32]. (Right) NotI fragments as telomeric probe I+L, C and M were separately used to distinguish C and M band, where C+M band was overlapped with I+L band. **(D)** The restriction site map of NotI of *S. pombe* chromosomes. Chromosomes I, II, and III (Ch. I, Ch. II, and Ch. III) are shown and the scale represents 1Mbp

2.3.3. *pot1*Δ and the 9-1-1 complex double mutant cells are sensitive to Fudr

Circular chromosomes have topological stress that may cause more replication stress. To assay the sensitivity of *pot1*Δ *hus1*Δ and *pot1*Δ *rad1*Δ double mutant cells to replication stress, I treated cells with agent causing replication stress, hydroxyurea (HU) and methyl-methanesulfonate. I performed serial dilution of wild-type, *pot1*Δ*, rad1*Δ*, hus1*Δ*, pot1*Δ *hus1*Δ and *pot1*Δ *rad1*Δ strains. I found that *pot1*Δ *hus1*Δ and *pot1*Δ *rad1*Δ double mutant cells exhibited comparatively greater sensitivity to HU and MMS than that shown by each single mutant strain of *pot1*Δ, *hus1*Δ*,* and *rad1*Δ (Fig. 2.5. A). HU inhibits ribonucleotide reductase I, which is required for the synthesis of dNTPs [35]. HU treatment causes dNTP depletion which leads to replication arrest and genomic instability. Cell treated with MMS also causes replication arrest by modifying the bases guanine to 7-methylguanine and adenine to 3-methyladenine [36,37]. *pot1*Δ cells treated with MMS showed more sensitivity compared to the 9-1-1 complex single mutant. Then for searching other drugs that cause replication stress to *pot1*Δ and the 9-1-1 complex double mutant strains, I used Fudr and 5-FU. I found that the *pot1*Δ *hus1*Δ*, pot1*Δ *rad1*Δ and *pot1*Δ *rad9*Δ double mutant cells were more sensitive to Fudr than each single mutant (Fig. 2.5. A, B). This data suggested that Fudr causes replication stress to *pot1*Δ and the 9-1-1 complex double mutant cells like HU. However, 5-FU did not generate synthetic lethality either *pot1*Δ and the 9-1-1 complex double or single mutant cells (Fig. 2.5. A).

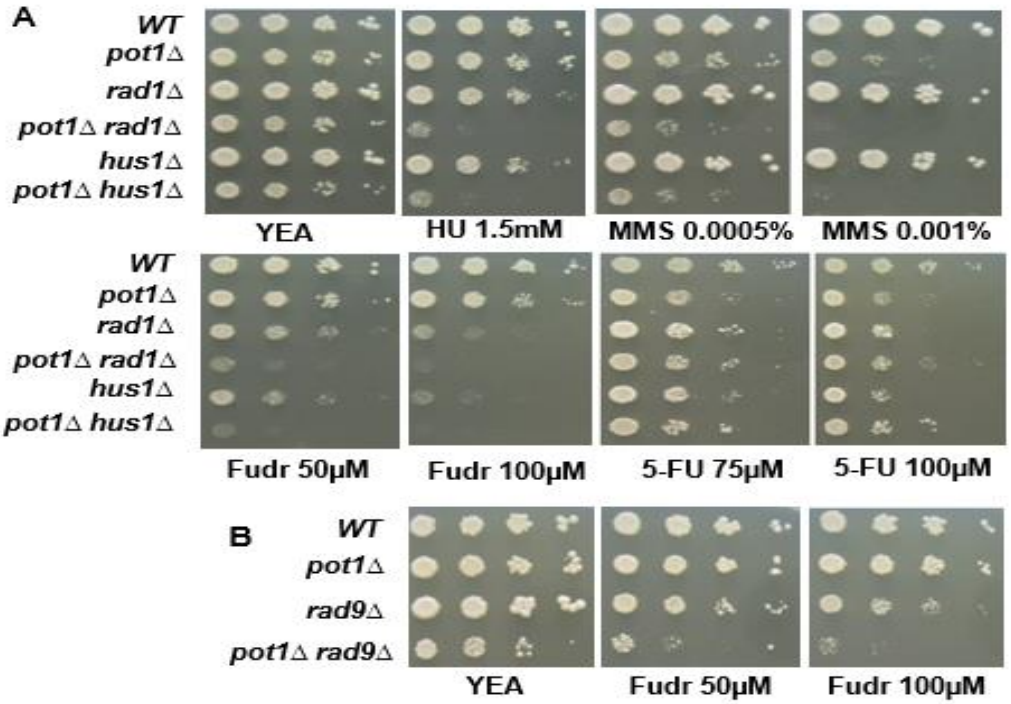

Fig. 2.5. *pot1*Δ and 9-1-1 complex double mutant strain are sensitive to HU, MMS and Fudr. **(A)** Wild-type, *pot1*Δ, *rad1*Δ, *pot1*Δ *rad1*Δ, *hus1*Δ, and *pot1*Δ *hus1*Δ cells were used to investigate the sensitivity by using serial dilution. Logarithmically *S. pombe* (2×10^7 cells/ml) growing cells of 10-fold serially diluted were used for spotting onto YEA plates as the control and on YEA plates containing HU, MMS, Fudr, or 5-FU at the mentioned concentrations. The plates were incubated at 30°C about four days and observed the cells growth. **(B)** Wild-type, *pot1*Δ, *rad9*Δ, and *pot1*Δ *rad9*Δ cells were used for checking the sensitivity using serial dilution (spotting assay) on YEA containing Fudr plate and incubated around 4 days at 30°C.

2.3.4. Fudr exposure causes replication arrest of *pot1*Δ *hus1*Δ cells

Researchers believe that Fudr does not work in fission yeast, because Fudr activation needs thymidine kinase where fission yeast does not express thymidine kinase. However, interestingly I found Fudr toxicity in *S. pombe* cells. I assumed that

Fudr could be converted to FdUMP in *S. pombe* which leads to DNA replication inhibition. To understand the mechanism behind the detrimental effects due to exposure to Fudr on *S. pombe* cells, I first performed FACS analysis of cell cycle progression in wild-type, *pot1*Δ, *hus1*Δ and *pot1*Δ *hus1*Δ cells from 0 h (no drugs) to 3 h treatment with 12 mM HU (Fig. 2.6. A). It is known that HU causes replication arrest in S phase. In fission yeast, cell spends about 70% to 80% of the cell cycle in G2 phase that shows 2C (2 set of chromosomes). As expected, cells from wild-type, *pot1*Δ, *hus1*Δ, and *pot1*Δ *hus1*Δ were arrested in S phase after treating with HU following the incubation period of 3 h. After that, I performed the same experiment using 300 μM Fudr following the incubation period of 3 h. I found Fudr also arrested the cell cycle in S phase in all strains like HU (Fig. 2.6. B). Therefore, these data suggested that Fudr causes DNA replication arrest in *S. pombe* cells.

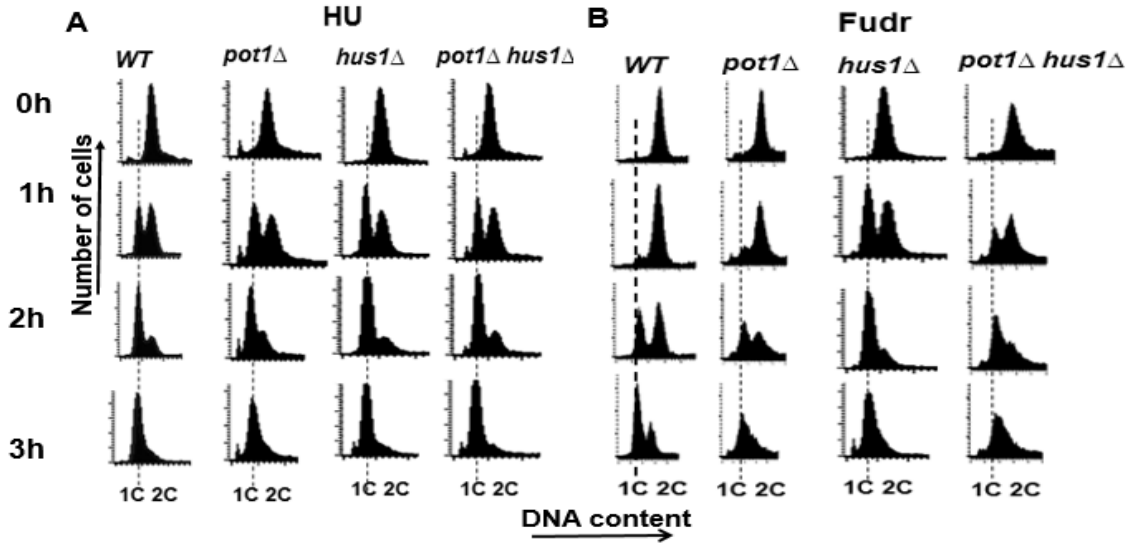

Fig. 2.6. (A, B) To analyze the cell cycle using FACS, wild-type, *pot1*Δ, *hus1*Δ, and *pot1*Δ *hus1*Δ cells were incubated with 300 μM Fudr and 12 mM HU following the incubation time 0 h, 1 h, 2 h and 3 h at 30°C. The x-axis indicates the number of cells and y-axis indicates the content of DNA.

2.3.5. *pot1*Δ *hus1*Δ cells treated with Fudr or HU resulted in a high frequency of chromosome segregation defects

I found that the double mutants were more sensitive to replication arrest. However, I did not know why they were more sensitive to replication arrest. Replication arrest may cause chromosome segregation defects and DNA damage. To study these, first I investigated the effect of Fudr and HU on chromosome segregation defects using RPA-mRFP (Rad11-mRFP)-expressing cells. Rad11 is a large subunit of RPA. RPA localizes to a hole nucleus [31]. I determined the percentage of chromosome segregation defects in wild-type, *pot1*Δ*, hus1*Δ and *pot1*Δ *hus1*Δ double mutant cells in the presence of 300 μM Fudr, and 12 mM HU following incubation time of 0 h to 3 h at 30°C. The chromosome segregation defects of *hus1*Δ and *pot1*Δ *hus1*Δ cells were increased by 8.9 fold and 9.5 fold, respectively, compared to *pot1*Δ cells and 23.2 fold and 25.7 fold, respectively, compared to wild-type cells due to treat cells with Fudr. Similarly, I found that chromosome segregation defects of HU treatment are almost same as Fudr treatment (Fig. 2.7. B, C). Therefore, $hus1^{+}$ is required for the proper chromosome segregation of both in circular and linear chromosomes when replication is arrested.

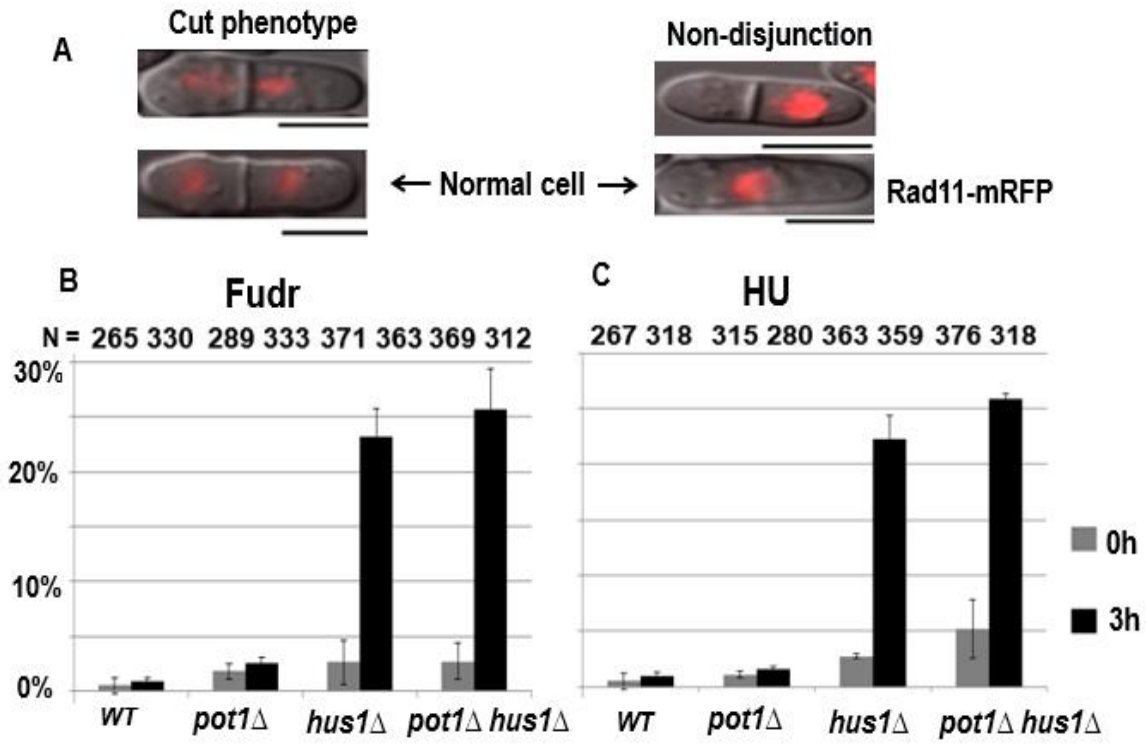

Fig. 2.7. Fudr induces chromosomal segregation defects (A) To analyze chromosome segregation defects, wild-type, *pot1*Δ, *hus1*Δ, and *pot1*Δ *hus1*Δ cells were incubated with 300µM Fudr for 3 h at 30°C. Chromosome segregation defect of the Rad11-mRFP cell was shown, like cut phenotype where septum bisects the nucleus (left side) and non-disjunction where chromosome fails to separate in equal amount in each pool (right side). Scale bar under the images represent 5µm. **(B-C)** To analyze the chromosome segregation defects, Rad11-mRFP wild-type, *pot1*Δ, *hus1*Δ, and *pot1*Δ *hus1*Δ mutant cells were incubated with 300 µM Fudr and HU for 3 h at 30°C. All types of segregation defects were counted together. Percentages of defects in chromosome segregation in cells at time 0 h and 3 h following the exposure to Fudr and HU were shown. The segregation defect was monitored in following two independent experiments and the bar charts indicated the average values ± standard error. The y-axis indicated the percentage of cells that showed chromosome segregation defects among the total number of cells. The total number of cells were examined (N) in this experiment mentioned above of the chart.

2.3.6. *pot1*Δ *hus1* cells treated with Fudr or HU resulted in the increase of DNA damage

Next, I conducted the experiment to determine the level of DNA damage using RPA-mRFP (Rad11-mRFP)-expressing cells. Usually, RPA accumulates at the damaged DNA [31, 38] (RPA binds to ssDNA) and more RPA formation denotes the more DNA damage [39]. I used wild-type, *pot1*Δ, *hus1*Δ and *pot1*Δ *hus1*Δ double mutant cells in the presence of Fudr 300 μM and HU 12 mM following incubation time of 0 h to 3 h at 30° C. The percentage of cells containing RPA foci of *pot1*Δ *hus1*Δ double mutant cells treated with Fudr, and HU after 3 h incubation is found to increase 3 fold and 2 fold, respectively, compared to *hus1*Δ single mutant (Fig. 2.8. A, B). Detection of RPA foci likely represents an increase in DNA lesions. I thereby concluded that Fudr and HU cause S-phase related DNA damage in checkpoint-defective and circular chromosome containing (absence of $pot1^{+}$) cells.

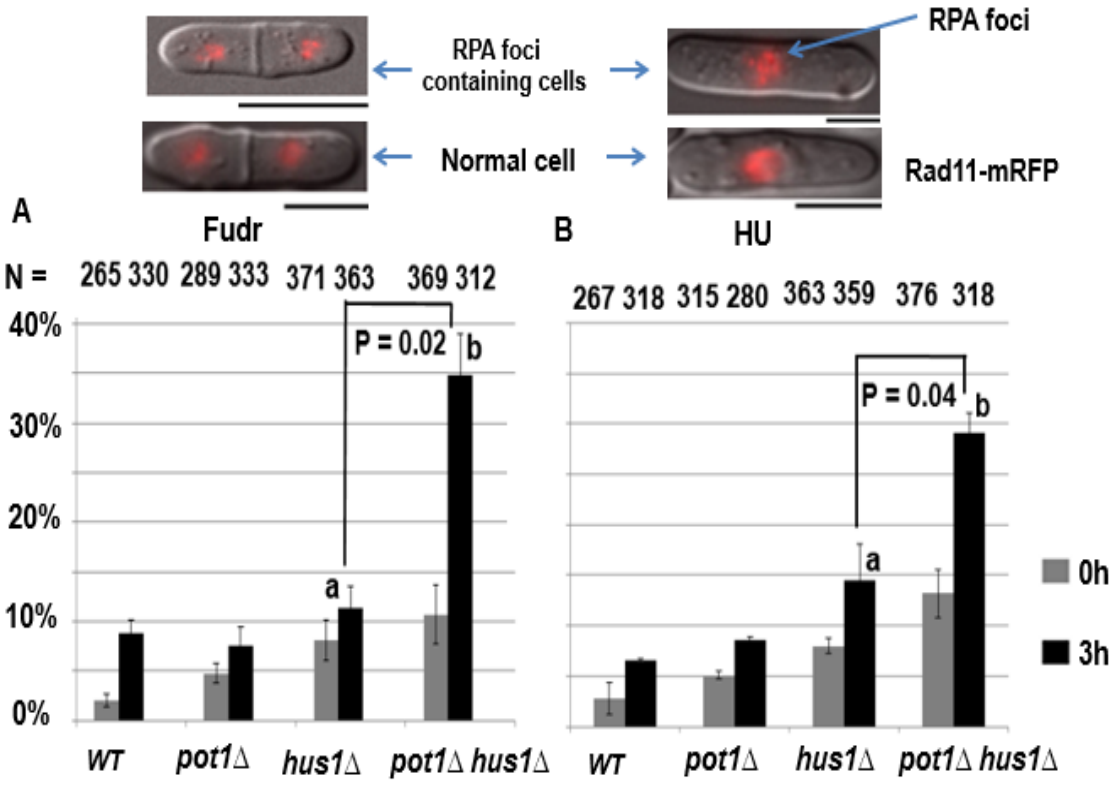

Fig. 2.8. Fudr treatment results in more ssDNA. The representative images of normal and RPA foci containing of Rad11-mRFP cells are shown. The bar under the image corresponded to 5µm. **(A-B)** Rad11-mRFP carrying wild-type, *pot1*Δ, *hus1*Δ, and *pot1*Δ *hus1*Δ mutant cells were incubated with 300 µM Fudr and 12 Mm HU following 3 h at 30°C. Percentages of RPA foci in cells at the time point 0 h and 3 h following the exposure to Fudr and HU were counted. Two independent experiments were used and the bar charts indicated the average values ± standard error. The y-axis indicated the percentage of cells that resulted in RPA foci among the total number of cells. The total number of cells that were examined (N) in this experiment mentioned above of the chart.

2.3.7. Fudr treatment causes chromosome segregation defects after S phase progression.

I found that Fudr causes chromosomes segregation defects and DNA damage. However, I did not know which one happened first. To study this, I synchronized cells using lactose gradient to obtain the early G2 phase cells. I used YEA liquid medium which contains 300 μM Fudr and following incubation time of 0 min to 300 min at 30°C. I analyzed wild-type, *pot1*Δ*, hus1*Δ and *pot1*Δ *hus1*Δ double mutant cells using diamidino 2 phenylindole (DAPI) staining in every 20 min. I found that the percentage of chromosome segregation defects in *hus1*Δ and *pot1*Δ *hus1*Δ cells did not increase in the first M phase in the presence of Fudr. But I found chromosome segregation defects increased in *hus1*Δ and *pot1*Δ *hus1*Δ cells in second M phase in the presence of Fudr. I did not find any chromosome segregation defects in wild-type and *pot1*Δ cells in the presence and absence of Fudr (Fig. 2.9). These data suggested that Fudr treatment induces S phase problem in both *hus1*Δ and *pot1*Δ *hus1*Δ cells and chromosome segregation defects happen after S phase progression.

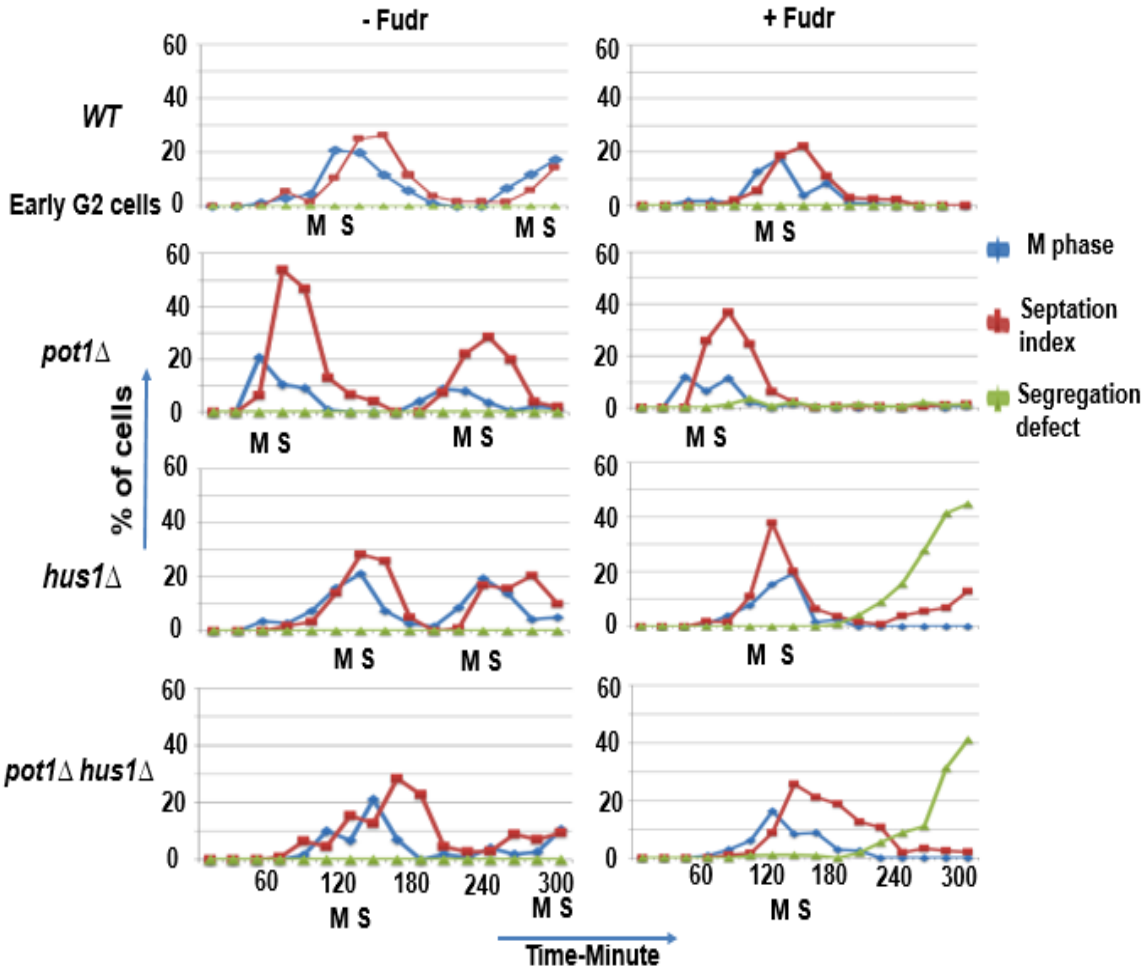

Fig. 2.9. Using lactose gradient wild-type, *pot1*Δ, *hus1*Δ and *pot1*Δ *hus1*Δ cells synchronized for obtaining early G2 phase cells. Cells were incubated in YEA liquid medium containing 300 µM Fudr and incubated 0 min to 300 min at 30° C and in every 20 min analyzed cells using diamidino 2 phenylindole (DAPI) staining and septation index. The y-axis indicated the percentage of cells that found M phase cells, septation index and chromosome segregation defects among the total number of cells, and x-axis indicated time (min).

2.3.8. Fudr treatment in *pot1*Δ *hus1*Δ cells resulted in DNA damage in S phase

Finally, I wanted to know when DNA damage increased in *pot1*Δ *hus1*Δ cells in the presence of Fudr. For this, I synchronized cells using lactose gradient to obtain

cells in early G2 phase. I incubated cells in liquid medium containing 300 μM Fudr and following incubation time of 0 min to 300 min at 30°C. I analyzed cells cycle progression in every 20 min. I found the percentage of cells with DNA damage multi foci increased at the time point 100 min (M or S phase) in *pot1*Δ *hus1*Δ double mutant cells in the presence of Fudr (Fig. 2.10. A). Next, to understand the exact phase for the increase of RPA foci (DNA damage), I analyzed the percentage of cells with bright clusters foci in M phase (no septum and bi-nucleate) cells and S phase cells (septated and bi-nucleate) separately during the time point 80 min and 100 min. Clusters of bright foci were found in S phase and treatment of cells with Fudr increased the bright clusters foci about 4 fold compared to unexposed cells (Fig. 2.10. C). Form these results, it is suggested that Fudr causes DNA damage in S phase of *pot1*Δ *hus1*Δ double mutant cells.

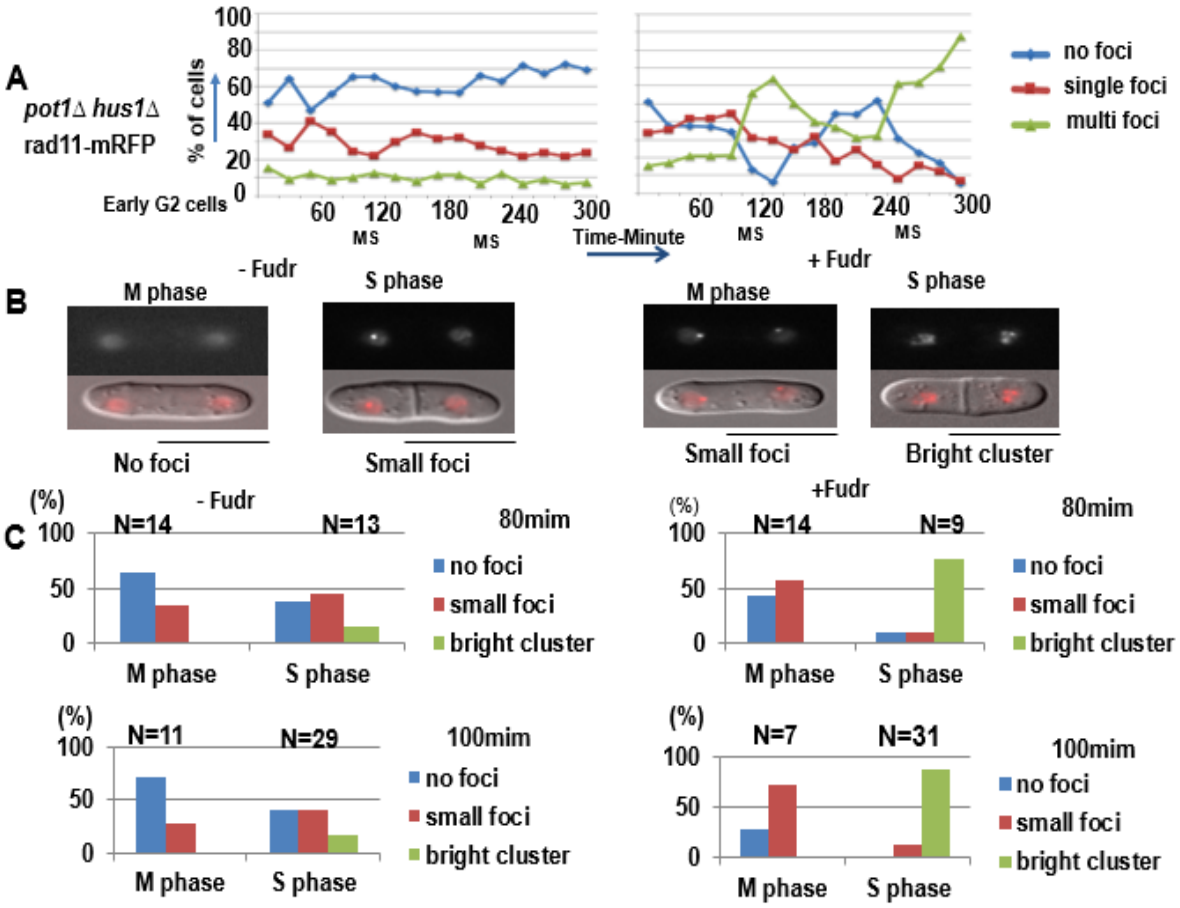

Fig. 2.10. (A) To analyze RPA foci, the *pot1*Δ *hus1*Δ cell was used that carrying Rad11-mRFP. Early G2 phase cells were obtained by using lactose gradient and cells were incubated in YEA liquid medium with 300 µM Fudr and following incubation time of 0 min to 300 min at 30° C. RPA Foci analyzed in every 20 min. The y-axis denoted the percentage of cells that showed no foci, single foci and multi-foci among the total number of cells. **(B)** M phase cells and S phase cells with no RPA foci, small foci and the bright cluster foci in the presence and absence of Fudr at the time 80 min and 100 min. The bar under the image's corresponded to 10 µm. **(C)** After release from early G2 phase cells, the number of no foci, single foci and bright cluster containing cells were counted.

2.4. Discussion

In human, circular chromosomes induce genomic instability, some types of cancer and other detrimental phenotypes. For example, atypical lipomatous tumors cells contain about 85% circular chromosomes and the cells of dermatofibrosarcoma protuberans contain about 70% circular chromosomes [40,14]. Therefore, it is important to find out the possible therapy that can kill cancer cells where most of the cells have circular chromosomes. However, the proteins involved in the stability of circular chromosomes are not known. Therefore, I made *pot1*Δ *hus1*Δ, *pot1*Δ *rad9*Δ and *pot1*Δ *rad1*Δ double mutant strains that have circular chromosomes. I found that *pot1*Δ and the 9-1-1 complex double mutant cells showed more sensitivity to agent causing replication arrest than each single mutant. This finding revealed that DNA damage checkpoints play important role in maintaining of circular chromosomes when DNA replication has been stalled.

The activity of Fudr in *S. pombe* cells yet are not properly understood. If *S. pombe* does not synthesize thymidine kinase, Fudr cannot be converted to FdUMP. I found that wild-type *S. pombe* cells showed only a slight decrease in growth rate on Fudr. On the other hand, cells cannot grow when thymidine kinase is produced. Thus, I used Fudr for counter selection to select the cells that have lost the plasmid expressing thymidine kinase [41]. Furthermore, it is reported that Fudr inhibits DNA synthesis and enhances the recombination frequency in *S. pombe* without causing ectopic expression of thymidine kinase [42,43]. These findings support my findings that Fudr affects *S. pombe* cells without expressing thymidine kinase. Both 5-FU and Fudr are used as an anticancer therapy. 5-FU is generally used for the treatment of colon and breast cancer.

Fudr is used for colon and ovarian cancer treatment [27,28]. 5-FU is intracellularly converted to FdUMP and fluorouridine triphosphate (FUTP) [44]. FdUMP affects the activity of TS and inhibits the DNA metabolism. Fudr also plays the same role as FdUMP [45,46]. In *S. pombe*, *pot1*$^{+}$ and the 9-1-1 complex double mutant cells were found to be more sensitive to Fudr but not 5-FU. This result suggested that Fudr affects DNA replication and repair but not RNA metabolism.

In comparison, in the bacterium *Escherichia coli*, dimeric chromosomes are transfigured to monomers by Xer site-specific recombination [47,48]. The defects of Xer mutants are suppressed by the inactivation of RecA (the Rad51 homolog in *E. coli*), suggesting that the RecA-dependent creation of chromosome dimers by homologous recombination (HR) is toxic in *E. coli* [49,50]. Rad51 assists in the repair of double-strand DNA break by promoting HR [51]. However, in circular chromosomes, HR between sister chromatids may induce crossing-over, creating a chromosome dimer and resulting in chromosome segregation defects [30]. In current study, I found that the deletion of *rad51*$^{+}$ did not suppress the synthetic lethality of *pot1*Δ *rad9*Δ double mutants in the presence of Fudr. This suggests that other possible factors may underlie the lethality of *pot1*Δ *rad9*Δ cells aside from dimer formation. One such possibility may be incomplete DNA replication in the presence of Fudr. The incomplete DNA replication may introduce to single-stranded DNA. Incomplete DNA replication also may cause a chromosomal segregation problem because of the physical link between sister chromatids. Fudr exposure also resulted in multi RPA foci and bright cluster RPA foci in *pot1*Δ *hus1*Δ double mutant cells in S phase but not in M phase. These data suggested that Fudr treatment leads to ssDNA in *pot1*Δ *hus1*Δ double mutant cells in S phase in checkpoint defective cells with circular chromosomes.

Chapter 3. Mutation in fission yeast phosphatidylinositol 4-kinase Pik1 is synthetically lethal with defect in telomere protection protein Pot1

3.1. Introduction

The phosphatidylinositol 4-kinase (PI4K) is a member of the phosphoinositide family which catalyzes the phosphorylation of phosphatidylinositol (PI) to phosphatidylinositol 4-phosphate {PI(4)P}. Phosphoinositide (PI) differentiates according to phosphorylation status and the presence of inositol head group. PI can phosphorylate in 3, 4 and 5 positions of the inositol [52]. Among these inositol, PI(4)P is the main lipid determinants in Golgi [53]. The yeast phosphatidylinositol 4-kinase, Pik1p, is homologous to human PI 4-kinase IIIβ. Pik1p is found to associate with Golgi [54,55]. Pik1 is involved in sorting proteins in Golgi. Golgi acts as a processing unit in cells by receiving proteins from the Endoplasmic Reticulum (ER). It also acts as the principle director of macromolecules by trafficking them into the cells. The Golgi consists of two main networks. One is *cis*-Golgi network (CGN). CGN receives proteins in vesicle from the ER. Another one is *trans*-Golgi network (TGN). TGN transports proteins from vesicles to the target organelles [56]. Golgi in cell acts like a post office. Golgi packages and labels proteins (post-translational modification) and then sends to the different specific parts of the cell or to the extracellular space. Functional defects in Golgi can result in congenital glycosylation disorders, muscular dystrophy and also may lead to diabetes, cancer and cystic fibrosis [57]. In *S. cerevisiae,* Pik1 is found in the nucleus and Golgi, and it is required for the maintenance of Golgi structures, protein secretion, endocytosis and cytokinesis [55]. On the other hand, *S. pombe* Pik1 is appeared to associate with Golgi and the role of $pik1^{+}$ in the maintenance of circular

chromosomes are not reported [58,59]. Telomere is the protective part of eukaryotic DNA which protects DNA from its degradation. Telomere protection protein Pot1 is one of the most important subunits of shelterin complex. Pot1 is required for the maintenance of telomere from its degradation. By loss of telomere, chromosome joins to its end to end and initiates the formation of circular chromosome. Circular chromosomes are unstable in eukaryotic cells which can result in genomic instability of the cells. Circular chromosomes are found to be associated with some types of cancer cells and some genetic diseases in human. The reasons behind the stability of circular chromosome are not clearly understood. I tried to identify the genes that are required for the maintenance of circular chromosome. The alkylating agent, ethyl methanesulfonate (EMS) induces mispairing with their complementary bases. EMS exposure leads to excessive point mutation [60]. Therefore, chemicals mutagenesis become a more effective method for genetic studies rather than the sophisticated transgenic technologies. To find the gene which is synthetically lethal with *pot1* deletion strain, *pot1*Δ strain which has *pot1*$^{+}$ expressing plasmid is treated with EMS. From here, I obtained an unknown EMS-induced mutation. The EMS-induced mutation showed synthetic lethality with *pot1* deletion strain. After that, a browser-accessible tool Mutation discovery (Mudi system) was used and identified that this unknown EMS-induced mutation is *pik1* mutation [61]. I called this *pik1* mutation as *pik1-1*. There are two possible reasons for the lethality of *pot1Δ* and *pik1-1* double mutant. Either Pik1 is required for the formation of circular chromosome or the maintenance of circular chromosome. I analyzed the first possibility and found that *pik1* is not required for the formation of circular chromosome. Therefore, it could be possible that *pik1* is required for the maintenance of circular chromosome.

3.2. Materials and Methods

3.2.1. Strains construction

The strains used in this study are listed in Table 3.1. For introducing *pik1*$^+$ expressing plasmid or empty vector into candidate 27 (AS007), plasmids expressing *pik1*$^+$ (*pAL-KS-FYG66_i21*) and empty vector (*pREP1)* were introduced into AS007 to obtain strain AS481 and AS491, respectively. *pot1Δ* cell (YI002) expressing *pot1*$^+$ from the plasmid *pPC27-ura4-pot1*$^+$*-HA* was mutagenized with ethyl-methanesulfonate (EMS) essentially as has been described previously [62]. Mutagenized *pot1Δ* cell (YI002) expressing *pot1*$^+$ from the plasmid was selected on EMM3+AL plate. Then cells were streaked on YEA plate containing Fudr to counter select the cell that had lost *pot1*$^+$ expressing plasmid [30]. The strain No. 27 (AS007) that cannot lose *pot1*$^+$ expressing plasmid was selected as a candidate of the mutant that is synthetically lethal with *pot1Δ*. The plasmid *pPC27-Leu-pot1*$^+$*-HA* (AGK004) was introduced into AS007 strain to result in the strain AS279. YEA plate containing 2 mg/ml 5-Fluoroorotic acid (FOA) at 25^{o}C was used to select for removal of YI002 (*pPC27-ura4-pot1*$^+$*-HA*) from AS279 to obtain strain AS280. After that, an amplified fragment including the downstream *pik1* which does not contain *pik1* mutation but carrying *ura4* using primer pik1-afmuT (CTTGATGCTGAAGGTAGCAG) and pik1-5b2 (CAAGTAATAATTCACCTCAGTTGG) was inserted into the strain that lost pPC27-ura4-pot1$^+$-HA from AS279 strain to create AS882 strain. Next, I amplified *pik1* mutated with *ura4* gene using primer pik1-mut-tra (TTTAAATAACAACCTTCCAGCCG) and pik1-5b2 (CAAGTAATAATTCACCTCAGTTGG) from genomic DNA of strain (AS892) as a template for PCR and was introduced into the PCR product into strain AS280 to create

strain AS882. Next, I amplified the DNA fragment containing *pik1-1* mutation and *ura4*$^{+}$ using primers pik1-mut-tra (TTTAAATAACAACCTTCCAGCCG) and pik1-5b2 (CAAGTAATAATTCACCTCAGTTGG) from the genomic DNA of AS882 as a template. The PCR product of mutated *pik1* with *ura4*$^{+}$ gene from AS882 strain transformed into the diploid wild-type cells (h^{+}/h^{-}) to obtain AS915. After that, I was introduced *pik1*$^{+}$ plasmid (*pPC27-leu-pik1*$^{+}$) into AS915 strain to obtain the strain AS926. The AS926 spore's strains containing *pik1-1* mutation and *pPC27-leu-pik1*$^{+}$ (SH012) were streaked on Fudr containing YEA plate for obtaining the strains SH013 after losing the *pik1*$^{+}$ expressing plasmid. The PCR fragment that containing *pik1-1* mutation with *ura4*$^{+}$ obtained from strain AS882 was transferred to strain AGK004 resulting in *pot1Δ pik1-1* cells containing the *pot1*$^{+}$ expressing plasmid *pPC27-Leu-pot1*$^{+}$*-HA* (AS902). To obtain plasmids pGFP-2xPH(PLCδ) and pGFP-PH(FAPP1), I fused genes of green fluorescent protein (GFP) and two tandem copies of the phospholipase Cδ1 (PLCδ1) pleckstrin homology (PH) domain or four-phosphate-adaptor protein 1(FAPPI) PH domain into the *S. pombe* expression vector, pTN54 containing nmt41 promotor and *LEU2* marker, which is used in *S. cerevisiae*. Then I introduced these plasmids into the *S. pombe* wild-type strain (AS892) or the *pik1-1* mutant (SH013) strain to result in the SH015, SH016, SH017 and SH018 strains. After that, I introduced pAU-Gms1-mCherry and pGFP-PH(FAPP1) into the wild-type strain (5A) to generate SH020. The strain LU001 was constructed by amplifying *ura4-294* from strain SH014 using primers ura4 bottom (GTGATATTGACGAAACTTTTTGAC) and ura4 top (AGCTACAAATCCCACTGGCTA) to be then introduced into strain NH001. The strain was selected from PMG + uracil plates containing 5-fluoroorotic acid (FOA) at

30ºC, on which only strains do not contain *ura4*$^{+}$ can survive. The strain LU001 was mated with strain SH012 to obtain the strain LU002 containing *pot1::sup3-5-nmt81-pot1*$^{+}$*-IAA17::ura4-294ade6::ade6*$^{+}$*-Padh15-skp1-AtTIR1-2NLS-9 myc, pik1-1* mutation and *pik1*$^{+}$-plasmid (*pPC27-leu-pik1*$^{+}$). The strain LU002 was selected on EMM3 + histidine plate at 25ºC and *nmt-pot1-aid* confirmed by PCR using primer Pot1 r2 and Pot1 c. The *nmt-pot1-aid* phenotype was also tested by streaking candidates onto EMM3 + Leucine + Histidine + Thiamine (15µM) + Auxin (0.5mM) at 25º C. Similarly, the *pik1-1* mutation was confirmed by using pik1-5b2 and int-2 primer. The strain LU003 was obtained from LU002 by removing *pik1*$^{+}$-plasmid (*pPC27-leu-pik1*$^{+}$*)* streaked on EMM3 containing 5-fluorodeoxyuridine (Fudr) (100µM) + Leucine + Histidine plate and confirmed by PCR using primer pik1-5b2 and pik1-4t of 52º C.

Table 3.1. *S. pombe* strains used in this study are listed bellow

Strain	Strain genotype	Source
YI002	*h⁻ pot1::kanMX6 leu1-32 ura4-D18 ade6-M216 (pPC27- pot1⁺-HA)*	[30]
SH011	*h⁺ pot1::kanMX6 leu1-32 ura4-D18 ade6-M210 (pPC27-pot1⁺-HA)*	Lab stock
JY741	*h- ade6-M216 leu1-32 ura4-D18*	Lab stock
D1	*h⁺ leu1-32 ura4-D18 his2-245 ade6-M216*	Toda
SH012	*h⁺pik1-1::term-nmt::ura4(pPC27-leu-pik1⁺)leu1-32ura4-D18 his2-245 ade6*	This study
SH013	*h⁻ pik1-1::term-nmt::ura4 leu1-32 ura4-D18 his2-245 ade6*	This study
KTA037	*h⁻ leu1-32 ura4-d18 ade6 pot1::kanMX6*	[31]
AS892	*h⁺ pik1⁺::term^nmt1 ::ura4 ade6-M210 His3-D1 leu1-32 ura4-D18*	This study
AS007	*h⁻ pot1::kanMX6 leu1-32 ura4-D18 ade6-M216 (pPC27-pot1⁺-3HA)* EMS27	This study
SH014	*h⁻ leu1-32 his3-D1 ura4-294 lys1-131::dis1-mcherry-lacI(lys1⁺) erg7::lacOrepeat(ura4⁺)arg3::HOsite-natMX(arg3-)ars::pJR-41XH+ HO (his3⁺) rad22-2xCFP(hphMX)top3-YFP(Leu1⁺)*	Lab stock
NH001	*h⁻leu1-32ura4-D18pot1::sup3-5-nmt81-pot1⁺-IAA17::ura4⁺ade6::ade 6⁺-Padh15-skp1-AtTIR1-2NLS-9myc*	Lab stock
LU001	*h⁻leu1-32ura4-D18pot1::sup3-5-nmt81-pot1⁺-IAA17::ura4-294ade6:: ade6⁺-Padh15-skp1-AtTIR1-2NLS-9myc*	This study
LU002	*h⁻pik1-1::term-nmt::ura4(pPC27-leu-pik1⁺)leu1-32ura4-D18 pot1::sup3-5-nmt81-pot1⁺-IAA17::ura4-294 ade6::ade6⁺-Padh15-skp1-AtTIR1-2NLS-9myc*	This study
LU003	*h⁻pik1-1::term-nmt::ura4leu1-32ura4-D18pot1::sup3-5-nmt81-pot1⁺-I AA17::ura4-294 ade6::ade6⁺-Padh15-skp1-AtTIR1-2NLS-9myc*	This study
N1550	*h⁺/h⁻pik1/pik1::term-nmt::ura4ade6-M210/ade-M216 his3-D1/his3-D1 leu1-32/leu1-32 ura4-D18/ura4-D18*	[59]

AS902	*h⁺ leu1-32 ura4-D18 ade6-M210 pot1::kanMX6 pik1-1ura4 pPC27-leu-pot1⁺-HA*	This study
AGK004	*h⁺ leu1-32 ura4-D18 ade6-M210 pot1::kanMX6 pPC27-Leu1-pot1⁺-HA*	Lab stock
5A	*h⁻ leu1-32 ura4-D18 ade6-M210*	Toda
AS915	*h⁺/h⁻ leu1-32/leu1-32 ura4-D18/ura4-D18 ade-M210/ade6-M216 his2⁺/his2-245 pik1⁺/pik1-1-ura4⁺*	This study
AS926	*h⁺/h⁻ leu1-32/leu1-32 ura4-D18/ura4-D18 ade-M210/ade6-M216 his2⁺/his2-245 pik1⁺/pik1-1-ura4⁺ pPC27-leu-pik1⁺*	This study
AS481	*h⁻ ade6 leu1-32 ura4-D18 pot1::kanMX6 pPC27-pot1⁺-HA pAL-KS-FYG66_i21 EMS 27*	This study
AS491	*h⁻ ade6 leu1-32 ura4-D18 pot1::kanMX6 pPC27-pot1⁺-HA pREP1 EMS 27*	This study
AS882	*h⁻ ade6 leu1-32 ura4-D18 pot1::kanMX6 pik1-1-ura4 pPC27-leu-pot1⁺-HA EMS 27*	This study
AS279	*h⁻ ade6 leu1-32 ura4-D18 pot1::kanMX6 pPC27-pot1⁺-HA pPC27-leu-pot1⁺-HA EMS 27*	This study
AS280	*h⁻ ade6 leu1-32 ura4-D18 pot1::kanMX6 pPC27-leu-pot1⁺-HA EMS 27*	This study
SH015	*h⁻ pik1-1::term-nmt::ura4 leu1-32 ura4-D18 his2-245 ade6* pGFP-PH(FHPP1)	This study
SH016	*h⁻ pik1-1::term-nmt::ura4 leu1-32 ura4-D18 his2-245 ade6* pGFP-2xPH(PLCδ)	This study
SH017	*h⁺ pik1⁺::term-nmt1 ::ura4 ade6-M210 His3-D1 leu1-32 ura4-D18* pGFP-PH(FHPP1)	This study
SH018	*h⁺ pik1⁺::term-nmt1 ::ura4 ade6-M210 His3-D1 leu1-32 ura4-D18* pGFP-2xPH(PLCδ)	This study
SH019	*h⁻ leu1-32 ura4-D18 ade6-M210 pAU-gms1⁺-mCherry*	This study
SH020	*h⁻ leu1-32 ura4-D18 ade6-M210 pAU-gms1⁺-mCherry* pGFP-PH(FHPP1)	This study

3.2.2. Backcross

The strain No. 27 (*pot1Δ* cells with unknown mutation expressing *pot1*$^{+}$ from the plasmid) was backcrossed with *pot1Δ* cells (SH011) expressing *pot1*$^{+}$ from the plasmid and spores were grown on EMM3+AL to select the cells that maintained *pot1*$^{+}$ expressing plasmid. Then cells were streaked on YEA plate containing Fudr to counter select the cell that lost *pot1*$^{+}$ expressing plasmid. I obtained 30 backcross strains and found that 14 strains (mutant strains) could not grow on YEA containing Fudr plate and remaining 16 strains (parental strains) could grow on YEA containing Fudr plate.

3.2.3. Next generation sequencing

Next generation sequencing was done as has been described previously [61]. Briefly, 14 backcrossed strains that could not grow on YEA containing Fudr plate and 16 backcrossed strains that could grow on YEA containing Fudr plate were cultured on 3 ml YEA and incubated overnight. Then the culture of 14 backcross strains that could not grow on YEA containing Fudr plate were mixed and samples of their genomic DNA for NGS was prepared and using the Nextera DNA library preparation kit (FC-121-1030; Illumina Inc.) to be Nextera index kit (FC-121-1011; Illumina Inc.), and sequenced using an Illumina Miseq system with paired-end cycle (151 base x 2). Comparison of DNA sequence of mutant strains and parental strains using Mudi web page with the *S. pombe* reference genome (http://naoii.nig.ac.jp/mudi_top.html) suggested that C1438 in *pik1* cDNA is mutated to T corresponding to the mutation of amino acid Q480 to stop codon (DRA analysis data accession DRZ014304, mi_EMS27_14585201412814569_uniq_mutations_dupsort.txt). The sequencing analysis data were registered at DDBJ Sequence Read Archive (DRA) as accession DRA006289.

3.2.4. Spotting assay

The strains *nmt-pot-aid* and *nmt-pot1-aid-pik1-1* cells were cultured in 5 ml EMM3+L liquid medium overnight to reach log phase ($1x10^7$) cells. Then the culture was diluted to $1x10^6$ cells/ml and incubated 24 h in 3 ml EMM3+L containing of thiamine (15μM) to obtain $1x10^7$ cells/ml cells. Then in 1ml cells $1x10^7$ cells/ml were concentrated one tenth to obtain $1x10^8$ cells/ml cells and that were used for spotting on EMM3+L and EMM3+L + thiamine + auxin (0.5mM)) containing plate.

3.2.5. Analysis of telomeres

The *nmt-pot1-aid* and *nmt-pot1-aid-pik1-1* strains were used for overnight culture in 10 ml EMM3+L liquid medium to reach log phase ($1x10^7$ cells/ml) cells (-24 h sample). Then the cultured strains were diluted to $1x10^6$ cells/ml and incubated 24 h in 50 ml EMM3+L+thiamine (0 h sample). After that, the culture strains were diluted to $1x10^6$ cells/ml and cultured in 50 ml EMM3+L+thiamine+auxin for incubation of 6 h and 9 h, and used for Southern hybridization with an AlkPhos direct kit module (GE Healthcare) according to a previously described procedure in chapter 2.

3.2.6. Pulsed-field gel electrophoresis (PFGE)

The *nmt-pot-aid* and *nmt-pot1-aid-pik1-1* strain were cultured overnight in 10 ml EMM3+L liquid medium to reach log phase ($1x10^7$ cells/ml) cells. The culture of cell was then diluted to $1x10^6$ cells/ml and incubated 24 h in 50 ml EMM3+L+thiamine (15μM) (0 h sample). Then the culture strains were diluted to $1x10^6$ cells/ml and incubated in 50 ml EMM3+L+thiamine (15μM) + auxin (0.5mM) 12 h and this step was performed once more. After that, the culture strains were again diluted to $2.5x10^6$ cells/ml and incubated 24 h in 50 ml EMM3+L+thiamine+auxin containing liquid medium to obtain $1x10^7$ cells/ml cells (48 h sample). After that, PFGE was performed

as described previously in the chapter 2.

3.3. Results

3.3.1. Construction of synthetically lethal strain with *pot1*Δ

To obtain the strain which is synthetically lethal with *pot1*Δ, I treated *pot1*Δ strain carrying a *pot1*$^{+}$ expressing plasmid with EMS. After that, I treated EMS-induced cells with 5-fluorodeoxyuridine (Fudr). Fudr has been used for the counter selection to select strains that have lost the plasmid expressing thymidine kinase (*tk*). I obtained a strain called candidate 27. The candidate 27 contained an unknown EMS-induced mutation. The EMS-induced mutation showed synthetic lethality with *pot1* deletion strain (Fig. 3.1. A).

3.3.2. Investigation of the responsible gene that is synthetically lethal with *pot1*Δ strain

In this study, I used the Mudi (mutation discovery) system to investigate the gene that is responsible for the lethality with *pot1* deletion strain. To obtain the causative mutation, backcross was performed between the EMS-induced strain (carrying *pot1*Δ and *pot1*$^{+}$ expressing plasmid) and *pot1*Δ strain (carrying *pot1*$^{+}$ expressing plasmid). Backcross resulted in two groups of mutation (spore were sequenced). One group contained one common causative mutation. This causative mutation was found to be synthetically lethal with *pot1*Δ. Another group contained only EMS-induced mutation with background mutation. In Mudi system, I used a web tool that can easily identify the mutation points by applying the whole genome data of candidate strain and parent strain (Mudi; http://naoii.nig.ac.jp/mudi_top.html). Mudi

system is used to find out the causative mutations from the whole-genome sequence data [61]. I found that mutation in C1438 to T in the *pik1* cDNA which is most probably mutation for the synthetically lethal with *pot1* deletion strain. I named this mutation as *pik1-1*. The *pik1-1* mutation results in a stop codon in the position of 480 amino acid Glutamate. Conditionally, if the *pik1-1* mutation is synthetically lethal with *pot1*Δ, then introducing of wild-type $pik1^+$ expressing plasmid in candidate 27 strain must suppress the lethality. Thus, I introduced $pik1^+$ expressing plasmid into the candidate 27 strain to examine the suppression of the lethality in plate YEA media containing FOA. FOA is used as a counter selection of a strain that have lost the pot1 plasmid expressing $ura4^+$. I found that $pik1^+$ expressing plasmid of candidate 27 strain suppressed the lethality on plate containing FOA (Fig. 3.1 B). However, the strain carrying empty vector did not suppress the lethality on plate containing FOA. These results revealed that the *pik1-1* mutation is synthetically lethal with *pot1* deletion strain.

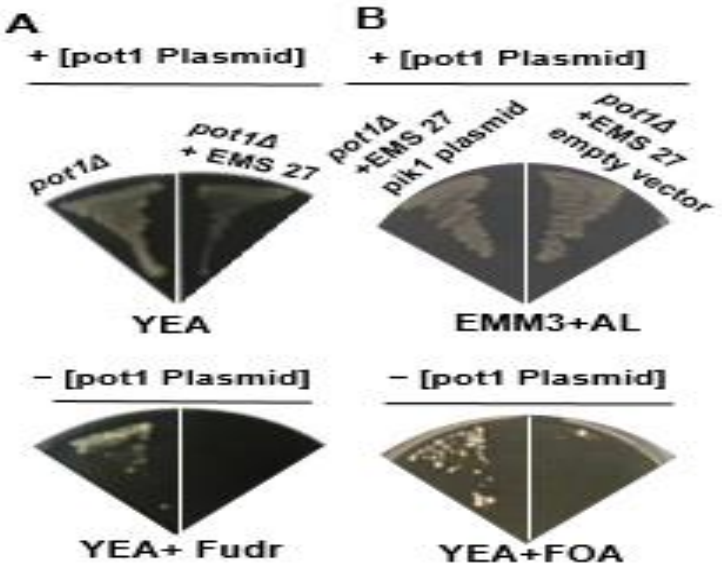

Fig. 3.1. EMS-induced mutation *pik1-1* synthetically lethal with *pot1Δ* (A) The *pot1Δ* with *pot1* expressing plasmid and candidate 27 were streaked on YEA plate and YEA containing Fudr plate. Candidate 27 lost the plasmid and showed sensitivity on YEA plus Fudr containing plate. (B) Candidate 27 that containing pot1 expressing plasmid and pik1 expressing plasmid, and pot1 expressing plasmid with empty vector both were streaked on plate EMM plus adenine and leucine (EMM+ AL) containing and YEA containing FOA plate. The lethality of candidate with *pik1*$^{+}$ expressing plasmid is suppressed the FOA lethality whereas loss of *pot1*$^{+}$ expressing plasmid of empty vector candidate showed the lethality on the plate in the presence of FOA.

3.3.3. The *pik1-1* single mutation is not lethal

It was reported that *pik1* deletion is lethal and kinase domain of *pik1* is required for its viability [59]. In contrast, candidate 27 which has *pik1-1* mutation is viable revealing that the *pik1-1* mutation is not lethal. However, candidate 27 which carries background mutations in addition to *pik1-1* mutation, that may suppress the lethality of *pik1-1* mutation. To test the lethality of *pik1-1* mutation, I constructed *pik1-1* single mutant which did not carry any background mutations. I amplified the downstream *pik1* which does not contain *pik1* mutation but carrying *ura4*$^+$ cassette was inserted into the 27 candidate strain. After that, I amplified *pik1-1* mutation with *ura4*$^+$ gene and inserted into the diploid wild-type cells. After that, I performed tetrad analysis. However, I obtained insufficient spore formation. I assumed that mutation in one copy of *pik1* gene results in haploinsufficiency. After that, I introduced *pik1*$^+$ expressing plasmid into *pik1+/pik1-1-ura4* cells and performed tetrad analysis. After segregation, I found 2 sets of spore formation. One set contains *pik1-1* mutation with *pik1*$^+$ expressing plasmid and other set contains wild-type *pik1*$^+$ with *pik1*$^+$ expressing plasmid (Fig. 3.2. B). Then the *pik1-1-ura4* strain with *pik1*$^+$ expressing plasmid were streaked in plate YEA containing Fudr to counter select the strains that have lost *pik1*$^+$ expressing plasmid. I found *pik1-1-ura4* strain is viable in plate YEA containing Fudr after removing *pik1*$^+$ expressing plasmid (Fig. 3.2. C). This result suggested that the *pik1-1* single mutant is not lethal.

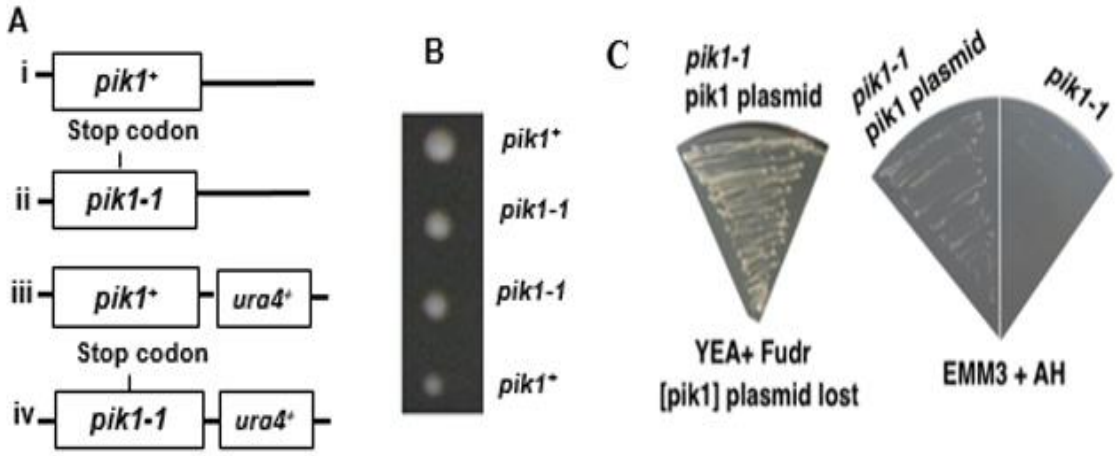

Fig. 3.2. (A) Schematic diagrams of wild-type *pik1*$^+$ gene (i), *pik1-1* mutation in candidate 27 (ii), wild-type *pik1*$^+$ with *ura4*$^+$ gene (iii) and *pik1-1* mutation with *ura4*$^+$ gene (iv) are shown. (B) *pik1*$^+$ plasmid was introduced into *pik1*$^+$*/pik1-1-ura4* cells and tetrad analysis on YEA was performed. After segregation 2 sets of spores were formed where one set containing *pik1* mutation and other set wild-type *pik1*$^+$. (C) The *pik1-1* mutant that containing the *pik1*$^+$ plasmid with marker *LEU2* and *tk*$^+$ was streaked on YEA plate with Fudr to counter-select the strain which lost the *pik1*$^+$ plasmid. The *pik1-1* mutant that expressing *pik1*$^+$ from plasmid with marker *LEU2* and *tk*$^+$ and the *pik1-1* mutant which lost the *pik1*$^+$ plasmid was streaked on EMM containing adenine and histidine (EMM+AH) with lack of leucine for confirming the loss of the plasmid that had the *LEU2* marker. If it contains pik1 plasmid only it can grow on this plate containing selective media.

3.3.4. The *pik1-1* mutation with *pot1Δ* strain is synthetically lethal

Next, I constructed *pot1Δ pik1-1-ura4* double mutant to examine the synthetic lethality. I amplified *pik1-1* mutation with *ura4*$^+$ gene and inserted into the *pot1Δ* carrying *pot1*$^+$ expressing plasmid for creating *pot1Δ pik1-1-ura4* strain (carrying *pot1*$^+$ expressing plasmid). Then the *pot1Δ pik1-1-ura4* double mutant strain was confirmed by PCR and plate containing EMM-uracil. I treated *pot1Δ pik1-1-ura4* double mutant

strain with Fudr. The *pik1-1-ura4* mutant showed synthetically lethal with *pot1Δ* strain (Fig. 3.3).

Fig. 3.3. *pot1Δ with* pot1 expressing plasmid, Candidate 27 and double mutant *pot1Δ pik1-1-ur4* containing pot1 expressing plasmid were streaked on YEA and YEA containing Fudr plate to counter select the strain that removed the pot1 expressing plasmid that containing tk^+. The double mutant *pot1Δ pik1-1-ur4* showed the synthetically lethal on Fudr containing plate.

3.3.5. *pik1-1* mutation influences PI(4)P level in Golgi

Pik1 kinase phosphorylates phosphatidylinositol (PtdIns) resulting in PtdIns 4-phosphate (PI4P). Pik1 is associated with Golgi in *S. pombe* cells [59]. To identify the effect of *pik1-1* mutation in living cells of PI(4)P level in Golgi, I checked the PI(4)P level. For analyzing this, FAPP1 PH domain was tagged with GFP where FAPP1 PH domain is found to bind both PI(4)P and PI(4,5)P2 [63]. The gms1 protein is also found to localize in Golgi [64]. I found FAPP1 GFP-PH domain and mcherry-Gms1 co-localization in wild-type *S. pombe* cells which represents the presence of PI(4)P and/or PI(4,5)P2 in Golgi (Fig. 3.4. A). After that, I inserted FAPP1 PH domain into the

pik1-1 mutant to analyze the PI(4)P and PI(4,5)P2 level. I found that the PI(4)P and/or PI(4,5)P2 in Golgi were sharply reduced in *pik1-1* mutant compared to wild-type strain (Fig. 3.4. B, C). To confirm which signal was reduced in *pik1-1* mutant. I introduced PLC PH tagging with GFP into both wild-type cells and *Pik1-1* mutant cells and monitored the existence of PI(4,5)P2 in Golgi. It was reported that PLC PH binds with PI(4,5)P2 [63]. I was unable to find out PI(4,5P2) signal in Golgi both in wild-type and *Pik1-1* mutant cells (Fig. 3.4. D, E). These results indicated that PI(4)P is associated with Golgi and PI(4)P level is reduced due to *pik1-1* mutant.

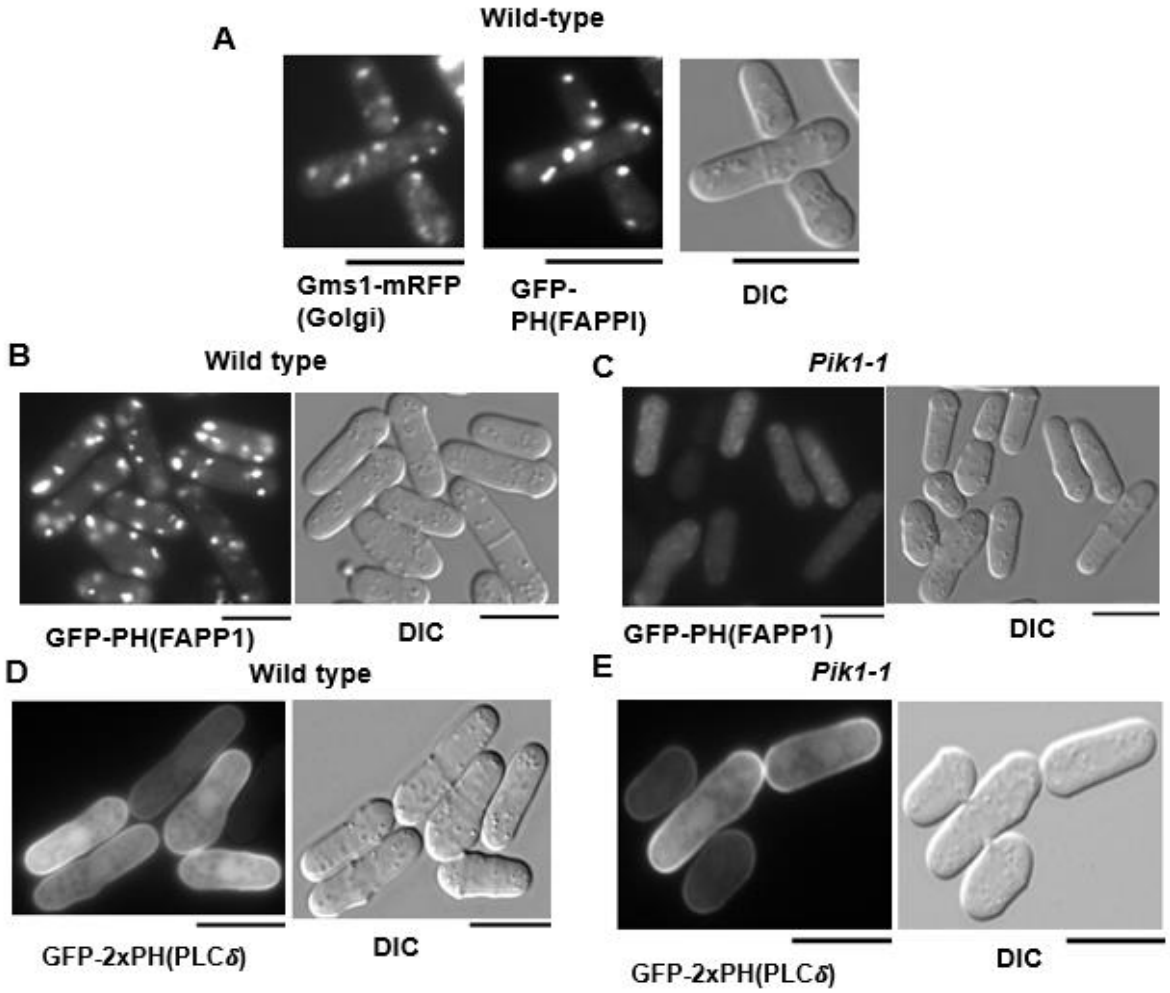

Fig. 3.4. PI(4)P level is decreased in Golgi due to *pik1-1* mutation. (A) PI(4)P and PI(4,5)P2 are localized in Golgi. The wild-type Gms1-mRFP, GFP-PH(FAPPI) and differential interference contrast (DIC) images are shown. The bar under the images represents 10 μm. (B) WT *pik1*$^{+}$ FAPP1 strongly binds with PI(4)P and PI(4,5)P2 in Golgi. The bar under the images represents 10 μm. (C) In *pik1-1* mutation strain, PI(4)P and FAPP1 binding in Golgi apparatus did not appear. The bar under the image represents 10 μm. (D-E) PH domain in PLC was used to be fused with GFP in *pik1-1* and wild-type cells. WT-PLC and *pik1-1*-PLC bind with PI(4,5)P2 in cell membrane and nucleus. The bar under the image represents 10 μm.

3.3.6. Telomre resection and chromosome circularization are not affected by *pik1-1* mutation

Deletion of *pot1*$^{+}$ causes immediate loss of telomere. Telomere loss leads to chromosome circularization by single strand annealing (SSA) and the gene associated with SSA is lethal with *pot1*$^{+}$ deletion strain [10]. I thought one possible reason of the lethality where *pik1*$^{+}$ may be involved in SSA. To investigate the possibility, I made *nmt-pot1-aid* and *nmt-pot1-aid-pik1-1* strain. *pot1*$^{+}$ function is shut-offed by using thiamine and Auxin. *pot1* shut-off strain can be used to examine the telomere resection and chromosome circularization [65]. On the other hand, use of *pot1* shut-off strain for analyzing synthetic lethality is not a good idea because *pot1* shut-off cannot stop the *pot1*$^{+}$ expression as obtained from a *pot1* deletion strain. To know whether *pik1-1* mutation is involved in suppression of telomere degradation or not, I examined the viability of *nmt-pot1-aid* and *nmt-pot1-aid-pik1-1* strain using *pot1* shut-off technique. I prepared overnight culture to obtain log phase cells. Then *nmt-pot1-aid* and *nmt-pot1-aid-pik1-1* cells were incubated in the presence of thiamine for 24 h and those were used for spotting on plate containing EMM3+L and EMM3+L + thiamine (15μM) + auxin (0.5mM)). The *nmt-pot1-aid* and *nmt-pot1-aid-pik1-1* cells were showed the same viability (Fig. 3.5. A). This result revealed that *pik1*$^{+}$ is not involved in telomere degradation. To confirm this possibility *nmt-pot1-aid* and *nmt-pot1-aid-pik1-1* strain were used to examine the telomere resection using genomic DNA digested with BglII. I incubated *nmt-pot1-aid* and *nmt-pot1-aid-pik1-1* cells in the presence of thiamine for 24 h to minimize the expression of *pot1*$^{+}$ (time point 0 h). After that, thiamine and auxin were added and cells were incubated for 6 h and 9 h. DNA was digested with BglII resulted in 15.4-kb telomere fragment and 8.3-kb internal fragment as a control. I found

that telomere degradation rate was similar after shutting of *pot1* in *pik1-1* mutant (Fig. 3.5. B). This result supported that *pik1-1* mutation does not affect telomere degradation.

Next, I examined the effect of *pik1-1* mutation on chromosome circularization using *nmt-pot1-aid* and *nmt-pot1-aid-pik1-1* strains by doing PFGE. The *nmt-pot1-aid* strain is used as the parental strain. Samples were collected at 0 h and 48 h after addition of drugs to shut off $pot1^{+}$ expression. The result showed that the most chromosomal end fragments namely I, L and M in both strains disappeared after 48 h incubation with drugs and chromosome still remained circular (detection of L+I band correspond to circular chromosome) in *nmt-pot1-aid pik1-1* mutant strain following *pot1* shut off (Fig. 3.5. D). This result supported that *pik1-1* mutation does not affect chromosome circularization.

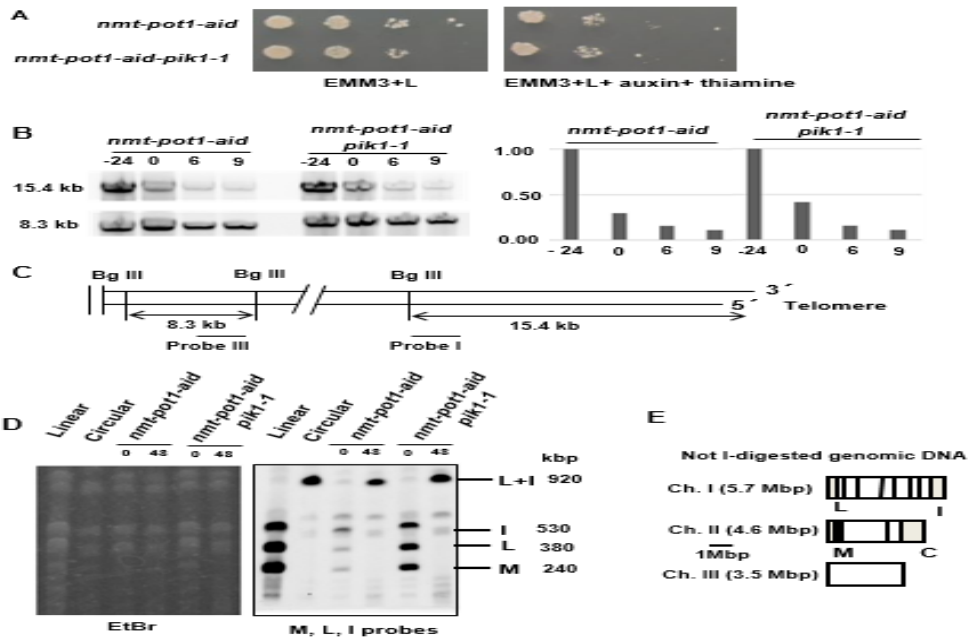

Fig. 3.5. Telomere degradation and chromosome circularization are not associated with Pik1.

(A) Serial dilution of *nmt-pot1-aid* and *nmt-pot1-aid pik1-1* cells were incubated on plate containing EMM3+L and EMM3+L + thiamine (15µM) + auxin (0.5mM)). The cells were pre-incubated with thiamine (15µM) for 24 h to minimize the *pot1*$^{+}$ expression. It was observed that viability of the *pik1-1* cell was not changed after *pot1* shut-off. **(B)** Wild-type cells and *pik1-1* cells telomere degradation was monitored using Southern hybridization and quantitated after shut-off *pot1*$^{+}$expression. Both *nmt-pot1-aid* and *nmt-pot1-aid pik1-1* cells were incubated with thiamine for 24 h (time point 0 h). After that, the telomere resection rate following incubation period 0 h, 6 h and 9 h with thiamine (15µM) and auxin (0.5mM) shutting off *pot1* was investigated. A 15.4kb telomere fragment and 8.3kb internal fragment were digested with *Bgl*II and these fragments were detected by using probe I and III, respectively [33]. The image was used for the quantification. For adjusting the loaded DNA, the intensity of telomere fragment (15.4kb) divided by the intensity of internal fragment (8.3kb) was used. In all strain, the band intensity at the time point 24 h was normalized as 1. **(C)** Chromosome I and II terminal region is on the *Bgl*II site [33]. The *Bgl*II situated on about 1.9 Mbp from the right side of the telomere end of chromosome II are also shown. The I and III probe positions were indicated which were used to detect incomprehension *Bgl*II-digested fragment are mentioned [65]. **(D)** *S. pombe* chromosomal DNA of *nmt-pot1-aid* and *nmt-pot1-aid pik1-1* strains were digested with NotI and analyzed using PFGE. The control was the parental *nmt-pot1-aid* strain. Samples were collected following 0 h and 48 h incubation after adding drugs to shut off *pot1* expression. Telomeric NotI fragment probes (M, L and I) were used. The digested chromosomal end fragment size by NotI, M, L, I and I+L are mentioned. EtBr stained PFGE agarose gel image is shown in bellow. (**E**) *S. pombe* chromosomes of NotI restriction site map is shown. Both chromosomes I, II, and III are shown. The using scale is corresponded to 1Mpb.

3.4. Discussion

Circular chromosomes are found in some types of cancer cells and genetic diseases in human. Therefore, screening for gene mutations that cause lethality in cells with circularized chromosomes can lead to potential therapeutic targets. To obtain a mutant that will be synthetically lethal with *pot1Δ*, I used mutagenic alkylating agent, EMS to mutagenize the *pot1Δ* strain carrying $pot1^{+}$ expressing plasmid and found a mutant called candidate 27. I used Fudr as a counter selection to remove $pot1^{+}$ expressing plasmid from the candidate and it is found to be lethal on plate containing Fudr after removing of plasmid. This result suggested that the unknown mutations of candidate 27 are synthetically lethal with *pot1Δ*. Later on, I used Mudi (mutation discovery) system to identify the gene responsible for the lethality with *pot1Δ* cells of candidate 27. This is a browser accessible web tool is used for recognizing the mutation point compared with whole genome data of candidate strain and parental strain [61]. I observed that *pik1-1* mutation looks like gene mutation that is responsible for the lethality with *pot1Δ* of candidate 27. After that, I introduced $pik1^{+}$ expressing plasmid into the candidate 27 strain and found $pik1^{+}$ expression suppressed the lethality. This result suggested that *pik1-1* mutation is responsible for the lethality. There may be two possible reasons for the lethality of *pot1* deletion and *pik1-1* double mutant strain. $pik1^{+}$ is either required for the formation of chromosome circularization or maintenance of chromosome circularization. To test the first possibility, I performed Southern blotting for telomere analysis and PFGE for knowing the phenotype of chromosome. I found that *pik1* is not required for the formation of circular chromosome. Therefore, it supported the second possibility where *pik1* is required for the maintenance of circular chromosome. I also found that PI(4)P level is reduced in *pik1-1* mutant cells in Golgi

compared to wild-type cells. PI(4)P is the main lipid determinant among the PIs in Golgi and trans-Golgi network (TGN). I assumed that the *pik1-1* mutation in *S. pombe* cell may hamper the transporting some proteins from Golgi to nucleus which is essential for the maintenance of circular chromosome. However, further research is essential to properly understand the mechanism behind the lethality between *pot1* deletion and *pik1-1* mutant strain.

Chapter 4. Conclusion

4.1. Thesis summary

Telomere usually protects cells from genomic instability and cellular senescence. DNA double strand break and telomere loss may result in circular chromosomes in human. Circular chromosome is unstable in human and this is the features of some types of cancer cells and genetic diseases. Cure of cancer is one of the most important tasks in biomedical research field because cancer is one of the main reasons for death. It is important to find out the new drug that specifically can kill cancer cells, because many drugs used for cancer therapy have strong side effects. If it is possible to find out a drug that can selectively kill cancer cell carrying circular chromosome, this drug obviously is expected to be no side effect to normal cells. Yet now, no modern advanced therapy has been discovered that can selectively kill the cancer cells without causing any side effect to normal cells. My research emphasizes to develop a therapy to kill cancer cells where most of the cells carrying circular chromosomes. But genes that are involved in the maintenance of circular chromosome are not known in human. I tried to find out the genes that are required for the maintenance of circular chromosome in *S. pombe*. Finding of these genes will be helpful to kill cancer cells that carry circular chromosomes. I have used *S. pombe pot1* deletion which has circular chromosomes. To identity genes that are involved in the maintenance of circular chromosome in *S. pombe*, I tried to find out a gene that will be synthetically lethal with *pot1* deletion strain.

I constructed *pot1*Δ and the 9-1-1 complex double mutant cells which have circular chromosomes. I found that *pot1*Δ and the 9-1-1 complex double mutant is

synthetically lethal in the presence of agent causing replication stress (Fudr, HU and MMS). These kinds of lethality will directly contribute to selectively kill cancer cells carrying circular chromosomes. The cells from atypical lipomatous and dermatofibrosarcoma protuberans tumors contain about 85% and 70% circular chromosomes, respectively. In these cases, therapies targeting the cells with circular chromosomes may facilitate the selective killing. In *S. pombe,* I found that the 9-1-1 complex when replication is arrested are required for the maintenance of circular chromosome. In *S. pombe* most of the genes that are related to chromosome maintenance are conserved in mammalian counterparts. Therefore, it is expected that the human homologue of *S. pombe* genes that are required for the maintenance of circular chromosome will be required for the maintenance of circular chromosome in human. The 9-1-1 complex checkpoint proteins Rad1, Rad9 and Hus1 are similar in both human and *S. pombe.* Consequently, it is expected that the human 9-1-1 complex checkpoint proteins (Rad1-Rad9-Hus1) will also be required for the maintenance of circular chromosome when replication is arrested. Thus, inhibiting the human 9-1-1 complex proteins when replication is arrested in cancer cells that have circular chromosomes would result in defect in the maintenance of their circular chromosomes. Therefore, the cancer cells will be died because their survival depend on the maintenance of circular chromosomes.

I also constructed *pot1Δ pik1-1* double mutant which has circular chromosomes. I found that *pot1Δ pik1*-1 double mutant is synthetically lethal. I also found that *pik1* could be required for the maintenance of circular chromosome. The *S. pombe* Pik1 is homologous to human PI(4)K IIIβ. Therefore, it is expected that human PI(4)K IIIβ will

also be required for the maintenance of circular chromosome. Thus, inhibiting the human PI(4)K IIIβ protein in cancer cells that have circular chromosomes would result in defect in the maintenance of their circular chromosomes.

4.2. Future perspective

Rad9-Hus1-Rad1 (9-1-1) complex is a cell cycle checkpoint protein, a sensor of DNA damage. However, the role of 9-1-1 complex on the maintenance of circular chromosome is not known. In this research, I found that *pot1*Δ and the 9-1-1 complex double mutant is synthetically lethal in the presence of agent causing replication arrest. These kinds of lethality will directly contribute to target kill of cancer, which carrying circular chromosomes. However, yet I still do not know why *pot1*Δ and the 9-1-1 complex double mutant is synthetically lethal in the presence of agent causing replication stress. In future, I would like to find out the suppressor mutant of *pot1Δ* and the 9-1-1 complex double mutant to understand the mechanism why circular chromosome shows sensitivity to agent causing replication arrest in the absence of 9-1-1 complex. Understanding the mechanism of their lethality will be helpful to identify more genes that are involved in this lethality. These findings will increase more molecular targets of killing cancer cells containing circular chromosomes. In addition, understanding the lethality between *pot1* deletion and the 9-1-1 complex double mutant will be helpful to understand the same phenotype expressing downstream regulator of DNA damaged checkpoint protein Rad3 and Cds1 (I constructed *pot1*Δ *rad3*Δ and *pot1*Δ *cds1*Δ double mutant).

Pik1 is involved in sorting proteins in Golgi network. Defects in Golgi function can result in congenital glycosylation disorders, muscular dystrophy, diabetes, cancer,

and cystic fibrosis. However, the role of *pik1*$^{+}$ in the maintenance of Golgi structures, nucleus and chromosome maintenance, protein secretion and the maintenance of circular chromosome are not reported. In this research, I found that *pot1*Δ *pik1-1* double mutant is synthetically lethal. However, I still do not know the reasons behind the synthetic lethality between *pot1*Δ and *pik1-1* double mutant. To understand the mechanism of their lethality, I would like to identify the suppressor mutant of *pot1*Δ and *pik1-1* double mutant strain. I expect that these finding will increase more molecular targets of killing cancer cells containing circular chromosomes. In addition, understanding the mechanism of their lethality will also be helpful to understand the new functions of *pik1*$^{+}$ which have huge scientific benefits in the fundamental research in life science.

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Acknowledgements

At first, I would like to express my gratitude to my supervisor Dr. Masaru Ueno for his invaluable guidance, continuous supervision, and constructive criticism during the entire period of research and invaluable insights and suggestions to improve my writing of the thesis. It was really a great opportunity for me to perform my doctoral work under his supervision and learn research expertise. I express my gratitude to our research collaborator Johanne M. Murray for her suggestion and constructive criticism. My sincere thanks to the reviewer committee of this thesis, Dr. Seiji Kawamoto, Dr. Nobukazu Tanaka and Dr. Tsunehiro Aki for their revision of my Ph.D. thesis and their insightful comments.

I owe my warm thanks to all of my lab-mates who help me by creating a suitable environment in Laboratory during my PhD thesis work.

I am very grateful to my beloved parents, my wife and brothers for their blessing and constant inspiration through the course of my study.

公表論文
（Articles）

(1) Fission yeast strains with circular chromosomes require the 9-1-1 checkpoint complex for the viability in response to the anti-cancer drug 5-fluorodeoxyuridine.
Hossain Mohammad Shamim, Yukako Minami, Daiki Tanaka, Shinobu Ukimori, Johanne M. Murray, Masaru Ueno
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(2) Mutation in fission yeast phosphatidylinositol 4-kinase Pik1 is synthetically lethal with defect in telomere protection protein Pot1.
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