Molecular Biology Research Communications 2018;7(3):97-106 DOI:10.22099/mbrc.2018.29705.1324 MBRC
Original Article Open Access

Pap1^{+} confers microtubule damage resistance to *mut2a*, an extragenic suppressor of the *rad26:4A* allele in *S. pombe*.

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ABSTRACT

The DNA structure checkpoint protein Rad26^{ATRIP} is also required for an interphase microtubule damage response. This checkpoint delays spindle pole body separation and entry into mitosis following treatment of cells with microtubule poisons. This checkpoint requires cytoplasmic Rad26^{ATRIP}, which is compromised by the *rad26:4A* allele that inhibits cytoplasmic accumulation of Rad26^{ATRIP} following microtubule damage. The *rad26::4a* allele also disrupts minichromosome stability and cellular morphology, suggesting that the interphase microtubule damage checkpoint pathway operates in an effort to maintain chromosome stability and proper cell shape. To identify other proteins of the Rad26-dependent interphase microtubule damage response, we used ultra violet (UV) radiation to identify extragenic interaction suppressors of the *rad26::4A* growth defect on microtubule poisons. One suppressor mutation, which we named *mut2a*, permitted growth of *rad26:4A* cells on MBC media and conferred sensitivity to a microtubulin poison upon genetic outcross. In an attempt to clone this interaction suppressor using a genomic library complementation strategy, we instead isolated *pap1*$^{+}$ as an extracopy suppressor of the *mut2a* growth defect. We discuss the mechanism by which *pap1*$^{+}$ overexpression may allow growth of *mut2a* cells in conditions that destabilize microtubules.

Keywords: DNA structure checkpoint; interphase microtubule damage response; extragenic suppressor; pap1; rad26^{ATRIP}

INTRODUCTION

DNA structure checkpoints assess genomic integrity to help ensure successful cell division [1]. These checkpoints operate as signal transduction pathway that detect structural perturbations of DNA and transduce its presence to effector kinases that influence cellular responses, including cell cycle delay [2]. In *S. pombe*, the phosphatidylinositol 3-kinase-related kinase **(**PIKK) family member Rad3^{ATR}, together with its regulatory subunit Rad26^{ATRIP}, are central components of DNA structure checkpoint pathways [3-5]. Also involved is Rad17, which positions the Rad9-Rad1-Hus1 checkpoint clamp onto DNA lesions that, along with the Rad3/Rad26 complex, detects DNA structural abnormalities[2, 6, 7]. Following detection, Rad3

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pISSN 2322-181X eISSN 2345-2005

kinase activity is stimulated to target transducing protein kinases including Mrc1 and Crb2. In turn, these transducers phosphorylate and activate effectors kinases Cds1 and Chk1 that target, among other things, mitotic regulators [8-11]. The ensuing delay to mitosis provides opportunity for necessary DNA repair.

Apart from this role in the DNA structure checkpoint, Rad26^{ATRIP} is also required for an interphase microtubule damage response [12, 13]. Upon detection of thiabendzole-(TBZ) or carbendazim-(MBC) induced microtubule damage, this response delays spindle pole body separation and entry into mitosis. It is clear that this is a response to microtubule damage because a benzimidazole-resistant -tubulin allele [14, 15] rescued the growth of *rad26Δ* cells on MBC [13]. Cytoplasmic Rad26^{ATRIP} is required for this response, which is compromised by the *rad26:4A* allele that inhibits cytoplasmic accumulation of Rad26^{ATRIP} following microtubule damage [13]. Importantly, the DNA structure checkpoint operates normally in *rad26:4A* cells and can therefore be genetically separated from the interphase microtubule damage response. The *rad26::4A* allele also disrupts minichromosome stability and cellular morphology, suggesting that the interphase microtubule damage checkpoint pathway operates in an effort to maintain chromosome stability and proper cell shape. A different group has discovered an interphase microtubule damage checkpoint pathway and found that it results in stabilization of Wee1, and universal mitotic inhibitor [16]. It has yet to be determined if the Wee1-dependent and Rad26^{ATRIP}-dependent pathways are one in the same.

To identify other proteins of the Rad26-dependent interphase microtubule damage response, we used ultra violet (UV) radiation to identify extragenic interaction suppressors [17] of the *rad26::4A* MBC growth defect. One suppressor mutation, which we named *mut2a*, permitted growth of *rad26:4A* cells on MBC media and conferred sensitivity to MBC upon genetic outcross. We attempted to clone this interaction suppressor using a genomic library complementation strategy and succeeded in identifying a genomic plasmid that permitted growth of the *mut2a* mutant on MBC plates. Of the genes in this genomic insert, we determined that *pap1*$^{+}$ was responsible for rescuing the MBC sensitivity of *mut2a*. However, genomic sequencing results and linkage analyses confirmed that *mut2a* was not allelic to *pap1*$^{+}$. Instead, we suggest that *pap1*$^{+}$ is an extracopy suppressor of the MBC growth defect caused by the *mut2a* mutation. We discuss the mechanism by which *pap1*$^{+}$ overexpression may allow growth of *rad26:4a* cells in conditions that destabilize microtubules.

MATERIALS AND METHODS

UV mutagenesis and isolation of *rad26:4a* extragenic suppressor mutations: Ultraviolet radiation induced mutagenesis was performed as described previously [18]. Briefly, *rad26::4a* cells were grown in liquid YE5s culture plated onto 50 YE5S plates (5,000 cells per plate). Plates were then UV-irradiated with 250 J/m^2 using a UV Stratalinker (Stratagene) and incubated at 30°C. The ten percent of *rad26::4a* yeast cells that survived and formed colonies after five days were replica plated to YE5S plates containing the viability indicator Phloxine B (Sigma Aldrich) with or without 8 μg/ml MBC. Five surviving colonies were isolated, streaked onto YE5S media and outcrossed to TE236 (leu1-32 ura4-d18 h$^-$; data not shown). The genotype of the *rad26::4A* strain (TW1279; [13]) is *rad26:4A-yfp (leu*$^{+}$ *G418*^{R}*) rad26::ura4+ leu1-32 ura4-D18 ade6-704 h*$^-$. In this strain, the *leu*$^{+}$ and *G418*^{R} markers are linked to the *rad26::4A* allele. Therefore, we determined if the suppressor mutations were extragenic by screening for recombinants that were ura-, leu- and sensitive to G418 by replica plating to EMM drop out plates and YE5S complete plates containing G418. Only one of the UV surviving colonies, which we named *mut2a*, contained a single genetic trait that conferred sensitivity to MBC upon outcrossing. The genotype of this strain (TW1335) is *mut2a leu1-32 ura4-d18* and

was successfully outcrossed from the *rad26::4A-yfp* (*leu*$^{+}$*G418*^{R}) allele as well as the *rad26::ura4*$^{+}$ deletion allele (Fig. 1).

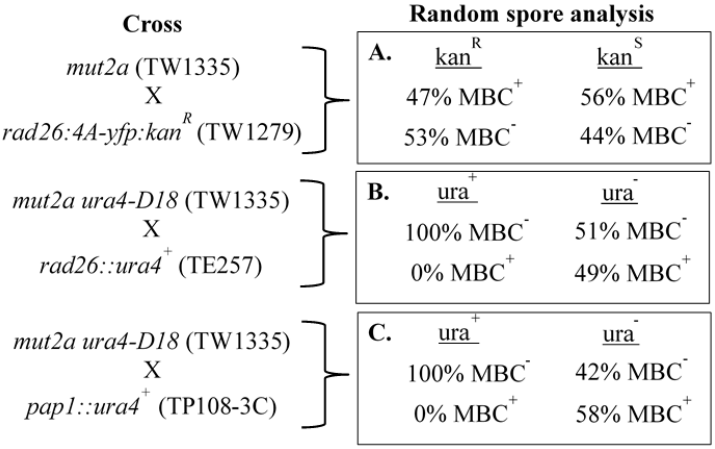

Figure 1: Random spore analyses of 200 progeny per cross. **A. *mut2a* suppresses the MBC sensitivity of *rad26:4A*.** Roughly half of the kan^{R}esistant colonies were able to grow on plates containing 8µg/ml MBC. These results also indicate that *mut2a* confers MBC sensitivity (MBC^{-}) following outcross from the *rad26:4A* allele. **B. *mut2a* does not suppress the MBC sensitivity of *rad26::ura*$^{+}$ cells.** The ura^{+} marker strictly co-segregated with MBC sensitivity (MBC^{-}). **C. The *mut2a* mutation is not allelic with *pap1*$^{+}$.** Roughly half (58%) of ura- progeny displayed wildtype levels of growth on MBC (MBC^{+}).

Transformation and Replica Plating: A lithium acetate transformation protocol [19] was used to transform the pTN-F2 *URA3* genomic library [20] into the mut2a strain. A single *mut2a* colony from YE5S complete media was inoculated in 50ml of YE5S liquid media containing 50µL of carbencillin (Sigma-Aldrich). This inoculated media was placed in an Incu Shaker Mini (Benchmark) at 116 rpm and 30ºC overnight to develop yeast culture. This culture was centrifuged for 5 minutes on Beckman Allegra ZIR centrifuge at 3000rpm at 4ºC. The pellet was suspended in 50 ml of sterile water and centrifuged at the previously mentioned cycle. The supernatant was carefully decanted and pellet was dissolved in the drop of water left in the tube. This content was transferred to 1.5ml Eppendorf tubes and centrifuged at 3000rpm for 20 seconds in a VNR galaxy mini. This final pellet was dissolved in 1ml of LiAc/TE solution and centrifuged again for 20 seconds at 3000rpm. After removing supernatant the pellet was gently mixed with 10 µL of pTNF2 yeast genomic library and 2µL of sheared herring sperm DNA and incubated at room temperature for 10 minutes. Control plates were made by spreading 10µL of this mixture on to EMM-ALH solid media plates. The remaining reaction tube solution was mixed with 260 µL of 4000PEG (Sigma) (40%w/v) and incubated at 116rpm and 30ºC for 1 hour in an incushaker. 43µL of DMSO was added to reaction tube and heat shocked for 5 minutes at 42 ºC. This reaction mixture was centrifuged again at 3000rpm for 20 seconds to obtain a pellet. This pellet was washed twice with EMM-ALH liquid media and centrifuged. The resulting pellet was dissolved in 200µL EMM-ALH liquid media and a 100µL of final cell volume was spread on EMM-ALH solid media plates using sterile glass spreader. These plates were incubated upside down at 30 ºC for 3 days. Transformants growing on EMM-ALH selective media plates were replica-plated using the stand and felt on to EMM-ALH-MBC (8µg/ml) solid media plates. These plates were incubated for 3 days at 30 ºC in an incubator. The viable transformant colonies from EMM-ALH media plates corresponding to the ones growing on MBC containing media were grid streaked on to EMM-ALH media plates and incubated at 30 ºC for 3 days.

Plasmid Complementation Test: To test if library complementation of the *mut2a* MBC-sensitive growth phenotype was due to a library plasmid or a genomic mutation that occurred during the transformation procedure, the transformant was streaked onto complete YE5S media and incubated for 3 days to obtain single colonies. These colonies were then replica plated on to EMM-AULH-MBC and EMM-ALH gel media for 3 days to determine if complementation activity was linked to the URA3 library plasmid.

Genomic DNA extraction: A single transformant viable colony with the desired plasmid was inoculated in 1ml of YE5S liquid media and incubated at 150rpm and 37 ºC for 12 hours. This culture was the centrifuged at 14K rpm for 1 minute. The pellet was then resuspended in 293µL of 50ml EDTA, 7.5µL of 75 units/µL lyticase (MP Biomedicals) and left at room temperature for 60 minutes. After an hour the reaction mix was centrifuges at 14K rpm for 2 minutes. 300µL of Nuclei lysis solution and 100µL of protein precipitation solution were gently mixed to the pellet after carefully removing supernatant. The mix was centrifuged again with same specifications to obtain a clear supernatant. This supernatant was transferred to a clean tube, mixed with 300µL of isopropanol and centrifuged at 14K rpm for 3 minutes. The supernatant was carefully discarded. The pellet was dissolved in 300µL of 70% ethanol. A last centrifugation was done to obtain DNA pellet after aspirating the ethanol. The DNA pellet was air dried and dissolved in 100µL of 1X TE. The Wizard® Genomic DNA Purification Kit (Promega) was used for Genomic DNA extraction.

Restriction digestion and gel electrophoresis: All restriction digestions were performed using 2µL of DNA, 16µL of molecular grade water, 2µL of the buffer and 0.5µL of appropriate restriction enzymes. A pre-set thermocycler program was used to run restriction cycle. Agarose solution (0.8%) was prepared by dissolving 0.32gm of Omnipure Agarose in 40ml 1X TAE buffer by microwaving it for 45 seconds to ensure thorough dissolving. 4µL of ethidium bromide solution was added to agarose mixture and the solution was allowed to cool for 10 minutes. This cooled solution was poured in prepared electrophoresis tank. The seals and well combs were carefully removed once the gel solidified. The gel was submerged in 1X TAE buffer. The wells were loaded with necessary restriction digestion mix and 1kb gene ruler ladder. 70mV current was used to run the gel for 45 minutes. The gel was observed in a UV transilluminator.

***E.coli* transformation:** The *E.coli* competent cells were thawed on ice after removal from the freezer. Purified genomic DNA (10µL) was added to 250µL of *E.coli* cells and incubated on ice for 10 minutes. The cells were heat shocked for 2 minutes at 42 ºC and incubated on ice for 1 minute. The cells were then incubated at 37 ºC and 200rpm for 1 hour after adding 1ml of SOC media. 100µL of this cell mix was spread on LB carb solid media plates. The rest of the cell mixture was centrifuged at 14K rpm for 1 minute and 800µL of SOC was aspirated. The cells were resuspended in the remaining SOC and spread on LB carb media plates and incubated at 37 ºC for 24 hours. The *E.coli* cells without DNA served as a control.

Alkaline lysis DNA extraction: Successfully transformed bacterial colonies were used to extract plasmid DNA by using Qiagen MIDI prep kit.

DNA Sequencing and Primer design Complementing library plasmid sequencing: The *S.pombe* genomic DNA was cleaved by BamHI to create pTNF2 genomic library so we utilized the nucleotide sequence flanking the BamHI site in the pBR322 vector backbone to design the sequencing primers below:

Forward primer- 5'GGC GAC CAC ACC CGT CCT GTG 3'

Reverse primer- 5'GCG TCC GGC GTA GAG GAT C 3'

Pap1 sequencing from *mut2a* strain: The genomic copy of *pap1*$^+$ present in mut2a strain TW1335 was amplified (Phusion DNA polymerase, Thermo Fisher) using five overlapping sets of primers, cloned into the pJet1.2 blunt-end PCR cloning vector (Thermo Scientific) and sent for sequencing (Integrated DNA Technologies, Coralville, IA). Sequencing reactions were carried-out using the pJET1.2 forward and reverse primers (IDT). The five primer pairs (5'- 3') used to amplify genomic *pap1*$^+$ follow:

Primer pair 1- CGTTAGCCCTCCTATGGCTG, ATGGATACATTTGCTCAGGGA
Primer pair 2- AGTTCCAAGCGGAAAGCTCA , AGCTAGGAGTCTCGGCGAT
Primer pair 3- ACATAACGACCGTAGTGCAGA, TCCGTACCACCAAAGGGACT
Primer pair 4- GGTCTTGATTTTCTGGAGAATGCC, TGGATACATTTGCTCAGGGAA
Primer pair 5- CGTTAGCCCTCCTATGGATG , AGATCAAACCAAGCAGGCCA

Sequencing reaction mix: Nanodrop 2000 (ThermoScientific) was used to measure the concentrations of the primers and the plasmid DNA. These were then diluted to final concentrations as required by the sequencing company's protocol (Functional Biosciences).

RESULTS

Isolation of *mut2a*, an extragenic interaction suppressor of *rad26 4A*: Ultraviolet radiation-induced mutagenesis of *rad26::4a* was used to identify an extragenic mutation that permitted the growth of *rad26:4A* cells on MBC medium (see Materials and Methods). We named this mutation *mut2a*. Random spore analyses data presented in Figure 1A show that *mut2a* suppressed that MBC-growth defect of a kanamycin (kan^R)-marked *rad26:4A-yfp* allele, and conferred sensitivity to MBC upon outcross. The results of Figure 1B show that the *mut2a* mutation did not suppress the *rad26::ura*$^+$ null allele, since 100% of *ura*$^+$ progeny in this cross retained MBC sensitivity. Together, these random spore analyses data indicate that *mut2a* is an extragenic interaction suppressor [17, 21]. We hypothesize that the product of the *mut2*$^+$ gene physically interacts with Rad26^{ATRIP} during the interphase microtubule damage response.

Genomic library complementation of *mut2a* cells: *mut2a* (ura-) cells were transformed with the Ura$^+$ pTN-F2 genomic library (Table 1). Transformants were replica-plated to uracil drop-out media (EMM+ALH) media containing 8μg/ml of MBC and the vital dye Phloxin B. Viable transformants on MBC formed larger colonies that were pinkish in color, while non-viable transformants turned darker red due to accumulation of the vital dye (Fig. 2).

Table 1: Fission yeast strains, plasmids and library

Strains		
TW1275	*rad26:4A (leu+) rad26::ura4+ leu1-32 ura4-D18 ade6-704 h+*	Herring *et al.*, 2010
TW1279	*rad26:4A-yfp:kan^R (leu+) rad26::ura4+ leu1-32 ura4-D18 ade6-704 h+*	Herring et al., 2010
TE236	*leu1-32 ura 4-d18 h–*	Kostrub *et al.*, 1998
TP108-3C	*pap1::ura4+ ura4 leu1 h-*	Toda *et al.*, 1991
TW1335	*mut2a leu1-32 ura4-D18 h+*	this study
TE257	*rad26::ura4 ade 6-704 leu1-32 ura4-d18 h-*	*Al-Khodairy et al., 1994*
Plasmids and Library		
SpENT48G01	ATP-dependent DNA helicase Hrp1	Riken
SpYFH40F06	transcription factor Pap1/Caf3	Riken
PTNF2	genomic DNA library URA3	National BioResource Project, Osaka City University

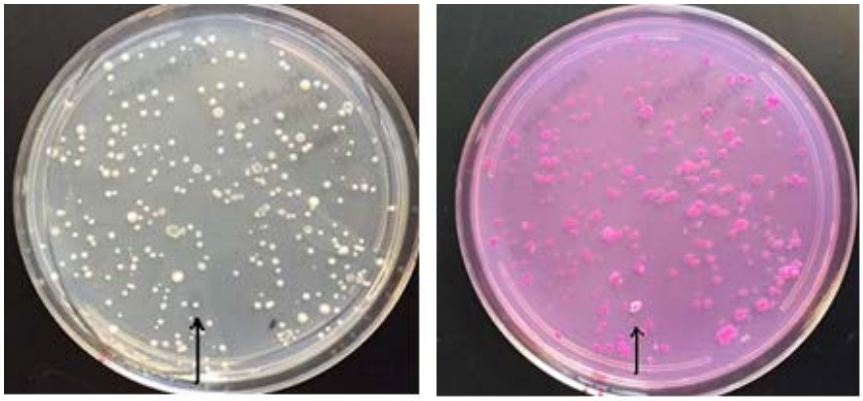

Figure 2: Genomic-library complementation of *mut2a*. The growth of *mut2a* transformants on the EMM-ura medium (left) and EMM-ura + MBC+ phloxinB medium (right) after 3 days. The growth of majority of cells lost viability while a single viable colony on the MBC medium (right) and its corresponding colony on the EMM media (left) is highlighted.

The pTN-F2 genomic library was constructed using a high-copy, autonomously replicating vector [20]. To determine if complementation was due to the library or an endogenous chromosomal mutation induced during the transformation procedure, transformants were picked from the EMM-ura plates and streaked onto fresh EMM-ura plates to maintain selection pressure. These colonies were then streaked onto complete medium containing uracil and replica plated to uracil dropout medium and complete medium containing MBC and phloxin B (Fig. 3). The results show that only colonies that grew on ura- medium also grew on MBC medium. This means that an autonomously replicating library plasmid was responsible for growth on MBC.

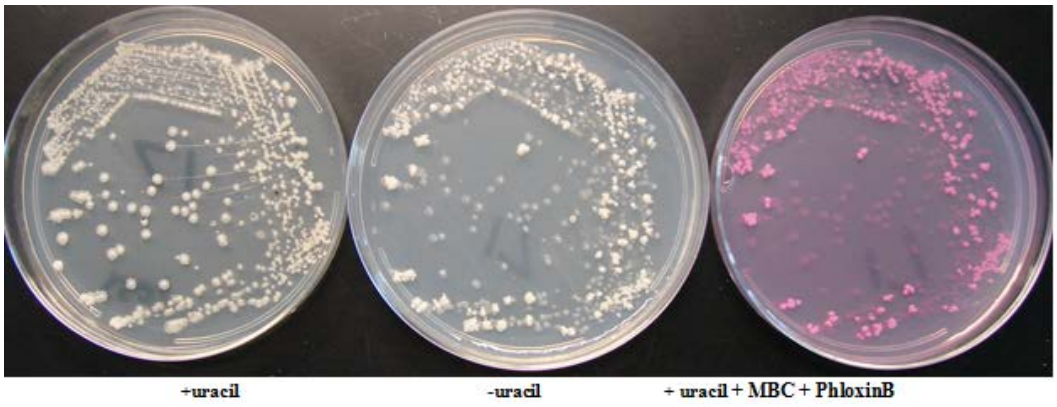

Figure 3: A Ura$^+$ library plasmid contained complementing activity. Cells were replica plated from complete medium (left) onto -ura (center) and -ura + MBC + phloxinB (right) plates. All the cells with plasmid conferring MBC resistance are viable and light pink on MBC media and grew on the –ura media. Cells that lost the plasmid did not form colonies on the –ura plates or the -ura MBC plates.

The complementing plasmid contained five open reading frames: The *S.pombe* genomic library was created by inserting BamHI genomic fragments in the plasmid pFL20 with pbr322 backbone (~8kb). Using restriction digest analyses (data not shown), we deduced the size of insert to be approximately 11 kb (Fig. 4).

The insert in the complementing library plasmid was sequenced using Functional Biosciences (Madison, WI; see Materials and Methods). The sequencing results returned by this facility were aligned using the nucleotide BLAST from the National Center for Biotechnology Information and showed that this plasmid contained *hrp1*$^{+}$, *pap1*$^{+}$, *atg12*$^{+}$, *rpl1502*$^{+}$ and SPAC1327.01c.1 (Fig. 5). These genes are present on the chromosome I of the *S. pombe* and stretches from nucleotide position 2194820 to 2208418 in the genome. Of immediate interest were *hrp1*$^{+}$ and *pap1*$^{+}$, since mutations in either confer sensitivity to microtubule destabilizing agents [22, 23].

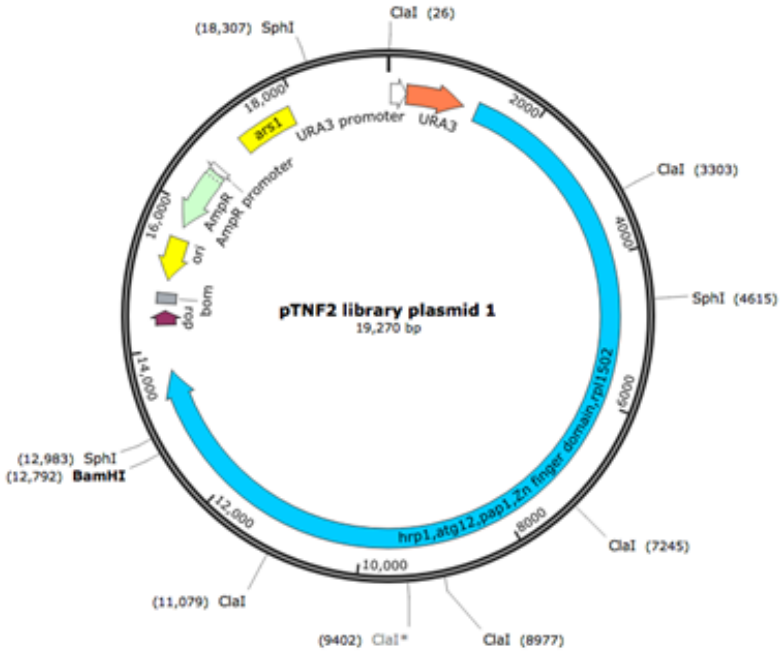

Figure 4: Map of the complmenting library plasmid

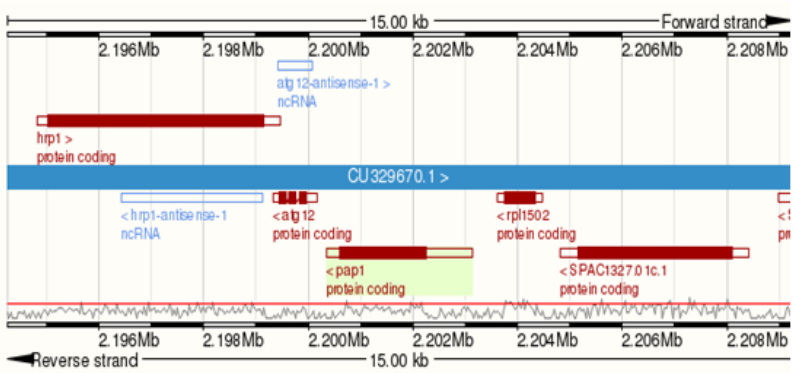

Figure 5: Physical map of the genes contained in the insert of the complementing library plasmid. BLAST results of the sequenced library insert showed that it contained *hrp1*$^{+}$, *pap1*$^{+}$, *atg12*$^{+}$, *rpl1502*$^{+}$and SPAC1327.01c.1.

***pap1*$^{+}$ rescued the MBC sensitivity of *mut2a* cells:** We requested the clones for the genes present in the insert from the Japan Yeast Genetics resources to determine if one was responsible for conferring MBC resistance to the *mut2a* cells. As apparent from Figure 6, the

cells that were transformed with *pap1*$^+$ gene alone provided complementation in the MBC media and showed similar growth and light pink color cells as the rescued library plasmid. The *hrp1*$^+$ transformed cells were smaller and darker red in color due to inhibited growth on MBC and accumulation of PB dye within cells. These results indicated that *pap1*$^+$ is responsible for rescuing the MBC sensitivity of the *mut2a* cells.

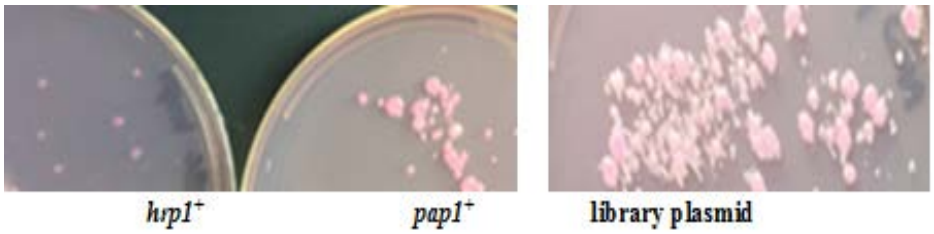

Figure 6: *Pap1*$^+$ rescued the growth of *mut2a* cells on MBC medium. Comparative images of the transformation results showed that *pap1*$^+$, contained within the insert of the library plasmid, was responsible for complementing activity.

***pap1*$^+$ is an extracopy suppressor of *mut2a*:** To if *mut2a* is a mutant allele of *pap1*$^+$, the *mut2a* strain was crossed to a *pap1::ura*$^+$ deletion strain [24] and random spore analysis was performed (Fig. 1C) [21]. Failure to recover recombinant colonies with wildtype levels of resistance to MBC would support to this hypothesis. However, Figure 1C showed that roughly half of *ura*$^-$ colonies exhibited wildtype levels of growth on MBC. Furthermore, DNA sequencing results demonstrated that the entire coding region of *pap1*$^+$ was normal in the *mut2a* strain (data not shown). These outcomes all together led us to conclude that *pap1*$^+$ and *mut2a* were not allelic. Rather, the expression of extra copies *pap1*+ from the autonomously replicating, high copy library plasmid was likely responsible, given that the pTN-F2 genomic library was constructed in a multicopy plasmid pFL20 [25], as was the *pap1*$^+$ plasmid obtained from Riken [26, 27]. We conclude that *pap1*$^+$ is an extracopy suppressor of the *mut2a* growth phenotype on MBC.

DISCUSSION

In an attempt to identify a gene encoding a Rad26 interacting protein required for the response to interphase microtubule damage, we identified a novel role of *pap1*$^+$ as an extra copy suppressor of *mut2a* sensitivity to MBC. Extra copy suppression of a phenotype occurs due to expression of multiple copies of a gene from a plasmid in addition to the inherent cellular gene. Pap1 and its budding yeast homolog Yap1 localize from the cytoplasm to the nucleus in response to stress where it regulates the expression of genes associated with cellular stress response [28]. Pap1 is required for upregulation of transcription of genes that respond to oxidative and peroxide stress and can impart multidrug resistance [24, 28, 29]. Indeed, it has been shown that expression of extra copies of *pap1*$^+$ from a multi-copy plasmid confers resistance to DNA damaging drugs in *chk1Δ* mutant cells [30]. Over produced Pap1 induced staurosporine (protein kinase inhibitor) resistance is hypothesized to be due to *pap1* - dependent upregulation of staurosporine targeted kinases or initiation of feedback mechanism resulting in activation of the same kinases [24].

We do not know the exact mechanism involved in extra copy *pap1*$^+$ dependent MBC resistance. One of the widely accepted mechanisms of *pap1*$^+$ induced resistance is the transcriptional upregulation of cellular efflux pumps [28]. Efflux pump upregulation by *pap1*$^+$ is

also involved in multidrug resistance and has been shown to be the mechanism for caffeine resistance in fission yeast [31].

In conclusion, we discovered that *pap1*$^{+}$ extra copy expression allows growth of *mut2a* cells on medium containing a microtubule destabilizing agent. The possibility that *pap1*$^{+}$ reduces intracellular drug accumulation to curtail cellular toxicity is a suggested mechanism of suppression.

Acknowledgement: The authors wish to thank Mr. Brent Wallace for laboratory support, the Partnership in Innovative Preparation for Educators and Students in the Center for Science, Technology, Engineering and Mathematics Education (CSTEME) at UCCS for financial support to R.W., and the Yeast Genetic Resource Center for strains and plasmids (http://yeast.nig.ac.jp/yeast/).

Conflict of Interest: The authors have no conflict of interest with CSTEME, the organization that sponsored this research.

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