RNF4's Role in Oncogene-Induced Replication Stress and Cancer-associated RNF4 Mutation G138*

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Abstract

RNF4 is a SUMO-targeted ubiquitin ligase known to play a role in DNA damage repair and maintain genome stability (Yin et al., 2012 & Galanty et al., 2012). G138* is a cancer-associated mutation in RNF4's RING domain, preventing RNF4 from functioning as a ubiquitin ligase. We transformed WT and *slx5*Δ *S. cerevisiae* with WT and G138* carrying RNF4 to confirm whether the G138* mutation negatively affects genome stability by preventing RNF4 from participating in DNA repair processes. Here we show that incorporating RNF4 into *slx5*Δ cells may encourage growth comparable to that of WT, and that incorporating G138* RNF4 may negatively affect genome stability. Additionally, we overexpressed RNF4 in breast cancer cell lines MCF-7 and MDA-MB-468 to observe whether the overexpression of RNF4 alleviates oncogene-induced replication stress and promotes cell proliferation. We hypothesized that RNF4 is able to share the functions of its homolog heterodimer, Slx5/Slx8, which alleviates replication stress in *S. cerevisiae* by ubiquitinating mitotic checkpoint proteins and preventing mitotic arrest (Thu et al., 2016). We show that the overexpression of RNF4 may alleviate oncogene-induced replication stress in MDA-MB-468 but fail to do so in MCF-7.

Introduction

Replication stress is a cellular phenomenon in which normal replication of DNA is impaired. Signs of replication stress includes an increase of stalled or collapsed replication forks that prevent replication from taking place (Horejsí et al., 2005). Studies of tumorigenic cells showed that activated oncogenes promote replication stress, inducing a DNA damage response leading to senescence or apoptosis (Hills and Diffley, 2014). These defense mechanisms serve as an inhibitor of tumor progression. Consistent with this idea, mutations in the DNA damage response pathway have been identified in cancer.

One of the proteins in mammalian cells that is believed to play a significant role in DNA damage repair is RNF4. RNF4 is an E3 SUMO-targeted ubiquitin ligase (STUbL) complex composed of three major domains: SIM (SUMO Interaction Motif), RING (Really Interesting New Gene), and ARM (Arginine Rich Motif) (Prudden et al., 2007 & Sriramachandran, 2014). It recognizes SUMO (Small Ubiquitin-like Modifier) conjugates, a post-translational modification that allows RNF4 to bind to and ubiquitinate sumoylated substrates (Uzunova et al., 2007). In DNA damaging conditions, SUMO chains accumulate in the cell (Hay et al., 2014) and RNF4 is recruited to the site of damage (Yin et al., 2012 & Galanty et al., 2012), suggesting a major role for RNF4 in DNA damage repair. Additionally, the deletion of the RNF4 gene in human cells results in an increased sensitivity to DNA damage agents (Yin et al., 2012), further highlighting RNF4's role in maintaining genome stability.

A homolog of RNF4 has been studied to alleviate replication stress in yeast cells. The Slx5/Slx8 heterodimer has been shown to mediate nuclear-pore associated repair of broken replication forks (Nagai et al., 2008). Additionally, genes that encode Slx5/Slx8 show a negative interaction with genes that allow normal replication fork elongation (Thu et al., 2016). Furthermore, Slx5/Slx8 has been shown to target mitotic checkpoint proteins for degradation, allowing cells to overcome mitotic arrest when replication conditions are suboptimal (Thu et al., 2016). These findings suggest a role for Slx5/Slx8 under conditions which compromise the ability of cells to accomplish faithful propagation of the genome.

Given Slx5/Slx8's role in alleviating replication stress in yeast cells (Thu et al., 2016), we want to confirm whether RNF4 serves a similar role in mediating oncogene-induced replication stress in cancer. Cancer cells chronically experience replication stress due to rapid proliferation triggered by oncogenes. Given RNF4's role in genomic stability and replication stress (Thu et al., 2016) (Yin et al., 2012 & Galanty et al., 2012), RNF4's function in cancer cells has not yet been thoroughly explored. Previous findings and the nature of RNF4 as a homolog of Slx5/Slx8 suggest a role for RNF4 in alleviating replication stress and allowing abnormal cell proliferation to continue.

To confirm RNF4's role in alleviating oncogene induced replication stress, we overexpressed RNF4 in two breast cancer cell lines: MCF-7 and MDA-MB-468. The two cells differ in oncogene expression and p53 deficiency. MDA-MB-468 has a higher oncogene expression (Eckert et al, 2004) and carries mutant p53, while MCF-7 carries WT p53. In cell lines with WT p53, replication stress induces G2 arrest. However, previous findings showed that in p53 deficient cell lines, replication stress induces mitotic arrest by activating mitotic checkpoint proteins (Nitta et al., 2004). Thus, in MDA-MB-468, which carries mutant p53, we hypothesize that the overexpression of RNF4 may target mitotic checkpoint proteins for degradation and allow mitosis to occur. The overexpression of RNF4 could alter cells to utilize the E3 ligase function of RNF4 and prioritize the RNF4 pathway to prevent mitotic arrest. This hypothesis aligns with RNF4's homolog Slx5/Slx8's role in alleviating replication stress in yeast cells (Thu et al., 2016). In MCF-7 cells, we hypothesize that the overexpression of RNF4 should not affect the proportion of cells in mitotic arrest since replication stress in the presence of WT p53 should induce G2 arrest (Nitta et al., 2004). To confirm RNF4's role in alleviating replication stress, we observed changes in the percentage of cells displaying signs of the mitotic index, metaphase-anaphase transition, and micronuclei under RNF4 overexpression. Our findings show that the overexpression of RNF4 decreases the proportion of MDA-MB-468 cells in mitosis but does the opposite for MCF-7.

In addition to studying the correlation between RNF4 expression and mitotic arrest, we studied cancer-associated mutations in RNF4. Due to its role in maintaining genomic stability (Yin et al., 2012 & Galanty et al., 2012), we predict that RNF4's function contributes to cancer. Multiple RNF4 mutations have been documented in various malignances. However, the significance of these mutations have not been explored. To study how these mutations affect the molecular function of RNF4, we used budding yeast as a genetic model. The mutation of interest is G138*, which replaces a glycine amino acid with a stop codon. The mutation resides on the RING finger domain, which is responsible for ubiquitinating sumoylated substrates (Uzunova et al., 2007). We hypothesize that yeast cells possessing the mutant RNF4 will show greater sensitivity to genotoxic agents because RNF4's main function as a ubiquitin ligase will be impaired, preventing RNF4 from participating in DNA repair processes. To confirm whether mutation G138* impairs RNF4's function in genomic stability, both WT and mutant RNF4 has been incorporated into WT and *slx5*Δ yeast cells. Transformed strains were exposed to genotoxic agents to create a condition similar to oncogene-induced replication stress. We were unsuccessful in incorporating WT RNF4 into *slx5*Δ yeast cells, but our findings suggest that the cancer-associated mutation G138* may impair genomic stability.

Results

WT RNF4

ATGAGTACAAGAAAGCGTCGTGGTGGAGCAATAAATTCTAGACAAGCTCAGAAGCGAACT
CGGGAAGCAACCTCCACCCCCGAGATCTCCTTGGAAGCAGAACCCATAGAACTCGTGGAA
ACTGCTGGAGATGAAATTGTGGACCTCACTTGTGAATCTTTAGAGCCTGTGGTGGTTGAT
CTGACTCACAATGACTCTGTTGTGATTGTTGACGAAAGAAGAAGACCAAGGAGGAATGCT
AGGAGGCTGCCCCAGGACCATGCTGACAGCTGTGTGGTGAGCAGTGACGATGAGGAGTTG
TCCAGGGACAGAGACGTATATGTGACTACCCATACTCCCAGAAACGCCAGGGATGAGGGC
GCTACAGGCCTCAGGCCCTCAGGTACTGTCAGTTGTCCCATCTGCATGGAC**GGA**TACTCA
GAGATCGTGCAGAATGGACGTCTCATCGTTTCCACAGAATGCGGCCATGTCTTCTGTAGC
CAGTGCCTCCGTGATTCCCTGAAGAATGCTAATACTTGCCCAACTTGTAGGAAAAAGATC
AACCACAAACGGTACCACCCCATTTATATAGTCGACTGA

G138*

ATGAGTACAAGAAAGCGTCGTGGTGGAGCAATAAATTCTAGACAAGCTCAGAAGCGAACT
CGGGAAGCAACCTCCACCCCCGAGATCTCCTTGGAAGCAGAACCCATAGAACTCGTGGAA
ACTGCTGGAGATGAAATTGTGGACCTCACTTGTGAATCTTTAGAGCCTGTGGTGGTTGAT
CTGACTCACAATGACTCTGTTGTGATTGTTGACGAAAGAAGAAGACCAAGGAGGAATGCT
AGGAGGCTGCCCCAGGACCATGCTGACAGCTGTGTGGTGAGCAGTGACGATGAGGAGTTG
TCCAGGGACAGAGACGTATATGTGACTACCCATACTCCCAGAAACGCCAGGGATGAGGGC
GCTACAGGCCTCAGGCCCTCAGGTACTGTCAGTTGTCCCATCTGCATGGAC**TGA**TACTCA
GAGATCGTGCAGAATGGACGTCTCATCGTTTCCACAGAATGCGGCCATGTCTTCTGTAGC
CAGTGCCTCCGTGATTCCCTGAAGAATGCTAATACTTGCCCAACTTGTAGGAAAAAGATC
AACCACAAACGGTACCACCCCATTTATATAGTCGACTGA

Figure 1. Sequence data of WT RNF4 and RNF4 containing G138* mutation. Primers including the G138* mutation were used to replicate HA-RNF4 of the pRK5 plasmid. Replicated plasmids containing G138* were sent in for sequencing. Highlighted sequences show the point mutation causing glycine codon to transform into a stop codon.

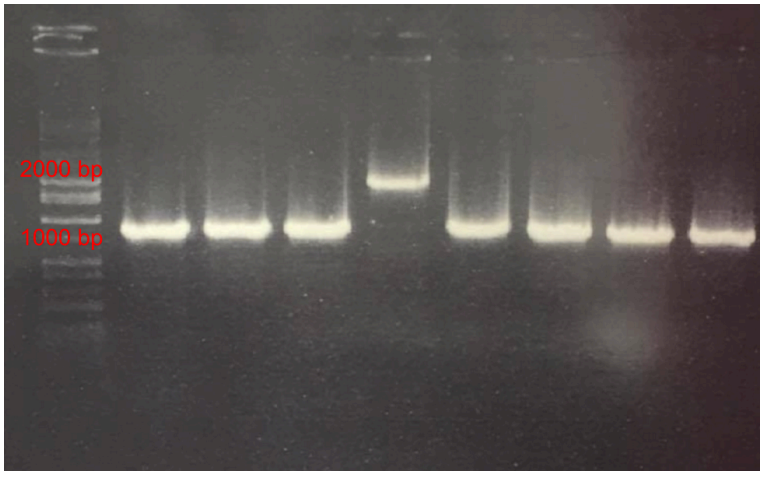

Figure 2. Successful incorporation of RNF4 into the endogenous locus of *SLX8* displayed on gel. Primers containing sequences 40 bp up and 20 bp down of Slx8 were used to confirm incorporation. HA-RNF4-HIS fragment is around 1800 bp, Slx8 is 825 bp.

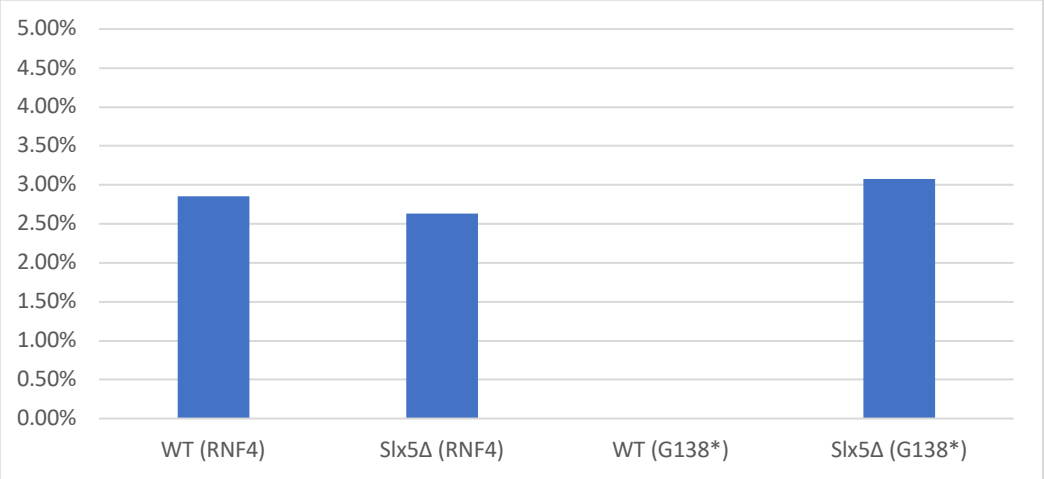

Figure 3. Frequency of successful incorporation of WT or G138* RNF4 in yb1894 (WT) and yb1985 (*slx5*Δ). Colonies screened: WT(RNF4) = 35, *slx5*Δ(RNF4) = 38, WT(G138*) = 109, *slx5*Δ(G138*) = 65.

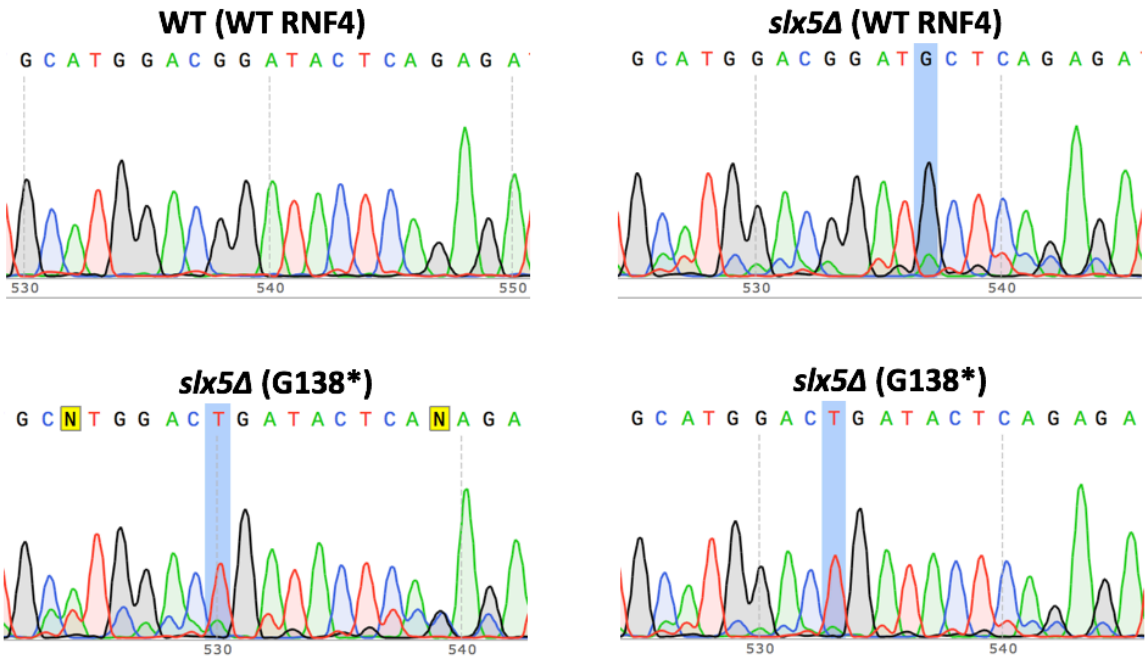

Figure 4. Sequencing data of transformed yeast strains. Primers 120 bp up of *SLX8* and down of RNF4 were used to sequence RNF4 in transformed yeast strains. WT (WT RNF4) sequence was identical to that of WT RNF4. *Slx5*Δ (WT RNF4) sequence indicated a point mutation from thymine to guanine. *Slx5*Δ (G138*) strains' sequences displayed the point mutation as intended.

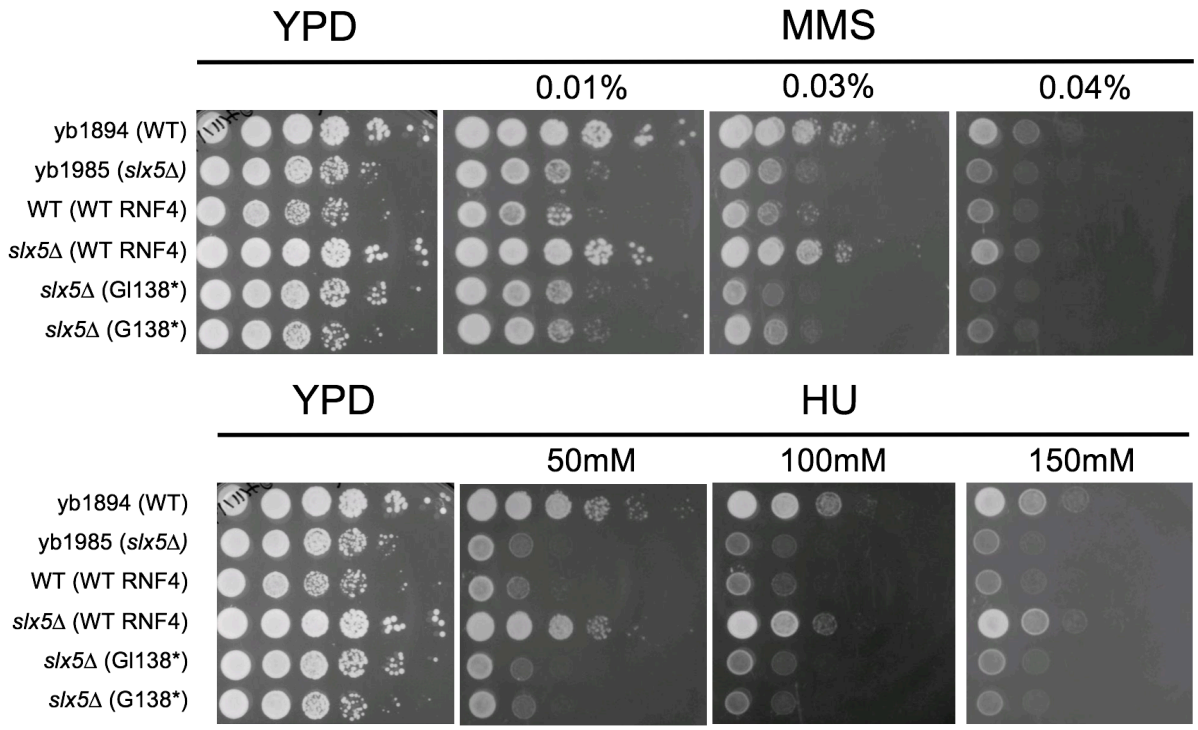

Figure 5. Spotting Assay of yb1894 (WT), yb1985 (*slx5*Δ), WT(RNF4), *slx5*Δ(RNF4) , *slx5*Δ(G138*) , *slx5*Δ(G138*). Listed genotypes were spotted on 50 mM, 100 mM, 150 mM HU, and 0.01%, 0.03%, 0.04% MMS to challenge growth and highlight the difference in phenotypes. Images were captured on day 2 of spotting.

WT RNF4 is more easily integrated into the endogenous locus than mutant G138* RNF4.

To generate a plasmid carrying G138* mutation, the pRK5 plasmid was replicated with primers containing the G138* mutation. The incorporation for our mutation of interest was confirmed by sequencing (Fig. 1). Using the pRK5 plasmid containing WT RNF4 and the newly synthesized plasmid containing G138* as templates, the RNF4 gene was replicated with PCR. The RNF4 fragment was annealed to another PCR product containing the HIS gene, which allows cells that successfully incorporate the gene to grow on histidine dropout media. The ends of the annealed fragment contained sequences that are homologous to 40 base pair upstream and downstream of the endogenous locus of *SLX8* gene. Subsequently, WT and *slx5*Δ yeast cells were transformed with the annealed PCR fragment to allow for integration of the RNF4/HIS fragment at the *SLX8* locus via homologous recombination. Successful transformations of yeast for both WT and mutant RNF4 were confirmed by PCR at the *SLX8* region (Fig. 2). Screening for successful incorporation of RNF4 at the endogenous locus showed that the frequency of success was very similar for WT RNF4 in WT (2.86%), *slx5*Δ (2.63%), and G138* in *slx5*Δ (3.08%) yeast cells (Fig. 3). However, incorporation of G138* in WT yeast cells has not been successful even after screening over 100 colonies (Fig. 3).

Incorporation of G138* RNF4 may impair genome stability

Once WT RNF4 or G138* was incorporated into WT and *slx5*Δ yeast cells, PCR products of screened colonies were sequenced to confirm successful integration at the correct endogenous

locus. Our sequencing data shows that WT (WT RNF4) was successfully transformed with WT RNF4. However, *slx5*Δ (WT RNF4) displayed a point mutation, changing a tyrosine to a cysteine residue. Additionally, both *slx5*Δ (G138*) strains were successfully transformed with mutant G138* RNF4 (Fig. 4). To confirm whether the cancer associated RNF4 mutation G138* affects genomic stability, yb1894 (WT), yb1985 (*slx5*Δ), WT (WT RNF4), *slx5*Δ (WT RNF4), and two *slx5*Δ (G138*) strains were spotted on plates containing various concentrations of MMS and HU, genotoxic agents that challenge cell growth by inducing DNA damage. By day 2 of growth on the spotting assay, *slx5Δ* transformed with RNF4 carrying the point mutation (Fig. 4) showed growth comparable to that of the WT yeast strain (Fig. 5), raising questions whether the point mutation contributed to the observed phenotype. It is also evident that *slx5*Δ yeast cells carrying the mutant G138* RNF4 do not grow as well as *slx5*Δ yeast carrying RNF4, suggesting that the G138* mutation decreases genomic stability (Fig. 5), although this cannot be confirmed due to the previously observed point mutation.

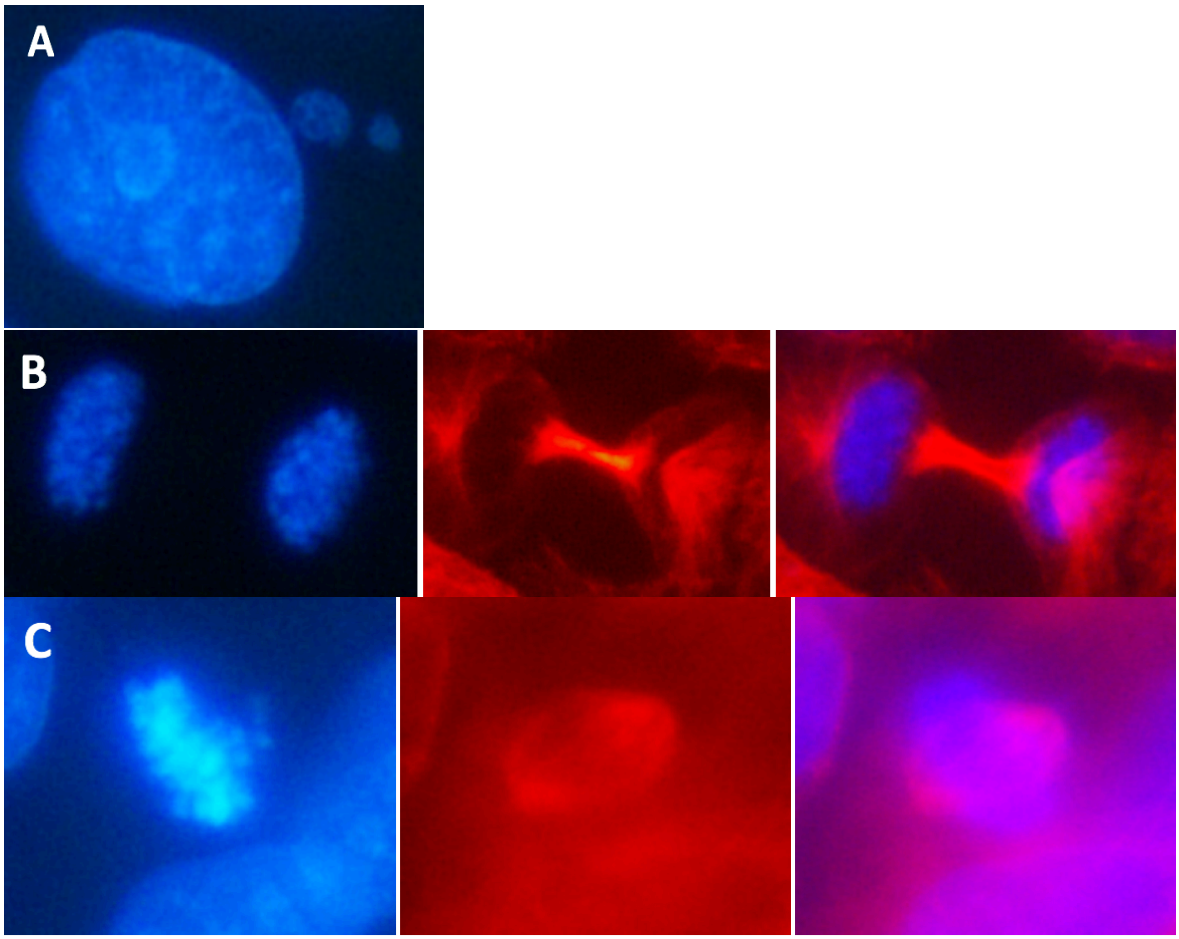

Figure 5. Example images of MCF-7 displaying phenotypes of interest. A) Micronuclei. B) Mitotic index. C) Metaphase-anaphase transition. Blue indicates DAPI staining, red indicates tubulin staining.

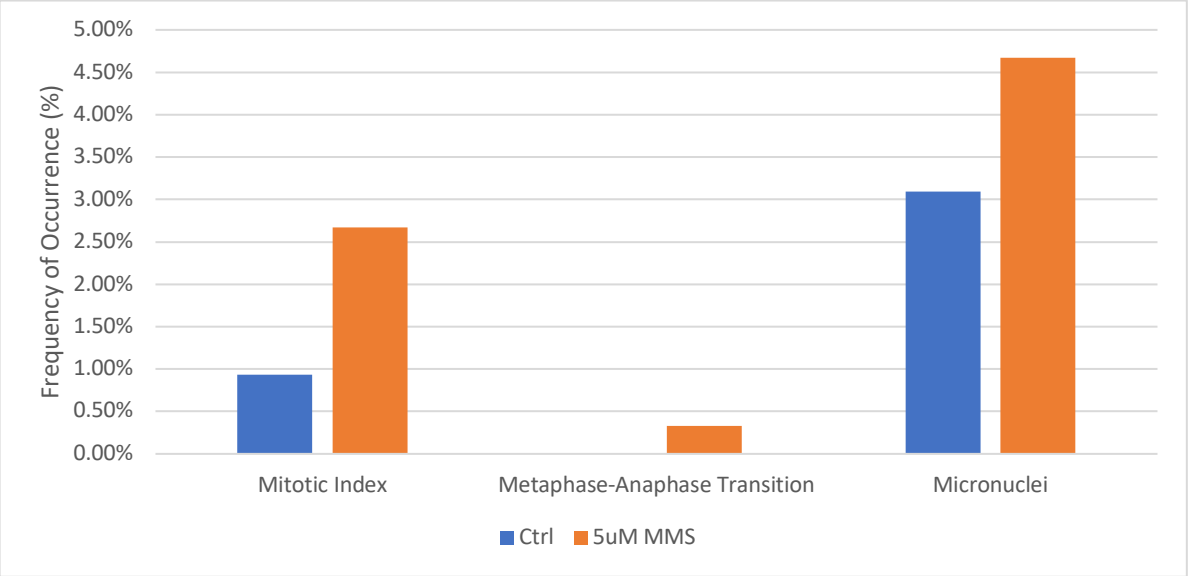

Figure 6. Preliminary study showing the frequency of occurrence for phenotypes of interest in MCF-7 cells exposed to 5µM MMS. Phenotype (Ctrl %, 5µM MMS %): Mitotic Index (0.93%, 2.67%), M-A Transition (0%, 0.33%), Micronuclei (3.09%, 4.67%). n=1.

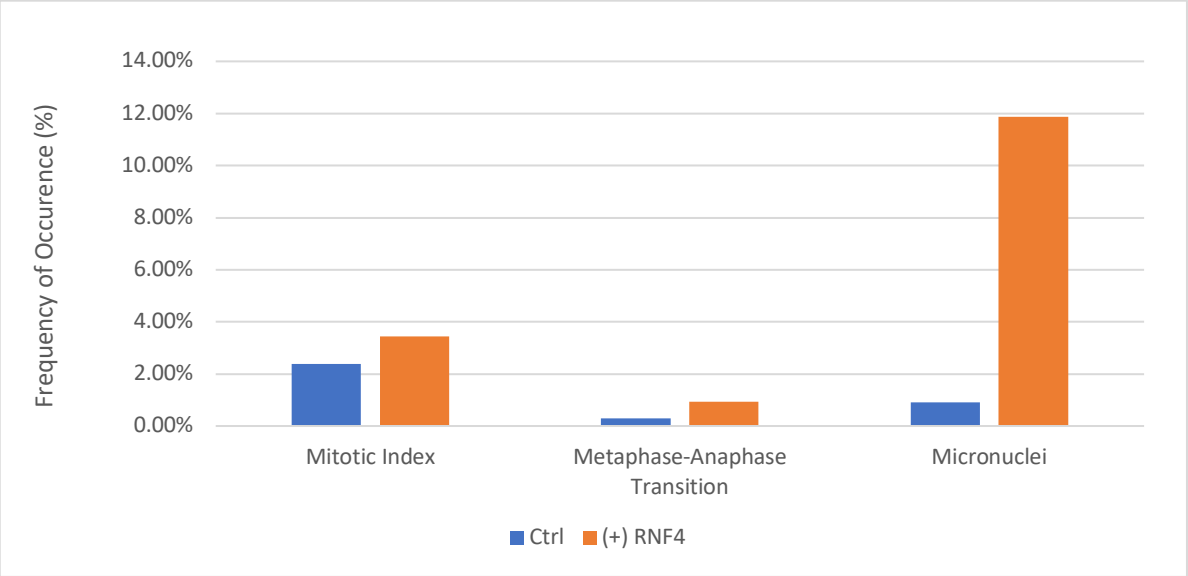

Figure 7. Frequency of occurrence for phenotypes of interest in MDA-MB-468 cells transfected with pRK5 (RNF4). Phenotype (Ctrl %, (+) RNF4 %): Mitotic Index (2.40%, 3.44%), M-A Transition (0.30%, 0.94%), Micronuclei (0.90%, 11.88%). n=1.

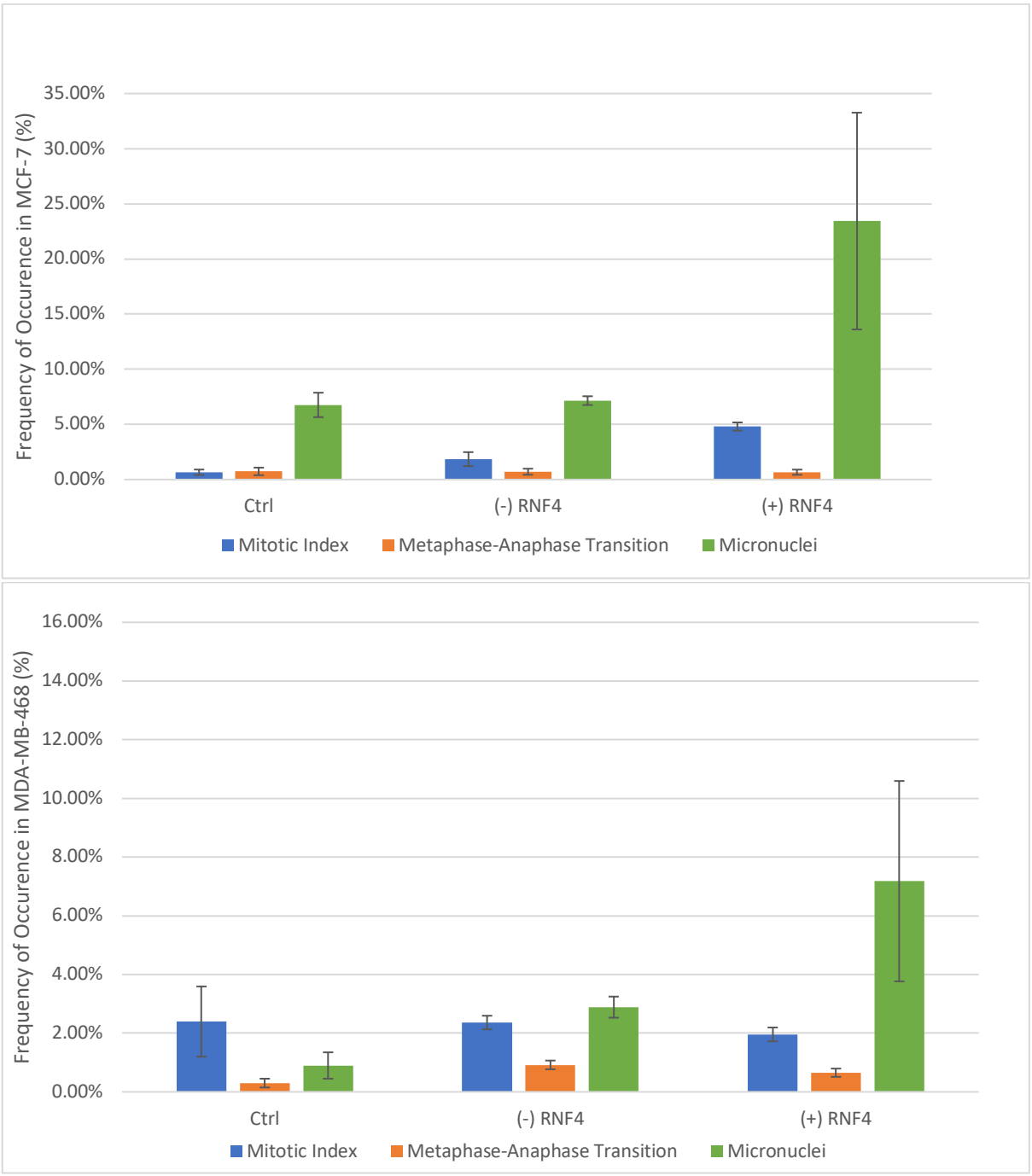

Figure 8. Frequency of occurrence for phenotypes of interest. MCF-7 and MDA-MB-468 cells were transfected with pRK5 plasmid to overexpress RNF4 and treated with 10 µM MMS for 18 hours. *Phenotype* (%, St. dev)*: MCF-7 Mitotic Index*: Ctrl (0.66%, 0.50%), (-) RNF4 (1.86%, 1.26%), (+) RNF4 (4.80%, 0.75%). *MCF-7 M-A Transition*: Ctrl (0.74%, 0.69%), (-) RNF4 (0.71%, 0.55%), (+) RNF4 (0.66%, 0.47%). *MCF-7 Micronuclei*: Ctrl (6.77%, 2.22%), (-) RNF4 (7.16%, 0.79%), (+) RNF4 (23.44%, 19.65%). *468 Mitotic Index*: Ctrl (2.40%, 2.40%), (-) RNF4 (2.36%, 0.47%), (+) RNF4 (1.96%, 0.47%). *468 M-A Transition*: Ctrl (0.30%, 0.30%), (-) RNF4 (0.92%, 0.30%), (+) RNF4 (0.65%, 0.28%). *468 Micronuclei*: Ctrl (0.90%, 0.90%), (-) RNF4 (2.89%, 0.72%), (+) RNF4 (7.18%, 6.83%). n=2, except for MDA-MB-468 Ctrl (n=1).

MMS exposure may increase the frequency of mitotic index, metaphase-anaphase transition, and micronuclei phenotypes.
To assess overexpression of RNF4 on genome stability and replication stress-induced mitotic arrest, we performed a preliminary study on MCF-7 cells. The percentage of cells that are in mitosis, exhibit metaphase-anaphase transition, and micronuclei were quantified. MCF-7 cells were stained with DAPI and tubulin staining. When exposed to 5 μM MMS, MCF-7 cells displayed an increase in percentage of cells in mitosis, metaphase-anaphase transition, and micronuclei (Fig. 6), confirming that exposure to genotoxic agents enhances our phenotypes of interest. Using these conditions, we asked if RNF4 overexpression could diminish these phenotypes by alleviating replication stress. However, it should be noted that the overexpression of RNF4 in the absence of MMS exposure may also increase the occurrence of these phenotypes, shown by our preliminary study conducted on MDA-MB-468 (Fig. 7).

Overexpression of RNF4 may increases frequency of mitotic index and micronuclei occurrence.
Based on the preliminary study, we decided to treat cells with MMS in order to enhance the phenotypes. Cells were transfected with pRK5 to overexpress RNF4 prior to MMS exposure in order to give enough time for sufficient RNF4 RNA translation. Our experiment shows that overexpressing RNF4 increases the percentage of mitotic MCF-7 cells but decreases the percentage of mitotic MDA-MB-468 cells. The percentage of cells in metaphase-anaphase transition slightly decreased for both cell lines (Fig. 8). An increase in micronuclei in response to RNF4 overexpression is the most prominent among all phenotypes. When RNF4 was overexpressed, there was a ~4-fold (7.16% to 23.44%, St. dev: 19.65%) and a ~2-fold (2.89% to 7.18%, St. dev: 6.83%) increase in the number of micronuclei in MCF-7 and MDA-MB-468, respectively (Fig. 8).

Discussion

Co-expression of RNF4 and Slx5 may interfere with normal growth.
Due to Slx5/Slx8's nature as a heterodimer, it was expected of *slx5*Δ strains to be unable to grow as well as WT strains. Previous studies show that when exposed to genotoxic stress, *slx5*Δ strains fail to grow as well as WT (Mullen et al., 2010). In *slx5*Δ strains, the *SLX5* gene is knocked out, leaving only the *SLX8* gene in the endogenous locus. Alone, Slx8 cannot accomplish the functions of the Slx5/Slx8 heterodimer, thus negatively affecting the growth of *slx5*Δ compared to WT (Fig. 5). Our experiments show that incorporating RNF4 may be able to rescue s*lx5*Δ strains. When RNF4 is incorporated into yeast strains, RNF4 replaces the *SLX8* gene in the endogenous locus. In WT cells, incorporation of RNF4 leaves the *SLX5* gene intact, raising the possibility of RNF4 heterodimerizing with Slx5 and failing to function normally. Expectedly, we saw that WT (WT RNF4) does not grow as well as WT when exposed to genotoxic agents (Fig. 5). However, *slx5*Δ (WT RNF4) displays equal levels of growth compared to that of WT (Fig. 5). We believe this is because *slx5*Δ strains eliminate the possibility of RNF4 heterodimerizing to Slx5. It is also possible that the point mutation observed in *slx5*Δ (WT RNF4) (Fig. 4) plays a role in rescuing the phenotype, and future experiments should address this observation. Overall, the incorporation of RNF4 seems to functionally rescues *slx5*Δ strains that are exposed to genotoxic stress.

Cancer-associated RNF4 mutation G138* may decrease genome stability.

The function of cancer-associated RNF4 mutation G138* is unknown. Our experiment does not demonstrate whether the mutation of interest is a gain or loss of function, however, we believe that the cancer-associated mutation G138* may contribute to genome instability, hindering the ability of cells to grow normally. Because our experiments failed to incorporate WT RNF4 into *slx5*Δ cells, we are unable to isolate the impact of the G138* mutation on growth, since it is possible that the point mutation observed in *slx5*Δ (WT RNF4) contributes to the phenotypes observed. However, comparing the growth between *slx5*Δ cells highlights an obvious decrease in growth for G138* incorporated cells (Fig. 5). The G138* mutation lies in the RING domain, which is responsible for ubiquitination of substrates (Deshaies and Joazeiro, 2009). The mutation replaces a glycine codon with a stop codon, preventing translation of sequences further down. This would leave the SIM and ARM domains intact, allowing recognition of sumoylated substrates (Miteva et al., 2010). As it was shown that Slx5/Slx8, a homolog of RNF4, can ubiquitinate mitotic checkpoint proteins to prevent mitotic arrest and allow mitosis to continue (Thu et al., 2016), RNF4 may perform the same function, especially when cells are exposed to DNA damaging agents such as MMS and HU. However, the G138* mutation would prevent cells from ubiquitinating substrates and escaping mitotic arrest when exposed to MMS/HU due to a loss of function in the RING domain. This phenomenon would explain the difference in growth between *slx5*Δ (WT RNF4) and *slx5*Δ (G138*) (Fig. 5). Unfortunately, due to the nature of this experiment being performed on *S. cerevisiae*, we can only observe the effects of G138* on cell growth. In breast cancer cells, in which G138* was observed, the mutation may be a precursor of tumorigenesis, a consequence of genome instability. However, to confirm this proposition, future experiments should attempt to incorporate mutant G138* RNF4 into breast cancer cell lines and observe any changes in growth rate that would suggest whether the mutation promotes tumorigenesis. The aggressiveness of the transformed cancer cells could also be measured by a soft-agar assay, or by observing whether G138* mutation increases the tumorigenic potential of breast cancer cells in mice.

Overexpression of RNF4 may increase genome instability in oncogene activated cell lines.

In both MCF-7 and MDA-MB-468, and in comparison to cells that were not transfected with RNF4, we observed a significant increase in the occurrence of micronuclei when RNF4 was overexpressed (Fig. 8). Micronuclei are an indication of genome instability (Mackenzie et al., 2017) and they result from fragmented chromosomes that become encapsulated in a small nucleus. We have shown that exposure to MMS, a genotoxic agent, increases the occurrence of micronuclei (Fig. 6). However, we have also shown that the overexpression of RNF4 in the absence of MMS exposure also produces the same observation (Fig. 7). The magnitude of increase in micronuclei frequency is greater when induced by the overexpression of RNF4 than the increase induced by MMS exposure (Fig. 6, 7). Thus, we believe that the overexpression of RNF4 is inducing genome instability that is independent of genome instability induced by MMS exposure. With activated oncogenes inducing replication stress, the DNA damage response pathway is activated (Hills, 2014) to prevent cell proliferation that could lead to abnormal replication of the genome. However, given RNF4's function as a ubiquitin ligase, there may be a selective pressure for RNF4 to ubiquitinate substrates in the DDR pathway and mitotic checkpoints to favor tumor progression, allowing DNA and cell replication to occur even under exposure to genotoxic agents and thus explaining the increase in micronuclei.

Overexpression of RNF4 may alleviate oncogene-induced replication in MDA-MB-468.

Given RNF4's known function in DNA repair as a SUMO-targeted E3 ubiquitin ligase (Yin et al., 2012 & Galanty et al., 2012), we predicted that the overexpression of RNF4 would decrease the percentage of cells in mitosis and those displaying metaphase-anaphase transition. Our results show that overexpressing RNF4 slightly decreases the percentage of cells in metaphase-anaphase transition, but increases the percentage of mitotic MCF-7 cells while decreasing the percentage of mitotic MDA-MB-468 cells (Fig. 8). The data supports our hypothesis for MDA-MB-468, but not for MCF-7. These two cell lines differ in oncogene expression and p53 deficiency. MDA-MB-468 has a greater oncogene (Ras) expression, characterizing the MDA-MB-468 as more malignant and aggressive in nature (Eckert et al., 2004). MDA-MB-468 also carries mutant p53. p53 is a tumor suppressor protein involved in protection against DNA damage (Cadwell, 2001) and a previous study showed that p53 deficient cell lines that experience replication stress enters mitotic arrest (Nitta et al., 2004). With the overexpression of RNF4, MDA-MB-468 cells could utilize the E3 ligase function of RNF4 and prioritize the RNF4 pathway to prevent mitotic arrest, similar to the function of its homolog Slx5/Slx8 ubiquintinating mitotic checkpoint proteins in yeast cells (Thu et al., 2016). This would allow cells to bypass mitotic arrest, explaining the decrease in the occurrence of mitotic cells and metaphase-anaphase transition. MCF-7, however, carries WT p53. p53-dependent mitotic checkpoints have been identified in the past (Lanni and Jacks, 1998), but the specific reason behind the increase in mitotic MCF-7 cells is unclear. It is possible that RNF4 interacts with p53 to induce mitotic arrest, but the mechanisms are unknown in this experiment. Given these interpretations, the overexpression of RNF4 seems to alleviate oncogene-induced replication stress in MDA-MB-468, but fail to do so in MCF-7. To confirm the role of p53 in RNF4-induced mitotic arrest, future experiments could attempt to silence WT p53 in MCF-7 and express mutant p53 in the hopes of observing a decrease in mitotic cells that agree to the pattern shown in MDA-MB-468.

Materials and Methods

Miniprep of pRK5 plasmid:

Procedure was adapted from Wizard®Plus SV Minipreps DNA Purification System. Plasmid purchased as transformed bacteria. Incubated in 37°C shaker for 18 hours in 5mL L-Broth including 100µg/mL ampicillin. Overnight culture was pelleted by centrifugation. Cells were resuspended, lysed, and treated with Alkaline Protease before neutralized. Plasmid DNA was isolated by binding to spin column, washed, and eluted with nuclease-free water.

Synthesis of Plasmid Containing Cancer-associated Mutation G138*

Customized primers containing the G138* mutations were used to replicate the pRK5 plasmid by QuikChange Lightning Site-Directed Mutagenesis Method. Amplified products were treated with *Dpn* I restriction enzyme and transferred into *E. coli* cells by heat shock. Cells were incubated in 37°C SOC media then pelleted by centrifugation. Pellet was resuspended, plated on L-broth containing Amp (100ug/ml), and incubated in 37°C.

Transformation of Yeast Cells

Yeast cells were grown overnight to reach an OD_{600} between 0.4 and 0.8 to confirm that cells are in log phase of growth. Cells were centrifuged and resuspended in 1XTE (10mM Tris-Cl, 1mM EDTA (pH 8.0))/1XLiAc buffer. Previously heated single-stranded salmon sperm DNA (10mg/mL) was added to the mixture with 1µg of DNA and 50% PEG/1XLiAc/1XTE solution. The mixture was incubated with shaking at 30°C and heat shocked in a 42°C water bath. Cells were pelleted by centrifugation and resuspended in sterile ddH_2O before plating. Plates were left in a 25°C incubator.

Genomic Prep of Transformed Yeast Cells
Screened colonies were grown in YPD for 18 hours then pelleted by centrifugation. Pellets were washed with H_2O and resuspended in Crude DNA isolation buffer (2% Triton X-100, 1% SDS, 100mM NaCl, 10mM Tris-Cl, pH 8.0, 1mM Na_2EDTA). Acid-washed glass beads were added and the mixture was vortexed. 1XTE and phenol:chloroform:isoamyl alcohol were added to the mixture and centrifuged. The aqueous top layer was transferred to 100% EtOH, centrifuged, and washed to isolate the DNA. DNA pellet was resuspended in 1X TE and treated with 10mg/mL RNaseA. Samples were stored at -20°C.

Gel Electrophoresis to Confirm Successful Transformations
Isolated yeast DNA were screened by gel electrophoresis in a 1% Agar gel composed of 300mL 1XTBE, 3g Agarose, and 30µL EtBr.

Conducting Spotting Assay with Genotoxic Agents
Yeast strains were inoculated in YPD at 25°C for 18 hours. Cells were pelleted by centrifugation and OD_{600} was measured to calculate the volume needed to plate $2X10^7$ cells. Cells were resuspended in sterile water and transferred to the first column of 96-well plate. Five 10-fold serial dilutions were made to have a total of 6 columns. Cells were plated on YPD plates containing 50mM, 100mM, 150mM HU, and 0.01%, 0.03%, 0.04% MMS. Cells grew for 3 days at 25°C.

Transfection and Immunofluorescence Staining of Cancer Cells
All cells were cultured in 37°C/5% CO_2 incubator. Cells were grown on cover slips in DMEM +10% fetal bovine serum + antibiotic/antimycotic drugs (complete media). Transfection procedures were taken from *Lipofectamine™ 3000 Reagent Protocol*. Volumes of reagents used were indicated in the 6-well column. Transfected cells were incubated for 24 hours and then treated with 10µM MMS and incubated for 18 hours. Post-treatment, cells were fixed in 3.7% formaldehyde solution and treated with blocking solution (1X PBS, 5% normal serum, 0.3% Triton X-100). A primary tubulin antibody (2µg/mL) was applied followed by a Rabbit anti-Mouse IgG (H+L) Cross-Adsorbed secondary antibody, Alexa Fluor 555 (2µg/mL). Coverslips were mounted by SlowFade Diamond Antifade DAPI on to a glass slide and sealed by nail polish the next day. Glass slides were stored in the dark at 4°C.

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