The Role of RNF4 in Genome Stability and Cancer

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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). Previously during the summer of 2017, we observed that the overexpression of RNF4 via transfection increased the frequency of micronuclei occurrence, an indication of genome instability (Stopper et al., 2003). To ascertain that it is the overexpression of RNF4 increasing micronuclei frequency, we transfected MCF-7 cells with empty vector EGFP and RNF4, and compared the relative increases in micronuclei frequency. We hypothesized that RNF4 transfections will show a greater micronuclei frequency due to RNF4's predicted ability to target mitotic checkpoint proteins for degradation and allow mitosis to continue in unfavorable conditions, such as genome instability. This predicted function was observed in Slx5/Slx8, a homolog of RNF4 found in *S. cerevisiae* (Thu et al., 2016). Our data suggests that the overexpression of RNF4 does not induce genome instability in MCF-7 cells.

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). Previous studies have shown that oncogenes promote replication stress in tumorigenic cells, inducing a DNA damage response leading to senescence or apoptosis (Hills and Diffley, 2014). These defense mechanisms serve as an inhibitor of tumor progression, encouraging mutations to form in the DNA damage response pathway in tumor cells.

A mammalian protein 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 proteins conjugated by SUMO (Small Ubiquitin-like Modifier), 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, deletion of the RNF4 gene in human cells results in an increased sensitivity to DNA damaging agents (Yin et al., 2012), further highlighting RNF4's role in maintaining genome stability.

The Slx5/Slx8 heterodimer, a homolog of RNF4 in yeast cells, 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 escape 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.

Although RNF4 and its yeast homologue Slx5/Slx8 has been shown to play a 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.

In the summer of 2017, we overexpressed RNF4 in two breast cancer cell lines: MCF-7 and MDA-MB-468. The frequency of occurrence of cells displaying signs of the mitotic index and metaphase-anaphase transition were quantified, and we observed that the frequency of metaphase-anaphase transition decreases for both cells lines, but the frequency of mitotic cells decreases for MDA-MB-468 and increases for MCF-7 cells. Our results suggest that the overexpression of RNF4 may alleviate replication stress in MDA-MB-468 but not in MCF-7. We believe this may be because MDA-MB-468 carries mutant p53 but MCF-7 carries WT p53. A previous study showed that in p53 deficient cell lines, replication stress induces mitotic arrest. However, replication stress induces G2 arrest in cell lines with WT p53 (Nitta et al., 2004). In MDA-MB-468, cells are more likely to enter mitotic arrest by due to a dysfunctional p53 in these cells. Therefore, the overexpression of RNF4 may allow these cells to target mitotic checkpoint proteins for degradation and allow mitosis to proceed and decreasing the number of cells in mitotic arrest. This claim aligns with RNF4's homolog Slx5/Slx8's role in alleviating replication stress in yeast cells (Thu et al., 2016). The overexpression of RNF4 also caused a significant increase in the frequency of micronuclei occurrence in both cell lines. This suggests that the overexpression of RNF4 may induce genome instability in cancer cells, as micronuclei are indicators of genome instability (Stopper et al., 2003). However, it is still uncertain whether it is RNF4 expression or the act of transfection that increases micronuclei occurrence. In this paper, we transfect MCF-7 cells with empty vector EGFP and RNF4 and quantify micronuclei occurrence in order to isolate RNF4's impact on producing micronuclei. Any significant results may suggest a role for RNF4 in encouraging tumorigenesis by introducing genome instability. We hypothesize that cells transfected with RNF4 will show a greater frequency of micronuclei occurrence, as RNF4 may be able to target mitotic checkpoint proteins for degradation and increase the formation of micronuclei by allowing mitosis to continue even under conditions of genome instability. Our results show that the overexpression of RNF4 does not increase the frequency of micronuclei, regardless of the quantity of DNA transfected, and therefore does not induce genome instability.

Results

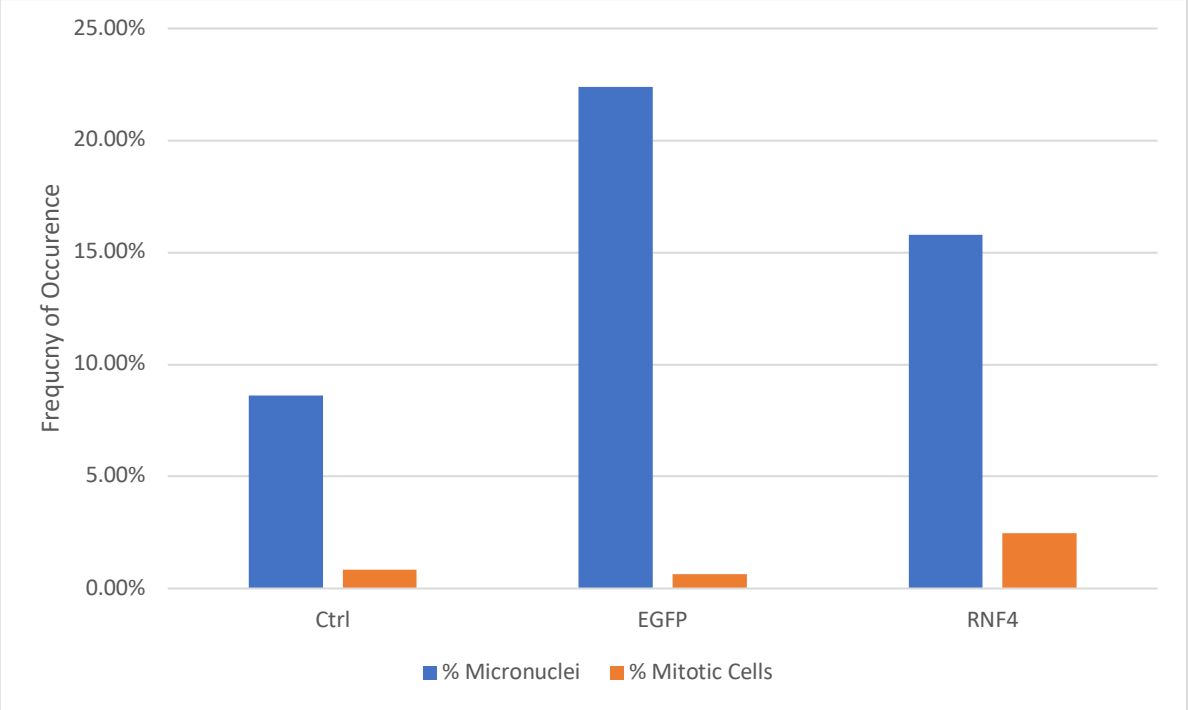

Figure 1. Frequency of occurrence of micronuclei and mitosis in MCF-7 cells. Cells were transfected with EGFP or RNF4. Treatment (% micronuclei, % mitotic): Ctrl(8.62%, 0.83%), EGFP(22.39%, 0.65%), RNF4(15.8%, 2.48%). n = 1.

A preliminary transfection control indicated a greater micronuclei frequency for MCF-7 cells transfected with EGFP (22.39%) than cells transfected with RNF4 (15.8%) (Fig. 1). This observation suggested that the overexpression of RNF4 alleviates the genome instability introduced by the act of transfection itself.

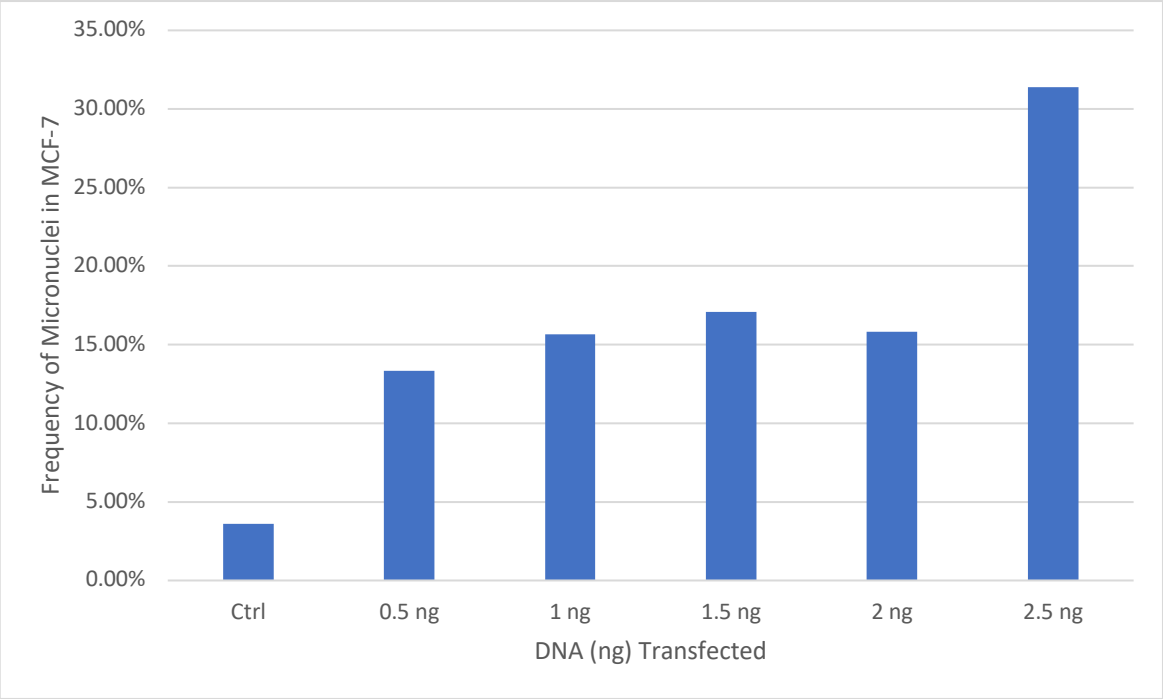

Figure 2. Frequency of micronuclei occurrence in MCF-7 cells transfected with varying quantities of EGFP. Transfected cells were treated with primary tubulin and secondary Rabbit anti-mouse antibodies. Type (% Micronuclei): Ctrl(3.59%), 0.5ng(13.33%), 1.0ng(15.67%), 1.5ng(17.1%), 2.0ng(15.8%), 2.5ng(31.37%). n = 1.

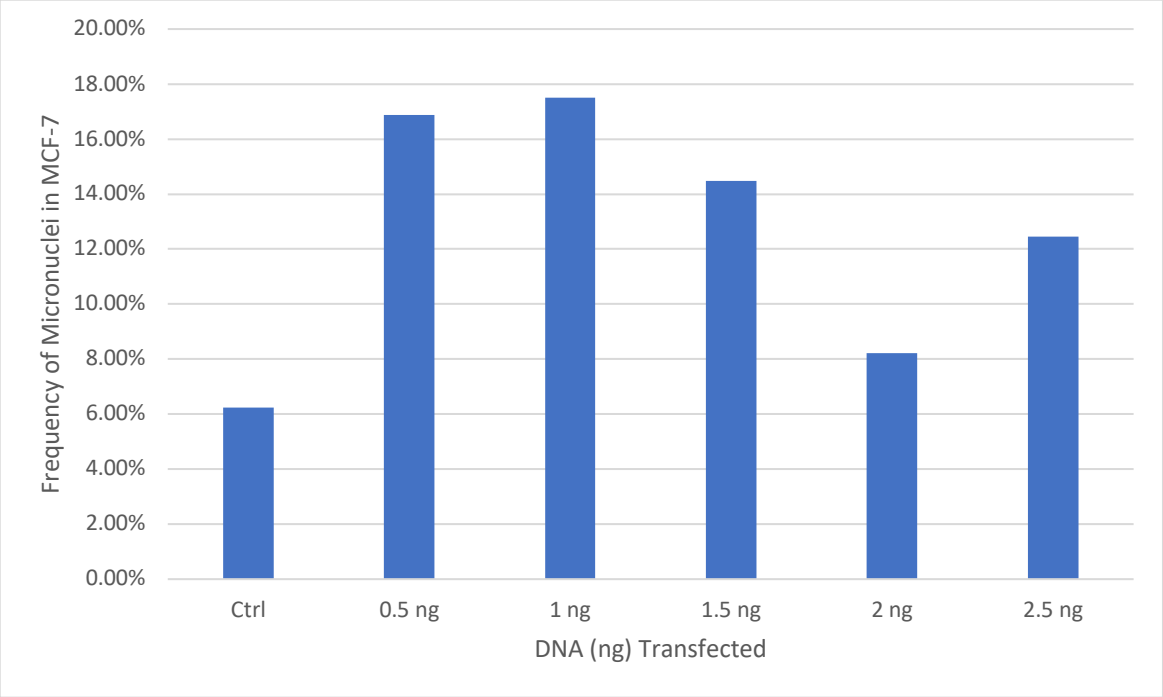

Figure 3. Frequency of micronuclei occurrence in MCF-7 cells transfected with varying quantities of RNF4. Transfected cells were treated with primary RNF4 and secondary Rabbit anti-mouse antibodies. Type (% Micronuclei): Ctrl(6.25%), 0.5ng(16.9%), 1.0ng(17.5%), 1.5ng(14.5%), 2.0ng(8.22%), 2.5ng(12.5%). n = 1.

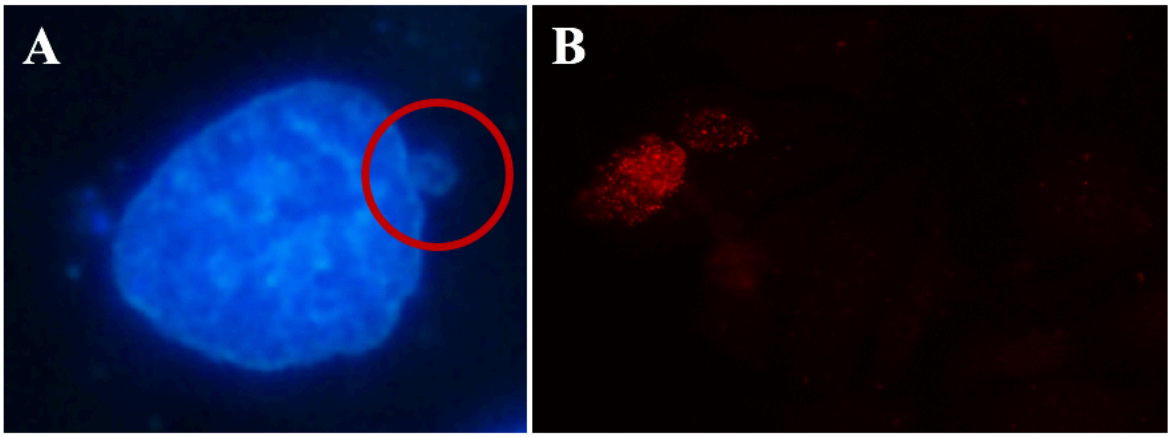

Figure 4. Immunofluorescence of MCF-7 cells transfected with RNF4. Cells were treated with primary RNF4 and secondary Rabbit anti-mouse antibodies. A) Micronuclei indicated by DAPI staining. B) RNF4 expression indicated by secondary antibody.

To verify the observations seen by the preliminary transfection control (Fig. 1), a dosage varying transfection control was conducted. Quantities of DNA transfected varied from 0.5ng to 2.5ng in 0.5ng intervals. The expression of RNF4 in transfected cells were confirmed by immunofluorescence (Fig 4).

MCF-7 cells transfected with EGFP show a direct relationship between the amount of transfected DNA and frequency of micronuclei occurrence. There is a steady increase from 0.5 ng DNA (13.33%) to 2.5ng (31.37%) (Fig. 2). RNF4 transfections, on the other hand, do not display any correlation between amount of transfected DNA and frequency of

micronuclei occurrence, with the highest frequency in 1.0ng (17.5%) that decreases in 2.5ng (12.5%) (Fig. 3). The 12.5% micronuclei frequency under 2.5ng agrees with the preliminary transfection control, which displayed a 15.8% frequency under the RNF4 transfection, when 2.5ug of DNA of used. It should also be noted that the micronuclei frequency for the two controls differ with 3.59% for EGFP and 6.25% for RNF4 transfections (Fig. 1, 2).

Discussion

Overexpression of RNF4 does not induce genome instability.

Although all RNF4 transfections display an increase in micronuclei frequency (Fig. 3), it cannot be confirmed that the overexpression of RNF4 induces genome instability, specifically due to EGFP transfections also displaying an increase in micronuclei frequency (Fig. 2). However, under smaller quantities of DNA, RNF4 transfections indicate a greater micronuclei frequency, suggesting that 0.5-1.0ng RNF4 transfections may induce genome instability independent of the act of transfection itself. However, because this experiment has only been conducted once, more trials are necessary to substantiate any findings. Additionally, compared to EGFP transfections, varying DNA quantities of RNF4 transfections does not indicate any relationship between DNA quantity and micronuclei frequency (Fig. 3). EGFP transfections, on the other hand, showed a steady increase in micronuclei frequency as the quantity of DNA increased (Fig. 2). These findings suggest that RNF4 may be functioning in a way to decrease genome instability. As a protein involved in DNA damage repair (Yin et al., 2012 & Galanty et al., 2012), it is possible that the overexpression of RNF4 allowed MCF-7 cells to maintain genome stability in oncogene activated cells. The mechanism, however, is unclear at this point, and future experiments should attempt to conduct the same experiment in a different oncogene-activated cell line that is similar to MCF-7 in order to confirm previous observations.

Materials and Methods

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 48 hours. Post-transfection, 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) for EGFP transfections. RNF4 transfected cells used a primary RNF4 Polyclonal antibody (PA5-35170) instead. 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 until they were ready to be examined by fluorescent microscopy.

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