Student Name: Jeonga Gim **Date:** August 8, 2014

Project Title: The oncofetal protein HMGA2 influences telomere stability in cancer cells.

Primary Supervisor Name: Dr. Sabine Hombach-Klonisch

Department: Department of Human Anatomy and Cell Science

SUMMARY: (no more than 250 words single spaced)

HMGA2 is a non-histone chromatin binding protein which binds to the adenine-thymine (AT) rich regions of DNA. HMGA2 is expressed in fetal tissues and its expression is downregulated through the microRNA let-7 in normal adult somatic tissues. Cancer cells frequently re-express the oncofetal protein HMGA2. HMGA2 has various functions related to neoplasm, including cancer transformation, epithelial-to-mesenchymal transition, aiding metastasis, and chemoresistance; all of which leads to poor prognosis for cancer patients. HMGA2 interacts with ataxia telangiectasia mutated (ATM) and ataxia telangiectasia and Rad3-related kinase (ATR) in the DNA damage signaling pathways and was shown to promote DNA damage repair. Thus, HMGA2 has a cytoprotective role against DNA damage and enhances cell survival. HMGA2 was shown to be present at telomeres, but its functional role at telomeres is not fully understood. Telomeres are nucleotide repeats located at the end of a chromosome. They are protected from being recognized as DNA damage sites by the shelterin complex consisting of six proteins. Preliminary data in the Hombach/Klonisch lab indicate that HMGA2 interacts with one of shelterin complex proteins.We hypothesized that HMGA2 has a protective function on telomeres and reduces telomere instability upon DNA damage. Telomere dysfunction was assessed by detection of anaphase chromatin bridges and micronuclei. We compared cancer cells with and without expression of HMGA2. Indeed, we have confirmed that HMGA2 helps to [redacted]erimental conditions did not show alterations in telomere [redacted]ge.

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Digitally signed by Sabine Hombach-Klonisch
DN: cn=Sabine Hombach-Klonisch, o=University of Manitoba, ou=Human Anatomy and Cell Science, email=hombach@cc.umanitoba.ca, c=CA
Date: 2014.08.07 18:53:36 -05'00'

Student Signature **Supervisor Signature**

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Introduction

HMGA2 is a non-histone chromatin binding protein which binds to adenine-thymine (AT) rich regions on the minor groove of nuclear DNA. It is expressed in embryonic stem cells and fetal tissues[1], but is absent or expressed in negligible amounts in normal adult tissues[2].

Many functions of HMGA2 are noted. Notably, when it comes to neoplasm, it participates in initiation of cancer transformation, epithelial-to-mesenchymal transition (EMT), metastasis support, and chemoresistance to therapies that are known to induce DNA modifications[3], all of the aforesaid correlates HMGA2 levels and poor prognosis for cancer patients. HMGA2 is also shown to have an intrinsic 5′-deoxyribose-5-phosphate (dRP) and apurinic/apyrimidic (AP) site lyase activity. It indicates that HMGA2 promotes the base excision repair machinery, which further bestows protective function upon HMGA2 against DNA damage, including chemotherapy[4]. HMGA2 also has a role in gene transcription[5]. Pygmy phenotype is observed in HMGA2 knockout mice models, indicating that HMGA2 has a function in cell proliferation, growth, and development[6].

HMGA2 is regulated through the microRNA let-7 as it binds to the 3'-untranslated region of the HMGA2 mRNA, and suppresses its translation. However, let-7 repression is frequently lost in cancer cells, and it may be a mechanism as to why HMGA2 is highly expressed in cancer cells[7].

Upon DNA damage, cells trigger signaling cascades in order to repair the damage by cell cycle arrest, or enter apoptosis. Ataxia telangiectasia mutated (ATM) and ataxia telangiectasia and Rad3-related kinase (ATR) are members of phosphatidylinositol 3-kinase-related protein family that function in the DNA damage response (DDR) pathways. ATM responds to double stranded breaks, and phosphorylates many downstream proteins of signal transduction cascades, including γH2AX and CHK2, to achieve cell cycle arrest to repair the damage. ATR responds to single stranded DNA damages, and phosphorylates CHK1 upon such damage, which results in cell cycle arrest. Both ATM and ATR are essential for cell survival[8].

HMGA2 was shown to induce ATM transcription (phosphorylated HMGA2 upregulates ATM expression), and binds to ATM and enhances the ATM-CHK2 DNA damage repair pathway[9] . Previous work in the Hombach/Klonisch lab has shown that HMGA2 also interacts with ATR and activated the ATR-CHK1 pathway. This resulted in prolonged cell cycle arrest and facilitated DNA repair. Thus, HMGA2 is shown to have a cytoprotective role via its sustained phosphorylation of ATR and its downstream target, CHK1, inhibiting apoptosis and helping in survival of stem cells and cancer cells[10].

Telomeres are regions of double stranded tandem repeats of nucleotide sequences, TTAGGG, with a single stranded 3' overhang, which are located at the ends of chromosomes. A complex of six proteins called shelterin protects telomeres from the cellular DNA repair machinery recognizing it as a site of DNA damage. Three of which directly recognize TTAGGG nucleotide sequences: telomeric repeat binding factor (TRF) 1, TRF2, and protection of telomeres (POT) 1.

The other three interlock the complex: TRF-interacting binding protein (TIN) 2, TPP1, and repressor-activator protein (RAP) 1[11].

Shelterin complexes affect telemetric structure in many ways: it is involved in generation of t-loops, has DNA remodeling activity, and maintains length of telomeres. Its inactivation can lead to senescence, telomere uncapping, chromosome fusions, and aberrant chromosomes[11]. Shelterin represses ATM and ATR, and protects from non-homologous ends joining (NHEJ) and homology directed repair (HDR), in which, if activated, would cause telomere dysfunction and result in chromosomal instability[12].

Telomere dysfunction leads to chromosome fusions with resulting problems for chromosome segregation during mitosis. Fused chromosome ends will not separate in anaphase and form chromatin bridges, referred to as anaphase bridges. Telomere dysfunction may also result I the formation of micronuclei, small chromosome fragments which are excluded from the daughter cell nucleus[13].

Unpublished novel data from the Hombach-Klonisch lab has shown that HMGA2 binds to the key shelterin protein TRF2. These findings suggest that HMGA2 has a function related to the shelterin complex, therefore affecting telomere functions.

Materials and Methods

Cell Lines and Culture Conditions

We used the undifferentiated thyroid cancer cell line UTC8505 and the lung cancer cell line A549 with and without expression of HMGA2 generated by stable transfections. UTC8505 mock clone 2 (empty vector transfectant), UTC8505 HMGA2 clone 4, and A549 HMGA2 clone 1.6 were cultured in DME-F12 medium (Thermo Scientific, Ottawa, Ontario) plus 10% fetal calf serum (FCS; Sigma-Aldrich, Oakville, Ontario) and 500μg/ml geneticin (G418, Life Technologies, Burlington, Ontario). A549 parental cell line was cultured without geneticin. All cell lines were kept in a humidified incubator under 5% CO2 at 37°C.

Induction of DNA Damage and Recovery

10^5 cells/well were cultured in six-well plates overnight. For DNA damage induction, cells were treated with methyl-methanesulfonate (MMS) (Sigma-Aldrich), and benzo[a]pyrene (B[a]P) (Sigma-Aldrich) for 30 minutes and 24 hours, washed thoroughly with 1x phosphate buffered saline (PBS) twice, lysed, and total protein collection was done. For recovery models, after 30-minute treatment, cells were given fresh medium and were incubated in a humidified incubator at 37°C for 24 hours to allow for recovery from DNA damage.

Immunoblot Analysis

Proteins were collected with 1x Laemmli protein lysis buffer following appropriate treatments and recovery time. Lysates equivalent to 30μg of total protein were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and blotted onto nitrocellulose membrane (Thermo Scientific). Blocking was done with 5% skimmed milk in 1x Tris-buffered saline and 0.1% Tween 20 adjusted to pH 7.6. Primary antibody and membrane were incubated overnight at 4°C in 5% bovine serum albumin in 1x Tris-buffered saline and 0.1% Tween 20 adjusted to pH 7.6. Secondary antibody in blocking buffer was incubated with membrane for an hour at room temperature. Detection of bands was done with the ECL kit (Pierce, Nepean, Ontario). Primary antibodies were rabbit polyclonal antibodies including γH2AX and HMGA2 (both from Cell Signaling Technology, Pickering, Ontario). Beta actin antibody was mouse (Sigma-Aldrich). Secondary antibody was HRP-conjugated anti-rabbit IgG (Cell Signaling) and anti-mouse IgG (Sigma-Aldrich).

Cell Viability Assay

5000 cells/well in a 96-well plate were incubated overnight at 37°C in a humidified incubator. Cells were then treated with MMS (Sigma-Aldrich), and B[a]P (Sigma-Aldrich) for 30 minutes, or were allowed to recover in fresh medium for 24 hours after a 30-minute treatment with DNA damaging agents. Cell proliferation reagent WST-1 (Roche, Mannheim, Germany) was added in 1:10 final dilution, and after 4 hours, the plate was read with the program SoftMax Pro 6.4 (Molecular Devices).

Fluorescence microscopy and Image Analysis

10^5 cells were cultured on coverslips (Fisher Scientific, Ottawa, Ontario) in a six-well plate overnight at 37°C in a humidified incubator. Cells were treated with MMS for 30 minutes, washed with 1x PBS once, and fixed with 3.7% formaldehyde (Fisher Scientific) in 1x PBS for 30 minutes. Cells were then washed with 1x PBS three times for 5 minutes each, and DNA was stained with 0.1 μg/ml 4',6-diamidino-2-phenylindole (DAPI) (Sigma-Aldrich) for 5 minutes. Cells were washed in 1x PBS for 5 minutes and the coverslip was mounted on glass slides (VWR International, Mississauga, Ontario) using Fluoromount anti-fade mounting media. Image acquisition was done using an AxioImager Z2 fluorescence microscope (Zeiss) and a 40x objective. All slides were consistently exposed for 300ms for imaging. For each experimental group, a total of 300 nuclei were imaged per individual experiment and the number of anaphase bridges and micronuclei were counted. Three independent experiments were performed and analyzed.

Statistical Analysis

Comparisons between the non-HMGA2 and HMGA2-expressing cell lines were conducted using t-test for independent samples. Values were considered statistically significant if $p<0.001$, $p<0.01$ or $p<0.05$; on figures, they are noted with three asterisks if $p<0.001$, two asterisks if $p<0.01$ and one asterisk if $p<0.05$.

Results

UTC8505 and A549 Cell Models Show DNA Damage upon MMS Treatment and HMGA2 Expression in HMGA2 Clones is Confirmed

Both cell lines were treated with MMS, a known DNA damaging agent, 2mM for UTC8505, and 5mM for A549. These concentrations were established in previous experiments in the Hombach/Klonisch lab as optimized concentrations of MMS that cause DNA damage in the respective cell lines. In both cell models, increased expression of γH2AX following MMS treatment was detected by Western blot analysis in the samples treated with MMS at two different time points selected: 30 minutes after the exposure to the toxin and 30 minutes of toxin exposure with subsequent 24 hours of recovery. γH2AX detection was used to confirm DNA-damage induced by MMS. HMGA2 expression in UTC8505 and A549 HMGA2 clones was confirmed via Western blot analysis as well (Figure 1, 2).

HMGA2 Significantly Decreases the Number of Anaphase Bridges

Presence of anaphase bridges between cells was detected by fluorescence imaging technique on cells treated with MMS for 30 minutes, or on cells treated with MMS for 30 minutes followed by a 24-hour recovery time. Three independent experiments have been completed for the UTC8505 cell model. HMGA2 expression decreased the number of anaphase bridges in UTC8505 cells, with statistical significance for both time points selected. There is no difference in the number of anaphase bridges between the MMS-treated cells and non-treated cells for both time points selected (Figure 3). Data analysis of three independent experiments of the A549 cell line is currently under investigation when this report was generated. These data will be available for the time of the oral presentation.

Lower Concentrations of B[a]P Reduces Cell Viability and Causes DNA Damage in the UTC8505 Cell Model

Cell viability assay was performed to establish dose concentration and time curve of B[a]P treatment. Decreased cell viability was shown in the UTC8505 cell line at lower concentrations of B[a]P including 0.1μM, 1μM, and 10μM, after 30 minutes of treatment with B[a]P. However, higher concentrations of B[a]P did not reduce cell viability. No reduction in cell viability was detected after a 24-hour recovery time following the 30-minute exposure to the toxin, as there was a minimal change in cell viability compared to the untreated controls (Figure 4). Immunoblot analysis on UTC8505 cells after a 30-minute exposure to B[a]P further demonstrated that DNA damage is caused in the cell line as there is increased expression of γH2AX at lower concentrations of B[a]P particularly with 1μM and 10μM B[a]P (Figure 5). γH2AX induction was less pronounced in the presence of HMGA2. Following 24 hours of treatment with B[a]P, γH2AX immunoblot analysis revealed a mitigated DNA damage response in the UTC8505 mock and the HMGA2 expressing clones (Figure 6).

The A549 Cell Line Did not show Significant Changes in Cell Viability and DNA Damage Upon B[a]P Treatment

Cell viability assay was performed on A549 parental and the HMGA2 expressing clone with different concentrations of B[a]P and with different time points. However, compared to the cells treated with MMS, which significantly reduced cell viability at 5 mM, no significant change in cell viability was detected in the cells treated with B[a]P, both after a 30 minute treatment and that with a succeeding 24-hour recovery time (Figure 7). Furthermore, the γH2AX immunoblot analysis of A549 cell lines treated with different concentrations of B[a]P for 30 minutes, and also for 24 hours showed no significant increase in the γH2AX expression (Figure 8, 9).

Discussion

In this study, we have demonstrated that HMGA2 significantly reduces the number of anaphase bridges in the human undifferentiated thyroid cancer cell line UTC8505. In the presence of HMGA2, the number of anaphase bridges was significantly decreased compared the mock cells lacking HMGA2. Thus, the presence of HMGA2 reduces telomere instability in this cancer cell model. Similar results have recently been published by our group using a fibrosarcoma cell model with endogenous expression of HMGA2[14]. It suggests that HMGA2 helps to stabilize telomeric ends and thus improves chromosomal stability, and helps in survival of HMGA2 expressing cancer cells.

Under our chosen experimental conditions, based on the induction of DNA damage and reduction in cell viability, treatment with methyl-methanesulfonate (MMS) did not cause a difference in the number of anaphase bridges compared to the untreated cell lines which suggests that DNA damage by this agent does not further affect telomere stability in UTC cells. It seems that γH2AX induction as detected by Western blot does not correlate with changes in the number of bridges as expression levels of γH2AX were increased as per the Western blot analysis, but minimal change was found in the number of anaphase bridges. It gives the impression that despite the DNA damage induction by MMS further telomere instability is not induced under our experimental conditions. We have not investigated longer time exposures to much lower MMS concentrations to test if cumulative DNA damage (versus short-term damage) affects telomere stability. Since we did not detect additional telomere instability under MMS treatment, we cannot exclude that HMGA2 may have protected from this additional instability.

However, the limitation of these experiments are that only one sample DNA-damaging agent was tested and only the 30-minute treatment time frame was chosen for this experiment. Different DNA damaging agents other than MMS can be used, and the exposure time to the drug can be varied as well. Rationale behind the preliminary decision of treating cell lines with MMS for 30 minutes was per previously published data from the Hombach/Klonisch lab that MMS induces DNA strand breaks under the concentrations used in this study. As the alkylating agent methyl methanesulfonate (MMS) MMS is known to cause single-strand DNA lesions and evokes the base excision repair machinery in which HMGA2 plays a role in, we have concluded that it would be a good choice of DNA damaging agent to preliminarily start the experiment and compare the effect of HMGA2 presence in the number of anaphase bridges. Although the number of anaphase bridges did not differ when cells were treated with MMS, both UTC8505

and A549 cell lines showed increased levels of γH2AX after 30 minutes of MMS exposure, further confirming that the concentrations of MMS used in this experiment causes DNA damage in both of the cell lines and can be continued to be used as a positive control for DNA damage.

Although we have demonstrated that HMGA2 helps to stabilize telomeres, the mechanism as to how it decreases the number of anaphase bridges is unknown and will need to be investigated.

B[a]P was chosen as a second DNA damaging agent as it is a polycyclic aromatic hydrocarbon with known mutagenic and carcinogenic potentials[15]. It is found in the natural environment, cigarette smoke, and through incomplete combustion of organic matters[15]. B[a]P is shown to increase the double strand break repair pathways, and when these repair pathways go wrong, it may be a mechanism of its genotoxicity[16].

Cell viability assay on the UTC8505 cell line did not show a statistical significant difference between untreated UTC mock and UTC mock cells treated with lower concentrations of B[a]P including 0.1μM, 1μM, and 10μM for 30 minutes as the standard error of untreated UTC mock was high. However, there was a significant decrease in cell viability in UTC HMGA2 clones treated with same low concentrations of B[a]P. If the experiment is repeated with diligence to decrease any errors, it may be possible that UTC mock cells will exhibit a statistically significant decrease in cell viability when treated with lower concentrations of B[a]P. According to the cell viability assay, 24 hours of recovery following insult seem to give cells ample time to recover from the damage. Interestingly, higher B[a]P concentrations of 25 μM, 50 μM and 100 μM did not reduce cell viability. One possible mechanism is that higher B[a]P may act through the aryl hydrocarbon receptor (AhR), in which may require more time of toxin exposure to exert its effect. At lower concentrations, B[a]P may act independently of the AhR to induce cytotoxic effects to reduce cell viability as shown in this experiment.

For Western Blot Analysis on UTC8505 cell lines treated with MMS for 30 minutes and B[a]P for 30 minutes (Figures 1, 5), we have demonstrated that DNA damage is induced by the increase in the level of γH2AX expression, especially with B[a]P concentration of 1μM and 10μM. For Western blot analysis on UTC8505 cell lines treated with B[a]P for 24 hours (Figure 6), baseline γH2AX damage in UTC mock was unexpectedly high and yielded difficulty to comment whether MMS, and/or B[a]P induced significant change in γH2AX levels. Many factors may have been a reason to the high baseline expression of γH2AX in the 24-hour cultured mock cells in this experiment including cell culture conditions, acidic medium, higher passage number of the cells or high cell confluency in the flask before seeding of the cells for the experiment. Repeating this experiment under optimized culture conditions is strongly recommended to confirm significant action of such DNA damaging agents. As well, longer exposure time allowing for AhR-dependent mechanisms (such as CYP induction) to occur should be tested.

It seems as though HMGA2 is protective after a 30 min treatment with lower concentrations of B[a]P as indicated by less expression of γH2AX on Western blot analysis (Figure 5). It is a

preliminary experiment and Western blot should be repeated before making a conclusion. If we were to optimize the B[a]P concentration, values anywhere between 0.1uM and 10uM may be chosen as they have showed an increase in γH2AX levels, thus indicating high levels of DNA damage caused by such concentration of B[a]P. The mechanisms of DNA damage induction by B[a]P following 30 min exposure is not identified. Further experiments to optimize the B[a]P concentration and exposure times are required to determine whether HMGA2 has a protective or repair-promoting effect under these conditions.

If this hypothesis, which HMGA2 protects against DNA damage caused by toxins, holds true, it calls for more investigation with other DNA damaging agents, including those clinically used for chemotherapy. These further studies will show whether HMGA2 may be an ideal target for cancer therapy in order to reduce the survival of cancer cells in patients.

We found that A549 cells, both parental and HMGA2 clones, are non-responsive to the B[a]P treatment under our experimental conditions. Cell viability and expression of γH2AX were not affected by B[a]P exposure, regardless of different concentrations and times exposed. Further repeated experiments to confirm this finding is warranted. More importantly, A549 cells are known to have the metabolic capacity to metabolize B[a]P[17], and DNA damaging effects may only be evident after longer exposure times to B[a]P. Lower concentrations of B[a]P were shown to increase cell proliferation and to cell migration in A549 cells which suggests other mechanisms to be activated in this cell line as well[18].

Conclusion

From this study, we conclude that HMGA2 is a factor in telomere stability in the UTC cell line. Further work, however, is required to determine how HMGA2 influences telomere stability. As our experimental conditions were not optimized to cause recognizable additional telomeric instability by DNA damaging agents, we declare that additional optimization is required in regards to the choice of toxins and duration of exposure for future experiments.

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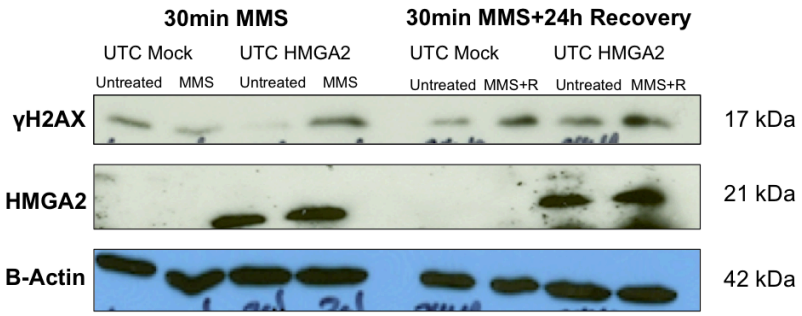

Figure 1. Representative immunoblot of UTC8505/mock and UTC805/HMGA2 clones treated with 2 mM MMS for 30 min only or for 30 min with subsequent 24-hour recovery time. 30µg of total protein was loaded for the detection of γH2AX and HMGA2. Beta-actin was detected as loading control.

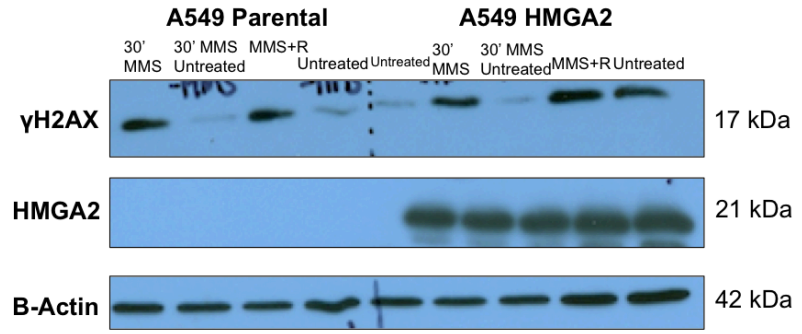

Figure 2. Representative Immunoblot of A549 parental and A549/HMGA2 clones treated with 5 mM MMS for 30 min only or for 30 min with subsequent 24-hour recovery time (MMS+R). 30 µg of total protein was loaded for the detection of γH2AX and HMGA2. Beta-actin was detected as loading control.

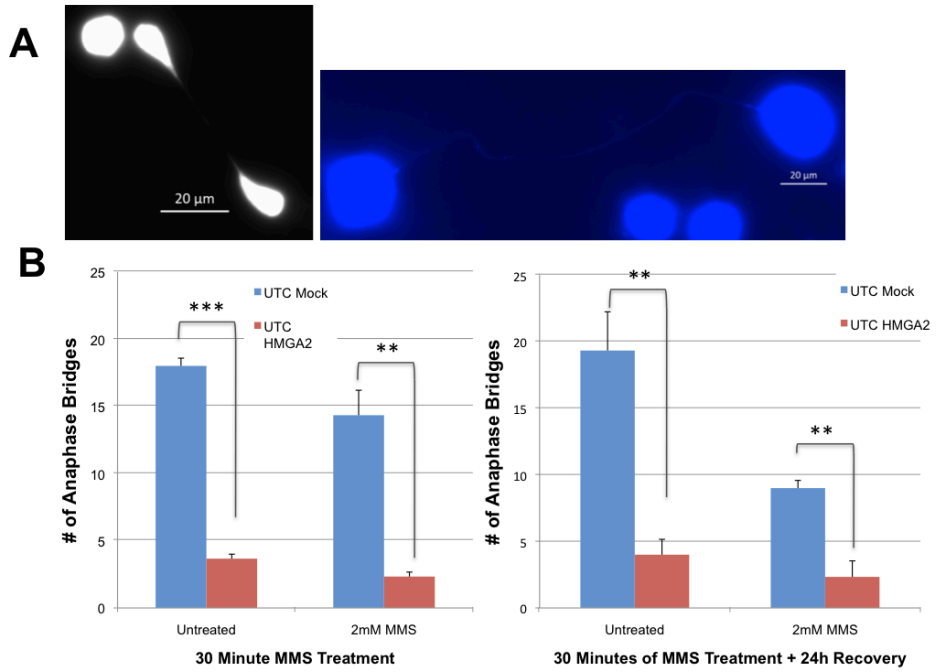

Figure 3. UTC8505 Mock and HMGA2 clones were stained with DAPI following a 30minute MMS treatment, or 30 minute MMS treatment with subsequent 24h recovery. (A) Two representative images of anaphase bridges of UTC8505 mock cells stained with

DAPI. (B) Quantification of anaphase bridges in UTC mock and HMGA2 clones with aforementioned treatment and recovery time. * p<0.05, ** p<0.01. Mean and standard deviation of three independent experiments are shown.

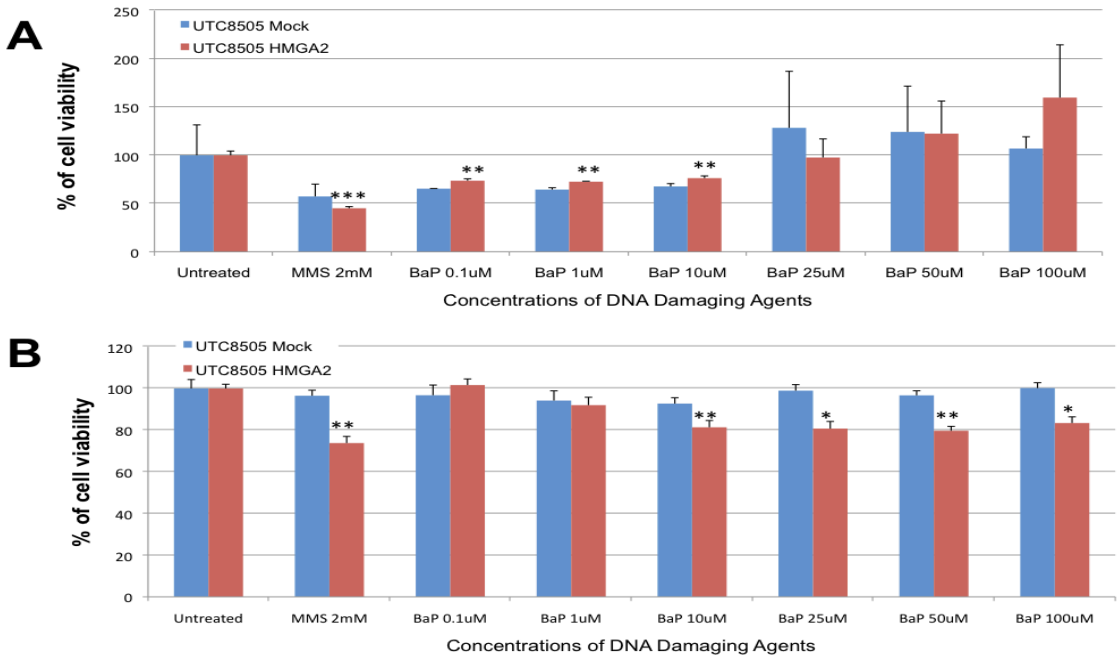

Figure 4. Cell viability assay on UTC8505 mock and HMGA2 clones after treatment with increasing concentrations of B(a)P for 30 minutes, or 30 minutes and subsequent 24h recovery. (A) Cell proliferation assay performed after 30 minutes of exposure. (B) Cell proliferation performed after 30 minutes of insult and 24h of recovery. *** p<0.001, ** p<0.01, * p<0.05.

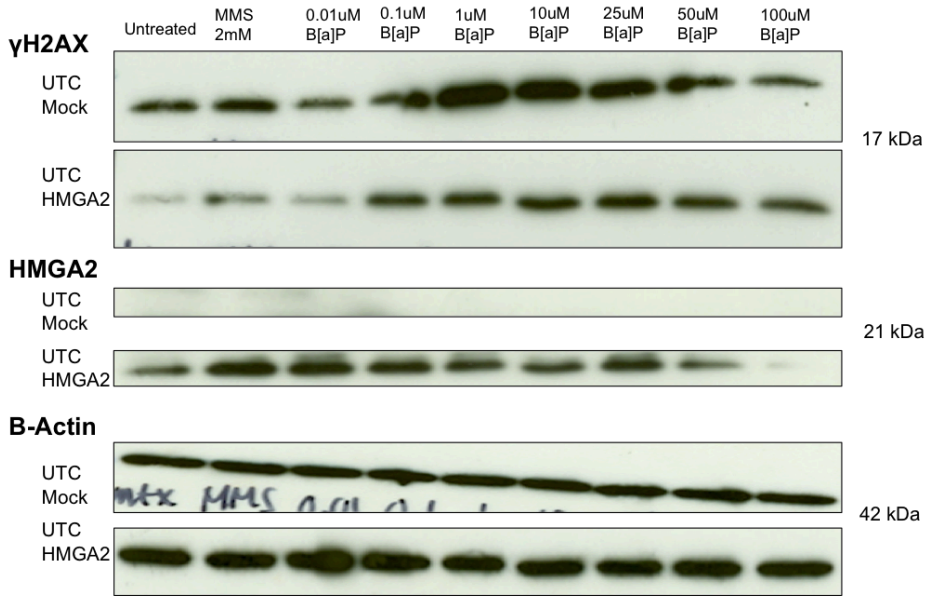

Figure 5. Immunoblot of UTC8505 mock and HMGA2 clones treated with DNA damaging agents 2mM MMS and different concentrations of B[a]P for 30 minutes is shown. γH2AX and HMGA2 proteins were detected, and B-actin was the loading control.

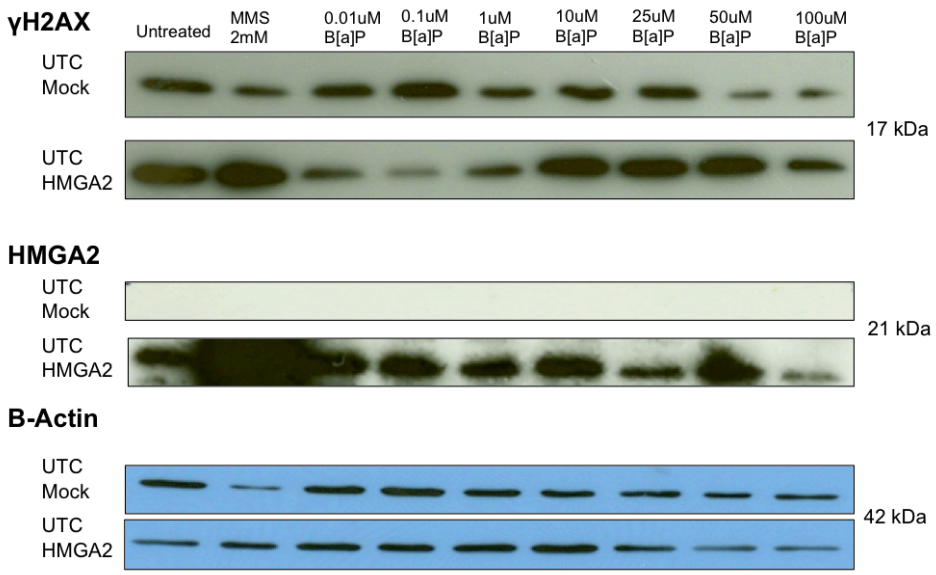

Figure 6. Immunoblot of UTC8505 mock and HMGA2 clones treated with DNA damaging agents 2mM MMS and different concentrations of B[a]P for 24 hours is shown. γH2AX and HMGA2 proteins were detected, and B-actin was the loading control.

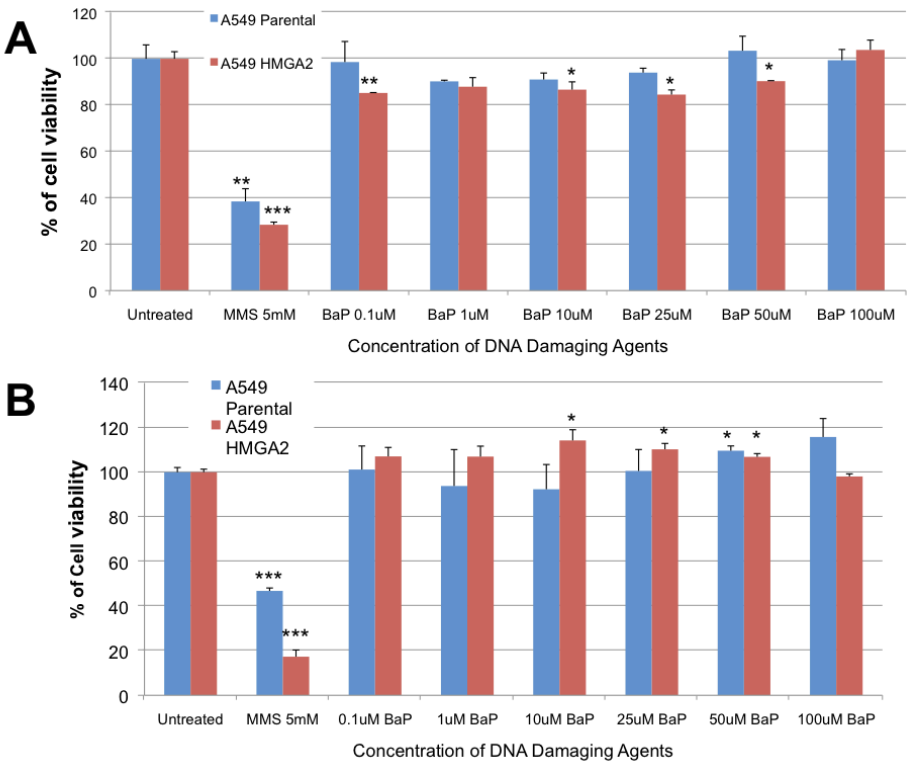

Figure 7. Cell viability assay on A549 parental and HMGA2 clones after treatment with different concentrations of B[a]P and 5 mM MMS for 30 minutes, or 30 minutes and subsequent 24h recovery. (A) Cell proliferation assay performed after 30 minutes of exposure. (B) Cell proliferation performed after 30 minutes of insult and 24h of subsequent recovery. *** p<0.001, ** p<0.01, * p<0.05.

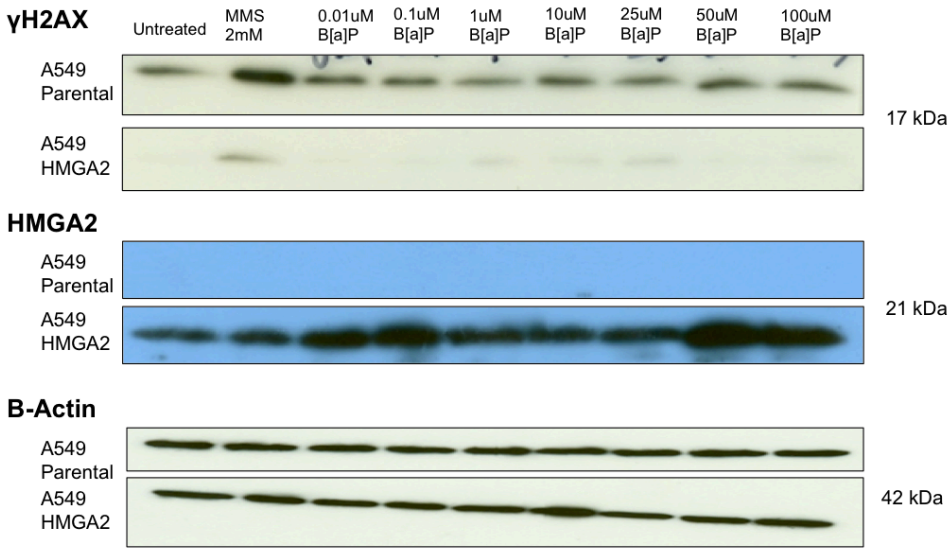

Figure 8. Immunoblot of A549 parental and HMGA2 clones treated with DNA damaging agents 5mM MMS and different concentrations of B[a]P for 30 minutes is shown. γH2AX and HMGA2 proteins were detected, and B-actin was the loading control.

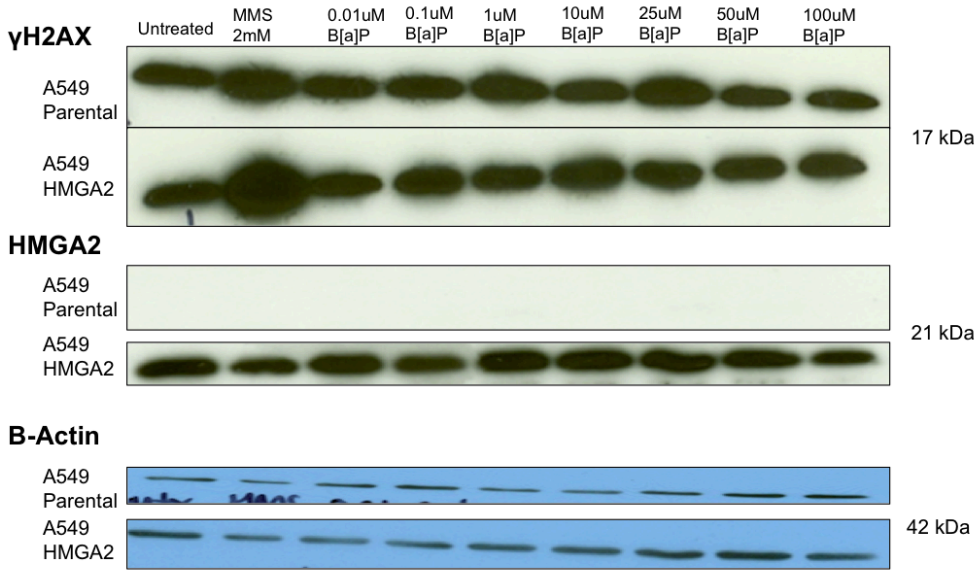

Figure 9. Immunoblot of A549 parental and HMGA2 clones treated with DNA damaging agents 5mM MMS and different concentrations of B[a]P for 24 hours is shown. γH2AX and HMGA2 proteins were detected, and B-actin was the loading control.