

ABSTRACT

Title of Document:

HOW SELENIUM MODIFIES CROSS-TALK
BETWEEN THE PIKK FAMILY AND
INSIGHTS ON THE REGULATION OF DNA-
PK_{cs}

Caroline Rocourt, Master of Science, 2009

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We recently found that ATM is required for a selenium-induced senescence response in non-cancerous cells. We hypothesize the selenium-induced DNA damage response modifies ATM and DNA-PK_{cs} cross-talk. Phospho-specific antibodies against ATM and DNA-PK_{cs} were used to follow the phosphorylation events after selenium treatment in normal human cells and two human cancer cell lines. Results from immunofluorescence analysis showed that selenium treatment induces hyperphosphorylation of DNA-PK_{cs} at T2647 and S2056 in non-cancerous MRC-5 cells but not in U-2 OS cancer cells. Further studies in MRC-5 cells treated with an ATM kinase inhibitor, KU 55933, showed attenuation of the selenium-induced DNA-PK_{cs} phosphorylation at both foci, whereas pre-treatment with a DNA-PK_{cs} kinase inhibitor, NU 7026, does not prevent ATM phosphorylation at S1981, an event leading to ATM pathway activation. These results give evidence that DNA-PK_{cs} and ATM have a cooperative role in the selenium-induced DNA damage response.

HOW SELENIUM MODIFIES CROSS-TALK BETWEEN THE PIKK FAMILY &
INSIGHTS ON THE REGULATION OF DNA-PK_{cs}

By

Caroline Rocourt

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Abbreviations used: non-homologous end-joining pathway (NHEJ), ataxia-telangiectasia mutated (ATM), DNA double strand break (DSB), mouse embryonic fibroblasts (MEFs), ataxia telangiectasia and Rad3 related (ATR), phosphoinositide-3-OH-kinase-related kinases (PIKKs), types of selenium; sodium selenite (SSe), methaneseleninic acid, 95% (MSA), Se-Methyl-selno-L-cysteine (SeC), aphidicolin (Aph) and reactive oxygen species (ROS)

Chapter 1: Literature Review

1.1 Cancer Prevention, Cancer Incidence and Nutrient Intervention

1.1.1 Cancer Prevention and Incidence

Cancer prevention is a relatively young field. Early studies of cancer prevention came from studies in pre-industrial Europe where chimney sweeps who worked naked, in order to avoid soiling their only set of clothes, were at greater risk of developing scrotal cancer than those who swept in clothes (LaMontagne AD, 2000). English physician Percivall Pott recommended chimney sweeps work with their clothes on to decrease their chances of developing cancer; Dutch sweeps who followed his recommendation did in fact have a lower incidence of cancer. These observational findings were later confirmed in animal studies, which showed absorption of polycyclic aromatic hydrocarbons from the soot were responsible for the cancer incidence (J Cook, 1933). Fast forward two centuries to the 1980's, and the molecular mechanisms of cancer promoting agents such as benzo(a)pyrene and aflatoxins were first being elucidated in colon cancer models and other epithelial neoplastic lesions (Vogelstein et al., 1988). In the decades since, in addition to increased elucidation of the molecular events leading to cancer, there has also been an increase in cancer prevalence, making it even more important to identify risk factors and discover possible novel cures.

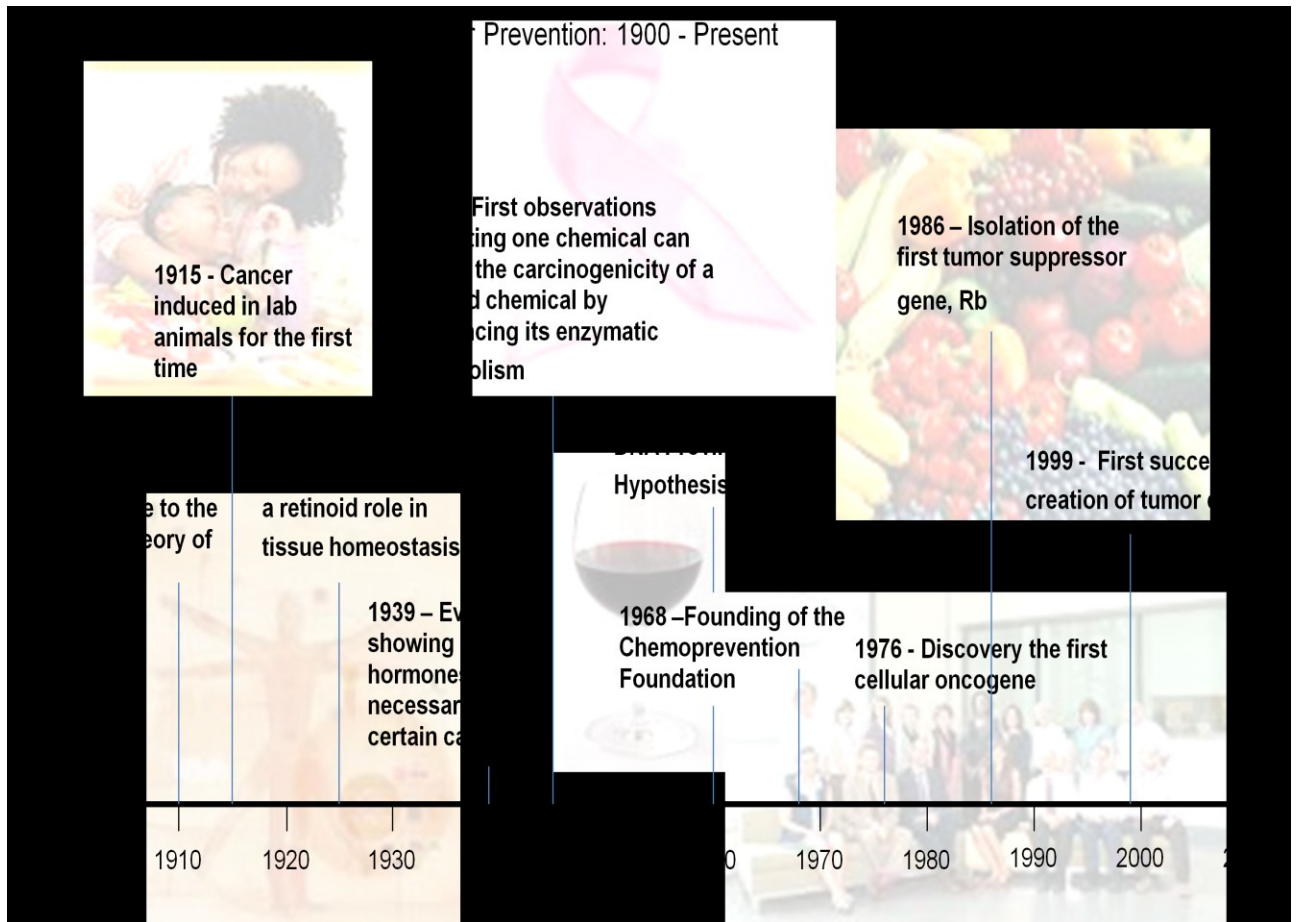


Figure 1.1: The History of Cancer Prevention

1.1.2 Nutrient Intervention

There have been incidences of nutrients and whole foods having a positive effect on the progression of tumorigenesis. Coumarins (Ito et al., 2005), garlic extracts (Nishino et al., 1989), green and black teas (Mehrabian, 2007), and resveratrol (Jang et al., 1997), have all been shown to have anti-carcinogenic effects. Most of the nutrient chemoprevention studies have been done in cell or animal models, and not in large-scale human trials. Although the data is promising, it wise to proceed with caution. The best possible outcome of these studies would be application to humans

based on their specific genotypes. The fields of nutrigenomics and personalized nutrition are gaining momentum quickly and are a promising approach for cancer prevention.

In 1983 The U.S. National Cancer Institute (NCI) founded the Division of Cancer Prevention and Control (later the Division of Cancer Prevention) and conducted the first large clinical chemoprevention workshop in 1984. The capstone of these trials is the SELECT Selenium and Vitamin E [prostate] Cancer Prevention Trial which had negative-neutral results (Lippman et al., 2009). The trial monitored over 35,000 men at 400 sites in the United States, Puerto Rico, and Canada taking oral selenium (200 µg/d from L-selenomethionine) and Vitamin E daily. It was found that selenium or vitamin E, alone or in combination, did not prevent prostate cancer in this population of relatively healthy men. However, other studies have shown that selenium has a role in preventing cancer. Many in the field of selenium chemoprevention point out weaknesses in the study such as; age of subjects, nutrient status, timing of data collection, and lack of immunohistochemistry or molecular techniques.

1.1.3 Selenium as an Antioxidant

Selenium is a micronutrient found in nuts, especially the Brazil nut, chicken, fish, turkey, crab, cereal and eggs. The recommended daily allowance for males and females is 55 and 70 µg/day, respectively, as established by Food and Nutrition Board (FNB) of the Institute of Medicine. This RDA is based on the amount of dietary selenium needed to maximize the activity of glutathione peroxidase, an antioxidant enzyme in plasma (Monsen, 2000). In 1997, Combs, Clark and colleagues showed a

role of selenium in cancer prevention (Combs et al., 1997). Specifically they showed dietary intake of 200 µg of selenium enriched yeast reduced total mortality, mortality from all cancers, and decreased the incidence of lung cancer, colorectal cancer and prostate cancer. This study tested the hypothesis that dietary selenium can prevent cancer primarily through its role as an antioxidant. Selenium is an essential trace mineral and is well known for its antioxidant activity, primarily through its incorporation into selenoproteins such as glutathione peroxidase and thioredoxin reductase, which are important oxidative enzymes that are able to scavenge free radicals.

By identifying a specific selenocysteine insertion RNA structures known as Sec insertion sequences (SECIS), Krukov and colleagues found there are 25 selenoproteins in the mammalian genome (Kryukov et al., 2003). Selenoproteins are also important in immune (McKenzie et al., 1998) and thyroid function, particularly its regulation of thyroid hormones (Arthur et al., 1992). One of the earliest signs of selenium deficiency is immune deficiency, and interestingly, selenium and/or vitamin E deficiency in the host can increase RNA virus' virulence, in particular coxsackievirus B3 (Levander, 1997).

1.1.4 Selenium as an Inducer of a DNA Damage Response

Selenium's role in chemoprevention is not yet fully elucidated; however, there have been many proposed mechanisms of how selenium can decrease the risk of developing cancer such as: initiation or attenuation of apoptosis (Cho et al., 1999; Santamaria et al., 2005; Jariwalla et al., 2009; Zeng et al., 2009), decrease of

angiogenesis (McAuslan and Reilly, 1986; Jiang et al., 1999; Lu, 2001; Mousa et al., 2007), induction of cell cycle arrest (Zu et al., 2006; Zeng et al., 2009; Zhao et al., 2009) increase in DNA damage repair and response (Kaeck et al., 1997; Sinha et al., 1999; Seo et al., 2002; Yin et al., 2004; Waters et al., 2005; Fischer et al., 2006; Traynor et al., 2006; Yu et al., 2006; Fischer et al., 2007), increased mitochondrial dysfunction and caspase activation (Guan et al., 2009), increased endoplasmic reticulum stress (Wu et al., 2005), increased activity of tumor suppressor proteins (Berggren et al., 2009), as an intracellular generator of ROS (Lanfear et al., 1994; Ip et al., 2000; Soto-Reyes et al., 2005; Last et al., 2006; Xu et al., 2007), initiation of a cytotoxic response (Reid et al., 2004; Olm et al., 2009), increase of cellular radio sensitivity (Shin et al., 2007) and induction of cellular senescence (Cheng lab, unpublished.)

To elaborate on the more well studied mechanisms of selenium chemoprevention, a collective body of research has demonstrated selenium as a powerful inducer of apoptosis, possibly due to its cytotoxicity, which can activate a signaling pathway in the cell that ultimately can lead to apoptosis in cancer cells (Cho et al., 1999). In a 2004 study it was shown selenite treatment on U-2 OS cells actually decreased the cancer cell's ability for DNA damage repair (Abul-Hassan et al., 2004). This inhibition of DNA damage in cancer cells could be beneficial because defective DNA damage repair might signal an apoptotic or senescence pathway in the cell; however, this study found selenite treatment increased the prevalence of dicentric chromosomes. Antioxidants other than selenium, such as silibinin, extracted from milk thistle, can activate the DNA-PK-p53 pathway and induce apoptosis which

could also be another potential mechanism for selenium to therapeutically target cancer cells (Dhanalakshmi et al., 2005).

Apart from selenium's potential to treat cancer, it has also been shown that selenium can prevent cancer. Epidemiological studies have shown humans taking 200 µg of selenium yeast have a decreased risk of cancer. The epidemiological studies do not fully explain the mechanism of selenium chemoprevention. We propose that a mechanism of selenium chemoprevention is its ability to activate early tumorigenesis barriers such as senescence and DNA damage response. Before genomic instability and malignant conversion, normal cells undergo activation of DNA damage response (Bartkova et al., 2005), which is a proposed tumor barrier. It is thought that cells in this state could potentially never progress to cancer, if the proper signaling pathways remain intact. It is hypothesized selenium's metabolites can induce a ROS response. This ROS response could activate certain signal transduction pathways that would lead to an increased DNA damage response, thus preventing the cells from further tumorigenesis. In this study we examine a novel role of selenium as an inducer of DNA damage response and show that selenium can up-regulate genes involved in the DNA double strand break (DSB) response, thus showcasing selenite as an excellent candidate for chemoprevention.

1.2 The DNA Double Strand Break Response

1.2.1 The PI3K-like protein kinases

DNA double strand breaks promote cell death or genomic instability. DSBs are a dangerous type of DNA damage that can occur within the cell. A family of proteins, phosphoinositide-3-kinase-related protein kinases (PIKK) are activated in response to DNA damage, in particular, two of three are activated in response to DSBs. Three important members of this family are Ataxia telangiectasia mutated (ATM), ataxia telangiectasia and Rad3-related (ATR) and DNA-dependent protein kinase catalytic subunit (DNA-PK_{cs}). ATR is activated in response to single-stranded DNA and stalled replication forks while ATM and DNA-PK_{cs} respond to DSBs (Falck et al., 2005). DNA damage, and DSBs breaks in particular, can be induced by exogenous and endogenous sources. An exogenous source of DNA damage would be ionizing radiation and an endogenous example would be innate cellular metabolism, which generates reactive oxygen species (ROS); both types of DNA damage have been known to cause DSBs (Khanna and Jackson, 2001). After a double strand break is formed, the cell responds by activating sensor proteins, which recognize the damage, then activate transducer proteins that relay and amplify the damage signal. Finally effector proteins are activated, which can modulate the cell cycle, reconstruct chromatin, and control DNA repair (See Figure 1.2).

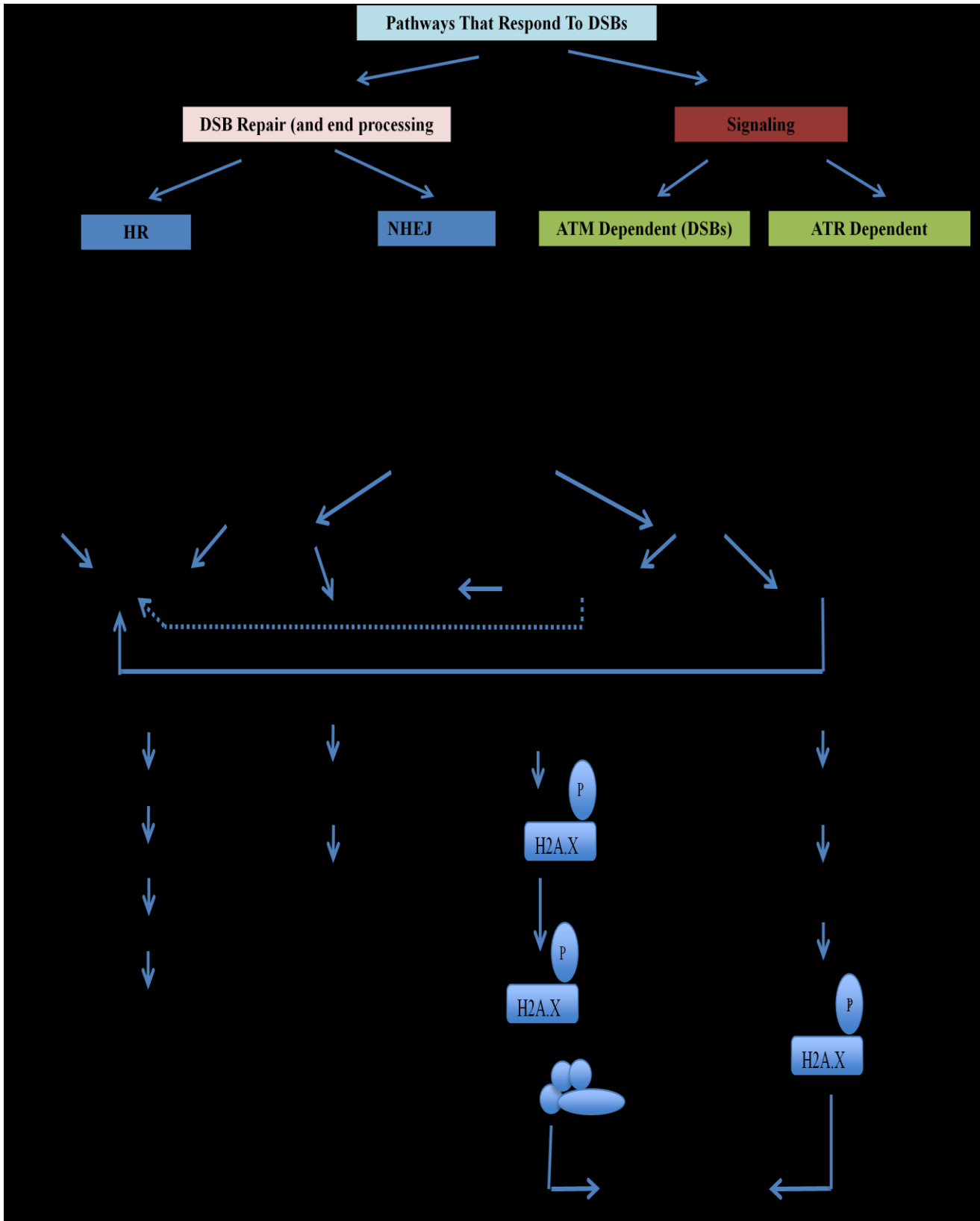


Figure 1.2 The DNA Double Strand Break Response.

1.2.3 Double Strand Break Signaling: ATM

The ataxia telangiectasia mutated protein is mutated in the genetic disorder ataxia telangiectasia. Patients suffering from this disease exhibit ataxia, immune defects, and cancer predisposition (Chan et al., 2000). ATM is a transducer protein. When ATM is activated in response to DSBs, it can promote cell cycle checkpoint arrest and allow the cell time for repair. It was shown in 2001 ATM is the major kinase involved in the phosphorylating histone 2A (H2A). They specifically found ATM can phosphorylate H2A *in vitro* and that ectopic expression of ATM in ATM^{-/-} fibroblasts restores H2A phosphorylation *in vivo*. This suggests ATM is one of the first proteins to be activated by DNA damage (See Figure 1.3) and one of the initial proteins to respond to DSBs (Burma et al., 2001). Molecular characteristics of ATM deficiency include delayed up-regulation of p53 in response to ionizing radiation. Substrates of ATM include Chk2, PHAS-I, the 32-kDa subunit of RPA and serine 15 of p53 *in vitro*; all of these sites are dependent on manganese concentration. In each case, phosphorylation was strictly dependent on manganese, and might be a common characteristic for all PIKK kinases (Chan et al., 2000).

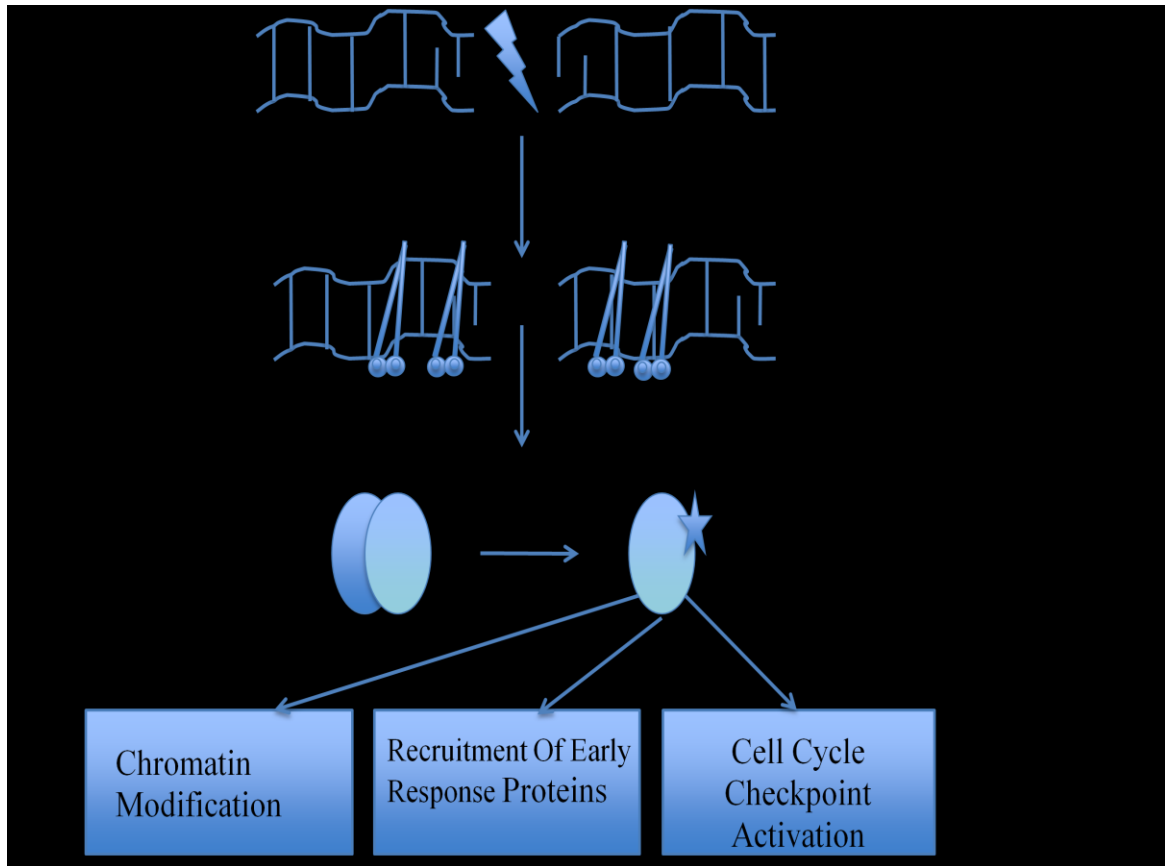


Figure 1.3 Activation of ATM. ATM is a master protein involved in the signal transduction pathway of the DNA double strand break response. It is recruited to site of DNA damage, in a conserved manner, similar to ATR and DNA-PK_{cs}, other PIKK proteins, by smaller effector proteins (Falck et al., 2005). This recruitment is facilitated by evolutionary conserved motifs in the effector proteins: Ku for DNA-PK_{cs}, ATRIP for ATR, and the MRN complex for ATM. These proper signaling pathways are necessary for the fidelity of the DNA double strand break response.

1.2.3 Double Strand Break Repair: DNA-PK_{cs} and NHEJ

In mammalian cells, the non-homologous end-joining (NHEJ) is the main pathway that repairs DNA double strand breaks. The proteins Ku70, Ku80 and DNA-PK_{cs} together form the holoenzyme DNA-dependent protein kinase (DNA-PK) which is activated in the presence of DNA *in vitro* and is required for proper NHEJ function (Chan et al., 2002). The NHEJ pathway is the predominate pathway that repairs

DNA double strand breaks in all stages of the cell cycle (Mao et al., 2008).

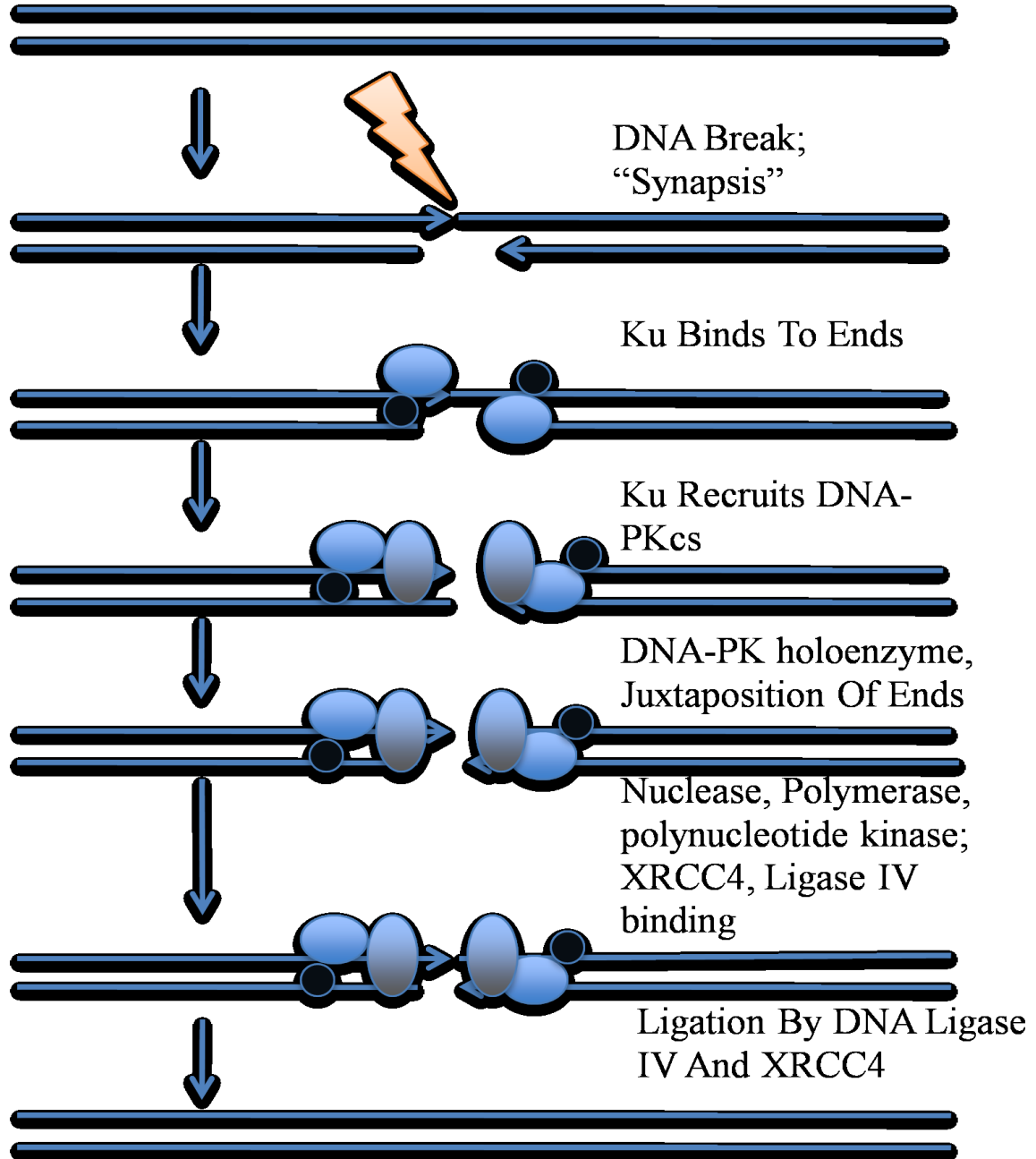


Figure 1.4 Simplified Mechanism of the Non-Homologous End-Joining pathway
This pathway, in contrast to the homologous recombination, is error prone because it does not invade a sister chromatid and gather the correct bases. Homologous recombination only takes place when there is a homologous template available, such as during S-phase. Although, the NHEJ pathway simply ligates the double strand break with minimal processing, it is actually the main pathway in the mammalian cell for repairing DNA double strand breaks (Mao et al., 2008).

DNA-PK_{cs} is the 460 kDa catalytic subunit associated with this pathway (See Figure 1.4 for more details). Unpublished research from our lab shows the responsibility of phosphorylating histone H2A.X at serine 139, surrounding regions of DNA damage, could be shared between ATM and DNA-PK_{cs}. The catalytic subunit DNA-PK_{cs} is rapidly phosphorylated at the Thr-2609 cluster and Ser-2056 upon ionizing radiation (IR). The threonine 2609 cluster includes the threonine 2647 site, and is phosphorylated *in vivo* in an ATM-dependent manner in response to DSBs, particularly after IR (Chen et al., 2007). The serine 2056 residue is autophosphorylated in response to DNA damage (Chan and LeesMiller, 1996), and its phosphorylation is required for the repair of DSBs by NHEJ (Chen et al., 2005). IR-induced DNA-PK_{cs} autophosphorylation is cell cycle dependent; there is less autophosphorylation of DNA-PK_{cs} in the S-phase (Chen et al., 2005). Interestingly, phosphorylated DNA-PK_{cs} colocalizes with both γ H2A.X and 53BP1 after DNA damage, demonstrating that DNA-PK_{cs} is present at sites of DNA damage (Chan et al., 2002).

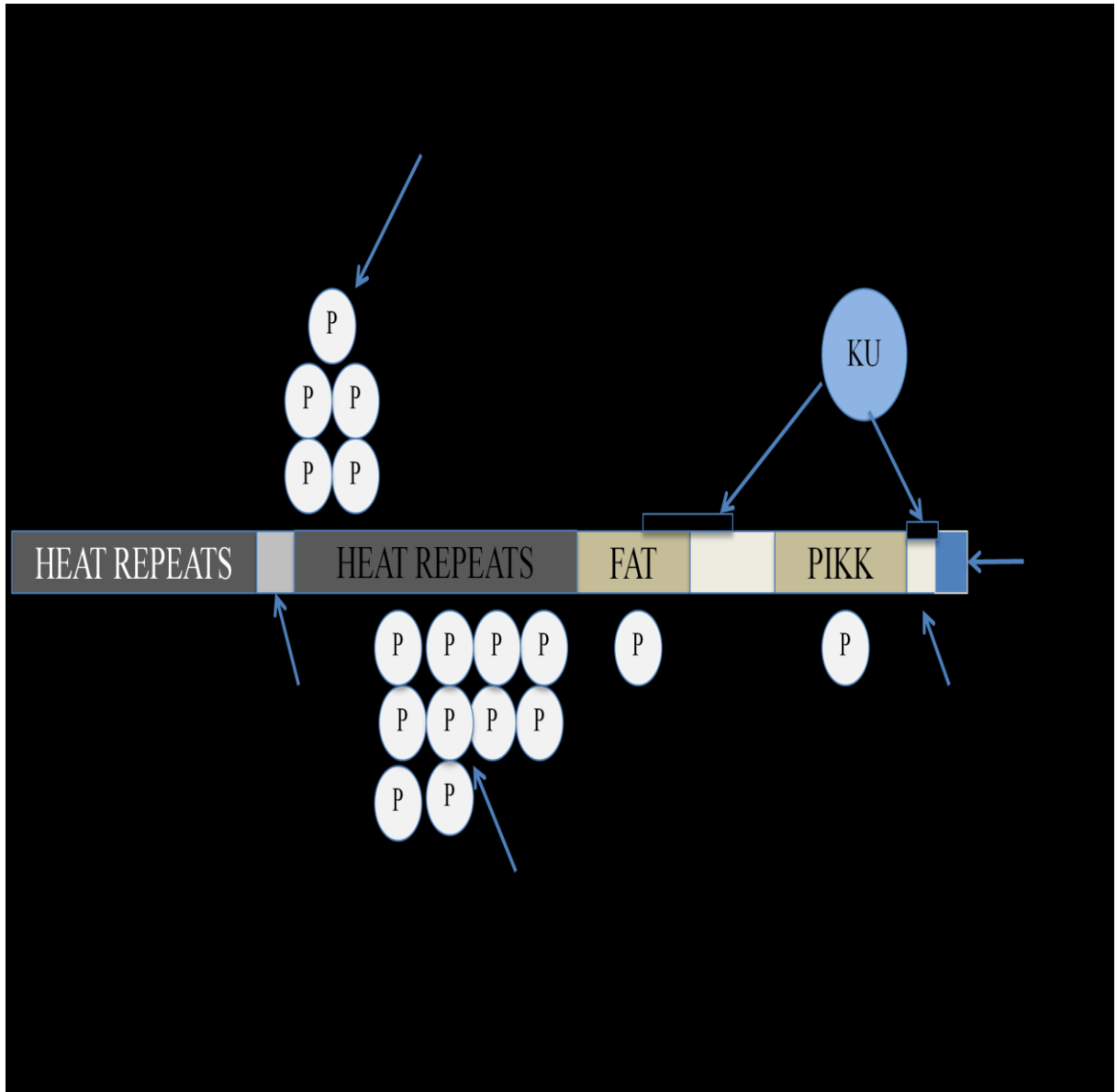


Figure 1.5 Major Features of DNA-PK_{cs}. The major of DNA-PK_{cs} are highlighted above, and many of the regions such as the FAT, PIKK, and HEAT repeats are evolutionary conserved across other members of the PIKK family, such as ATM and ATR. DNA-PK_{cs} is rapidly phosphorylated *in vivo* in response to DNA damage. There are many DNA-PK_{cs} phosphorylation sites, however, for the purpose of this work, threonine 2647, phosphorylated in response to ATR and ATM kinase activity and serine 2056, an autocatalytic phosphorylation site, are of particular interest.

1.2.4 Cross-talk between the proteins

It has been shown ATM is not fully responsible for decreased radio sensitivity in human malignant glioma cell lines (Chan et al., 1998) and when ATM kinase activity is inhibited in normal MRC-5 cells, the phosphorylation of histone 2A is only partially inhibited (Cheng, unpublished data). Also, it was shown in the Cheng lab that ATM is required for a selenium-induced senescence response. Therefore, this data led us to hypothesize that another protein, possibly a PIKK family member, is responsible for a normal radiosensitivity response, phosphorylation of histone sensing marker, H2A, and a normal selenium-induced senescence response. This protein could be DNA-PK_{cs}; its levels throughout the cell cycle are mostly consistent (see Fig 1.6), and its proper function is necessary for the correct repair of DNA double strand breaks. For this reason, we are interested in studying the cross-talk between PIKK family because this interaction could be crucial for proper DNA response and repair.

1.3 Hypothesis and concept

In the broad field of chemoprevention, selenium has not yet been selected as a prime target for induction of DNA damage repair. We hypothesize the chemopreventive properties of selenium are due to activation of early tumorigenesis barriers, such as induction of DNA damage response and senescence. We show here that selenium induces a DNA damage response in normal MRC-5 cells, but not in two cancer cell lines.

Chapter 2: Materials and Methods

2.1 Cell Culture and Drug Treatments

The non-cancerous MRC-5 human lung fibroblasts were cultured in α -minimum Eagle's medium supplemented with 15% fetal calf serum, 1% essential amino acids, 1% non essential amino acids, 1% vitamins, 0.5% amphotericin B, 1% penicillin-streptomycin and 5 μ g/ml plasmocin (Invitrogen, Carlsbad, California). The generation of ATM short-hairpin RNA (shRNA) and control cells using the U-2 OS osteosarcoma cell line have been described previously (Cheng et al., 2008). ATM shRNA U-2 OS and U-2 OS control cells were maintained in Dulbecco's Modified Eagle Medium supplemented with 10% fetal calf serum, 0.5% amphotericin B, 1% penicillin-streptomycin and 5 μ g/ml Plasmocin (Invitrogen). For immunofluorescence assays, exponentially growing cells grown on coverslips in 6-well plates were treated with a combination of the following chemicals; 1 or 2 μ M of sodium selenite (Sigma, St. Louis, Missouri) for 24 h, 100, 300, or 500 μ g/ml of neocarzinostatin (Sigma) for 10 min, 50 μ M NU 7026 for 24 h (Tocris, Ellisville, Missouri), 10 μ M KU 55933 (Tocris), for 24 h, and 1 μ g/mL aphidicolin (Tocris), for 24 h at the indicated concentrations at 37 °C.

2.2 Colony Formation

h-TERT immortalized mouse embryonic fibroblasts harboring deficiencies in the proteins making up the non-homologous end-joining pathway were grown to 80% confluency then seeded (10,000 for wild type mouse embryonic fibroblasts and 5,000

for DNA-PK_{cs}^{-/-}, Ku70^{-/-} and Ku80^{-/-}) into 6-cm dishes with 2 mL DMEM and incubated for two weeks in either 20% oxygen or 3% oxygen. In addition to the media 0, 1, 2, 5 μM Sodium Selenite, 0, 1, 2, 5, 10 μM Methaneseleninic acid, 95% and 0, 10, 20, 50, 100 μM Se-Methyl-selno-L-cysteine were added to the media. Media was changed one week after seeding. After two weeks, cells were fixed with methanol, stained with Coomassie blue dye and counted. Analysis was done by counting the number of colonies, then determining the percentage they represent of the control (percent of 100). Multiple independent experiments were done to generate error bars.

2.3 Detection of Reactive Oxygen Species (ROS)

Intracellular ROS were detected using 5-(and-6)-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate, acetyl ester (CM-H2DCFDA) (Invitrogen). DCFDA is a permanent indicator of ROS that is nonfluorescent until removal. The acetate groups are removed by intracellular esterases causing oxidation within the cell. Mouse embryonic fibroblasts and the corresponding NHEJ mutants were treated with 0, 1, 2, 5 μM Sodium Selenite, 0, 1, 2, 5, 10 μM Methaneseleninic acid, 95% and 0, 10, 20, 50, 100 μM Se-Methyl-selno-L-cysteine for 24 hours, then rinsed with PBS. They were then incubated with 10 uM DCFH-DA for 30 min at 37 °C. Lastly the cellular fluorescence intensity was detected using a fluorescence microplate reader (FLUOstar OPTIMA, BMG LABTECH, Cary, North Carolina) and a fluorescent microscope (Zeiss, Thornwood, New York).

2.4 Immunofluorescence and Antibodies

phospho-DNA-PK_{cs} T2647 (lot 903801) and phospho-DNA-PK_{cs} S2056 (lot 696143) polyclonal antibodies were purchased from Abcam (Boston, Massachusetts). Total DNA-PK_{cs} and anti- γ H2AX S139 (lot 41665603) monoclonal antibodies were also purchased from Abcam. Total ATM antibody (lot YF-10-17-02) was purchased from Epitomi_{cs} (Burlingame, California) and phospho-ATMS1981 (lot 20772) antibody was purchased from Rockland (Gilbertsville, Pennsylvania). MRC-5, ATM shRNA, and control shRNA cells were grown on slides to about 70% confluence and then treated with the chemicals described above. Cells were fixed in 4% paraformaldehyde for 15 min, permeabilized with ice cold methanol for 10 min at -20°C , permeabilized again for 10 min in 0.3% Triton X-100, and blocked in 10% normal goat serum / 0.3 M glycine in phosphate buffered saline (PBS) for 1 h to permeabilize the cells and block non-specific protein-protein interactions. The slides were incubated with the described antibodies overnight, washed in PBS, and incubated with Alexa Fluor 488-conjugated goat anti-rabbit and Alexa Fluor 594-conjugated goat anti-mouse secondary antibodies (Invitrogen) for 1 h at room temperature. Cells were then washed in PBS and mounted onto slides containing a drop of 4,6-Diamidino-2-phenylindole (DAPI) (Invitrogen) that stains the nuclei. The immunostaining was visualized by a Zeiss Axio Observer Z1m (Zeiss) and images were processed using deconvolution with the software AxioVision. To allow direct comparisons, all the cells were irradiated and processed simultaneously and all the images were obtained using the same parameters (brightness, contrast, etc.).

2.5 Quantification and Statistics of Immunofluorescence Data

An image was taken of each cover slip (one treatment) three times at 20x, then the nuclei were outlined using the spline function. The properties were measured and the mean densometric intensity was measured for each channel. The intensities for each nucleus were averaged over the samples and the phospho mean densometric intensity was divided by the nuclei mean densometric intensity, multiplied by a hundred to determine the intensity as a percent of the control. These numbers were averaged and then plotted on a graph. Multiple student's t-tests were done to analyze the means of various treatments.

2.6 Chemicals and Reagents

All chemicals and reagents (Appendix I), buffers (Appendix II), and commercial kits (Appendix III) can be found in the appropriate appendices.

2.7 Equipments and facilities used

All equipments and facilities were provided by Department of Nutrition and Food Science, University of Maryland, College Park. The inventory is shown Appendix IV.

Chapter 3: Results

3.1 Colony formation assay

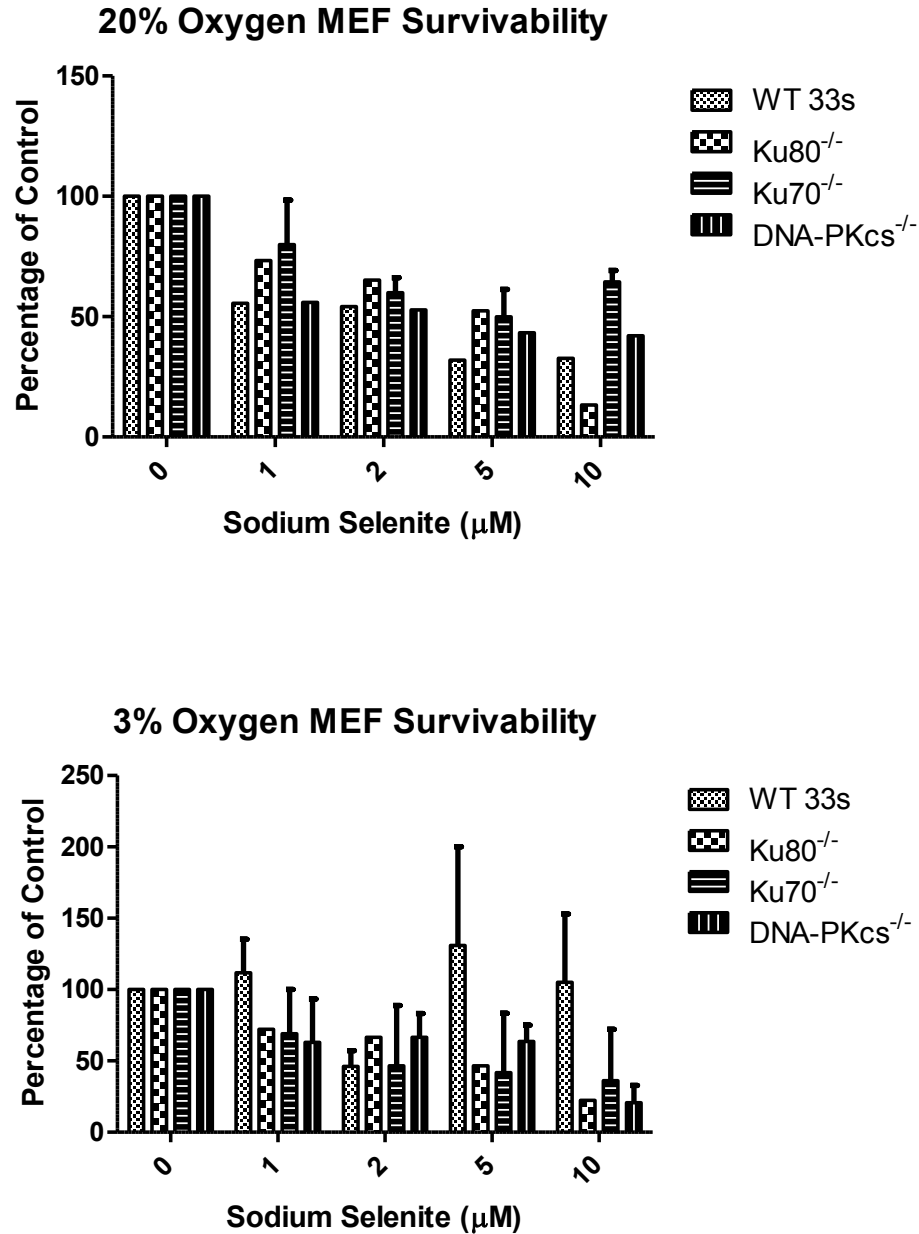


Figure 3.1 MEFs treated with Sodium Selenite Survival Data. The graph displays cell survivability of mouse embryonic fibroblasts treated with sodium selenite after a two week incubation in 20% and 3% oxygen. (20% oxygen: n=3; two way ANOVA p=0.99; bonferonni post tests showed no significance of mutants compared to wild types; 3% oxygen: n=3; two way ANOVA p=0.72; bonferonni post tests showed no significance of mutants compared to wild types. In both graphs, error bars are standard error of mean.)

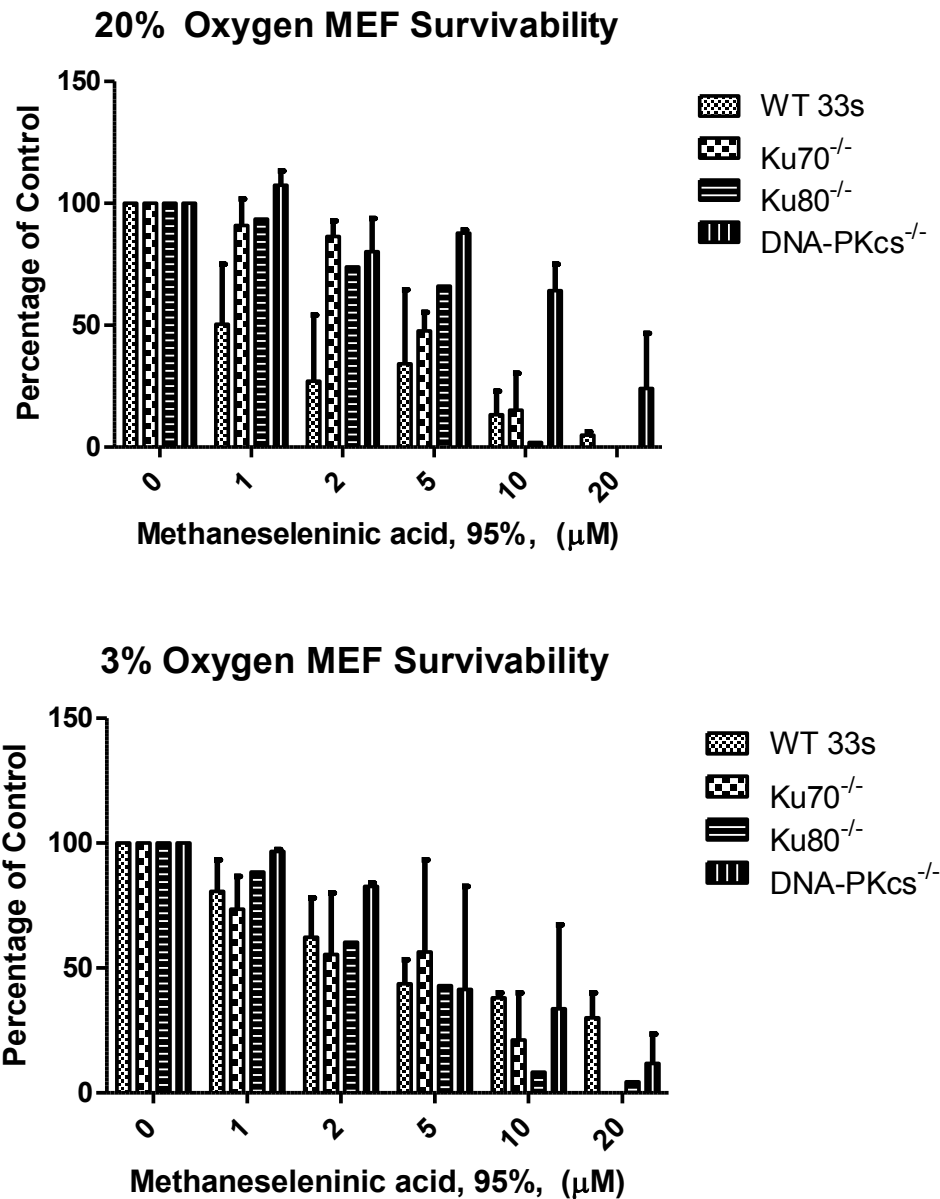


Figure 3.2 MEFs treated with Methaneselenenic Acid, 95% Survival Data. The graph displays cell survivability of mouse embryonic fibroblasts treated with methaneselenenic acid, 95% after a two week incubation in 20% and 3% oxygen. (20% oxygen: n=3, two way ANOVA p=0.1326; bonferonni post tests= Ku70^{-/-} treated with 2 µM methaneselenenic acid and DNA-PK_{cs}^{-/-} treated with 1, 2, 5, and 10 µM methaneselenenic acid are significantly different from the wild type control; 3% oxygen: n=3, two way ANOVA p=0.9740; bonferonni post tests showed no significance of mutants compared to wild types. In both graphs, error bars are standard error of mean.)

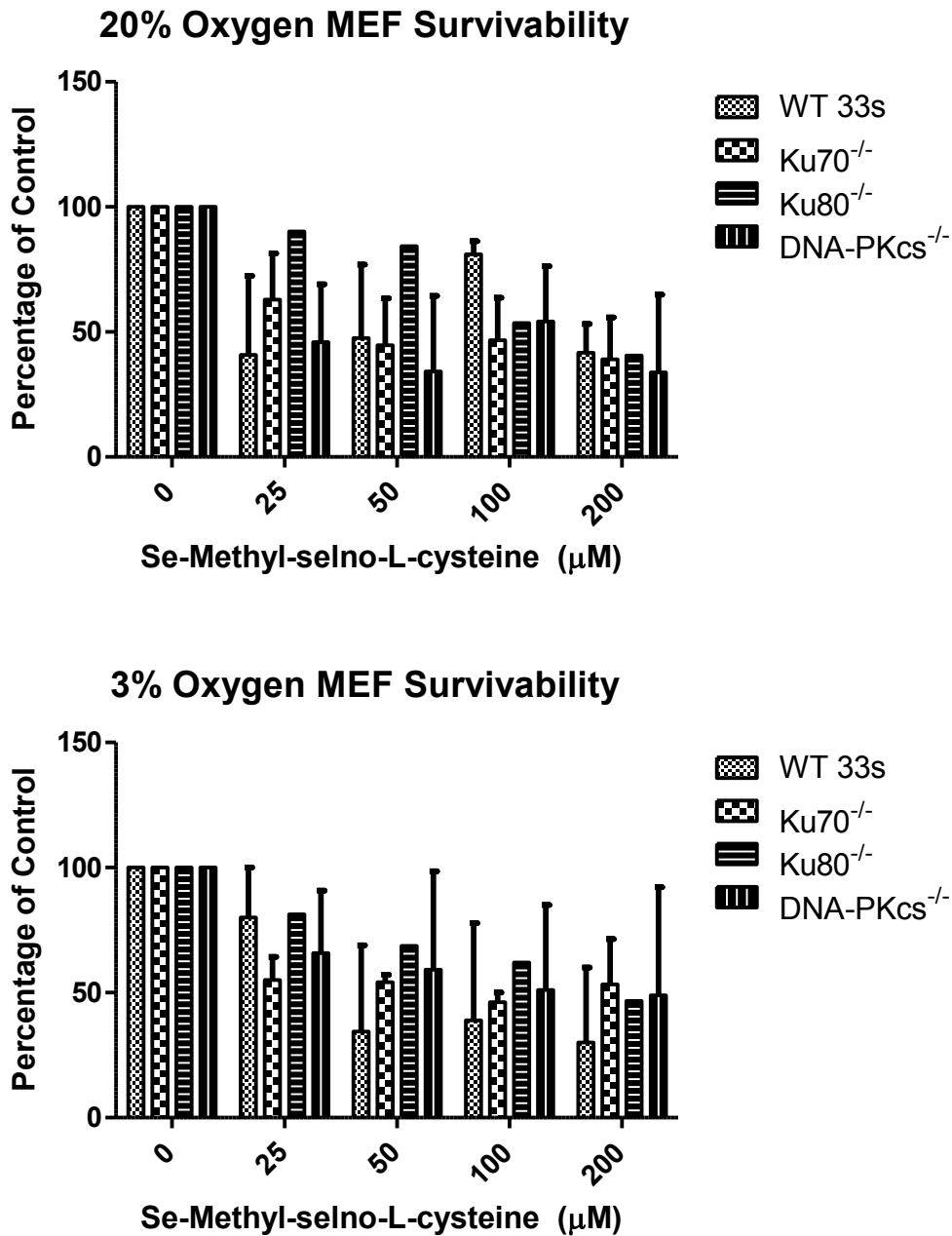


Figure 3.3 MEFs treated with Se-Methyl-selno-L-cysteine Survival Data. The graph displays cell survivability of mouse embryonic fibroblasts treated with Se-Methyl-selno-L-cysteine after a two week incubation in 20% and 3% oxygen. (20% oxygen: n=3; two way ANOVA p=0.754; bonferonni post tests showed no significance of mutants compared to wild types; 3% oxygen: n=3; two way ANOVA p=0.99; bonferonni post tests showed no significance of mutants compared to wild types. In both graphs, error bars are standard error of mean.)

After treatment of selenium to all cell lines, they were incubated for two weeks in either 20 % oxygen, normal cell culture conditions, or 3% oxygen, which is similar to *in vivo* oxygen concentrations (Li et al., 2009). The lower percentage of oxygen has been shown to generate less oxidative stress, which is favorable to extend the lifespan of cells cultured *in vitro* (Parrinello et al., 2003). Across all three types of selenium treatment (See Figures 3.1- 3.3), cells appear to be able to tolerate a greater dose of selenium when incubated in 3% oxygen. The cells with the highest survivability on average were the mouse embryonic fibroblasts deficient in DNA-PK_{cs}. However, it should be mentioned the mutant cells are immortalized with h-TERT, which can extend their replicative potential, and this could be a reason why there is little statistical significance when comparing the wild type survivability to the mutant survivability. If this experiment was repeated using primary cells with the core protein components of the NHEJ pathway knocked out, it is uncertain whether primary cells would have similar survivability responses to selenium treatment.

Figure 3.1 illustrates how sodium selenite affects survivability in mouse embryonic fibroblasts. Interestingly, the survivability of MEFs incubated with sodium selenite can be explained as function of ROS; decreased survivability of the wild type cells in 20% percent oxygen can be explained by their higher levels of ROS and the increased survivability of the mutants in 20% can be explained by their lower concentrations of ROS (see Figure 3.4). Another interesting trend see in cells incubated in physiological oxygen conditions (3%), and treated with sodium selenite, is the mutant cells offer protection against selenite induced cell death, presumably due to their decreased ROS response.

The highest dose of methaneseleninic acid, 20 μM , could be on the verge of toxic because no cell type thrived in this environment. For the Se-Methyl-selno-L-cysteine the highest dose chosen was conservative compared to highest dose chosen for sodium selenite and the acid. Se-Methyl-selno-L-cysteine is an organic form of selenium, and might explain why the highest dose is much less toxic than a lower dose of the inorganic selenium. Organic selenium is better tolerated *in vivo* because it is thought to be not as toxic as inorganic selenium. Furthermore, the selenium found in supplements and in whole foods is in the organic form, often complexed with yeast.

Although the results are slightly different for each type of selenium, it can be postulated Ku80, a protein that binds to DNA damage and recruits DNA-PK_{cs} (Spagnolo et al., 2006), is the most necessary of these three NHEJ proteins to combat ROS induced oxidative stress. When Ku80 mutant cells are challenged with increasing doses of different types of selenium, the Ku80 mutant cells are the most sensitive to selenium treatment and least likely to thrive. This is interesting because in mice models, the effect of Ku80 deletion is a much more severe phenotype than Ku70 deletion (Li et al., 2007; Li et al., 2009). To further understand why the protein components of the NHEJ pathway affect the lifespan of fibroblasts challenged with selenium, intracellular ROS was measured. Here we tested the hypothesis that selenium, in supranutritional doses, can generate ROS through its own metabolism.

3.2 Detection of ROS

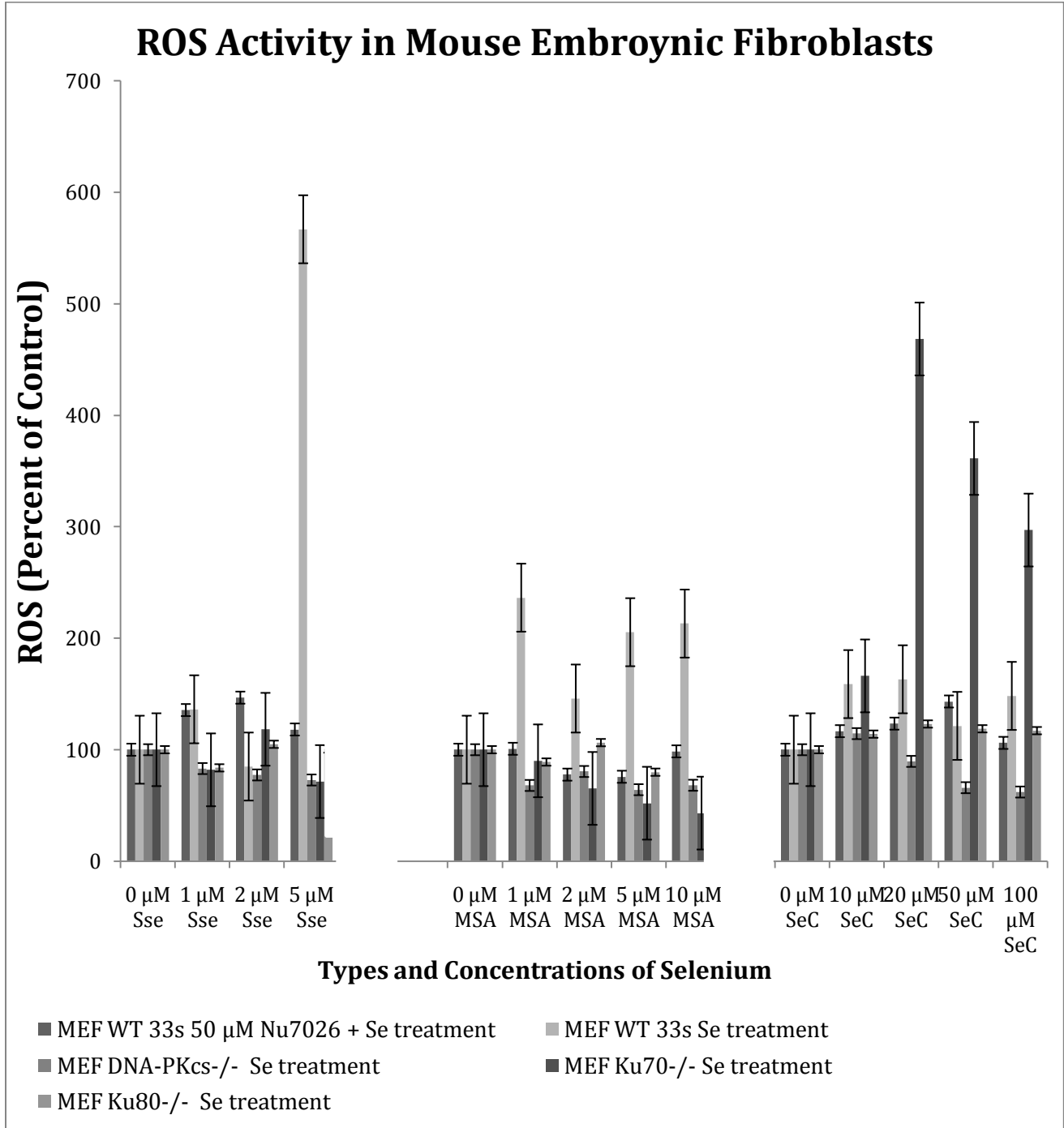


Figure 3.4 Reactive Oxygen Species in Mouse Embryonic Fibroblasts. Mouse embryonic fibroblasts with non-homologous end-joining components knocked down, treated with selenium for 24 h in 20% oxygen then measured for ROS using a fluorescent plate reader. Results were confirmed with a fluorescent microscope. Also shown is wild type mouse embryonic fibroblasts treated with a chemical DNA-PK_{cs} inhibitor, NU 7026 then measured for ROS production. The error bars represent stand error of the mean.

ROS production, the generation of free radicals containing an oxygen atom, were measured in mouse embryonic fibroblasts (MEFs) containing knockdowns of the important protein components of the non-homologous end-joining pathway. The same four cell types as used in colony formation experiments were used ; Wild type 33s, DNA-PK_{cs}^{-/-}, Ku70^{-/-} and Ku80^{-/-}. The cells were obtained already knocked down and all cell types were subjected to inorganic (sodium selenite) and organic selenium (Methaneseleninic acid, 95% and Se-Methyl-seleno-L-cysteine) then measured for ROS using a plate reading and fluorescent microscopy. The results of the ROS assay showed in order for a selenium-induced ROS response, the protein components of the NHEJ had to be present. This is a reasonable conclusion from the data because the wild type cells exhibited higher ROS levels than cells deficient in DNA-PK_{cs}^{-/-}, Ku70^{-/-} and Ku80^{-/-} (See Figure 3.4).

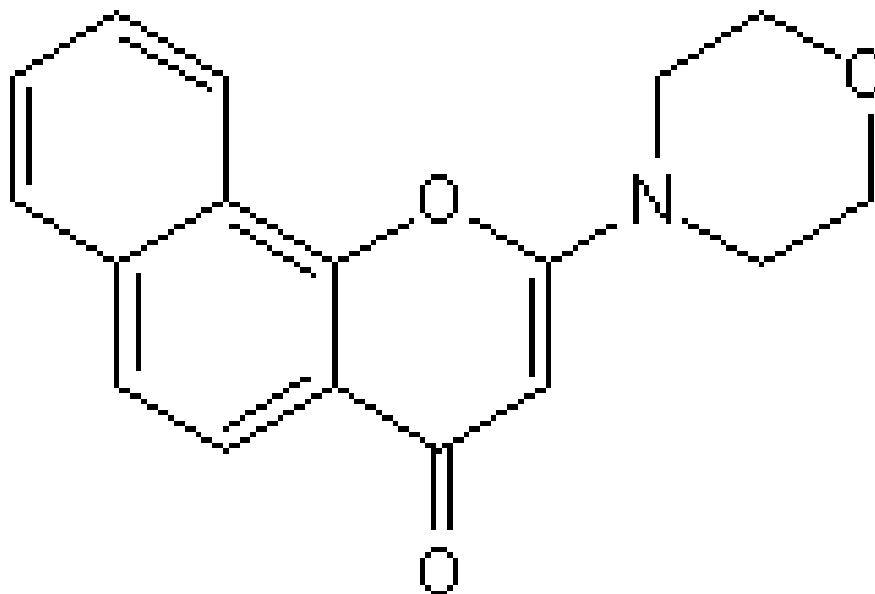


Figure 3.5 Chemical Structure of NU 7026 (2-(4-Morpholinyl)-4H-naphthol[1,2-b]pyran-4-one), an ATP-competitive inhibitor of DNA-dependent protein kinase that displays selectivity over other PIKK family enzymes.

To test the validity of the of the ROS results in the cells lacking protein components of the NHEJ pathway, in particular the activity of DNA-PK_{cs}, a chemical inhibitor of DNA-PK_{cs} was used to elucidate further the role of selenium in inducing a ROS response. The wild type fibroblasts, which showed significantly higher levels of ROS after selenium treatment, were pretreated with 50 μ M Nu 7026, a chemical DNA-PK_{cs} inhibitor, followed by selenium treatment. Results showed treatment with the chemical decreased the production of ROS significantly, similar to the effect of the DNA-PK_{cs} knockdown fibroblast (See Figure 3.6).

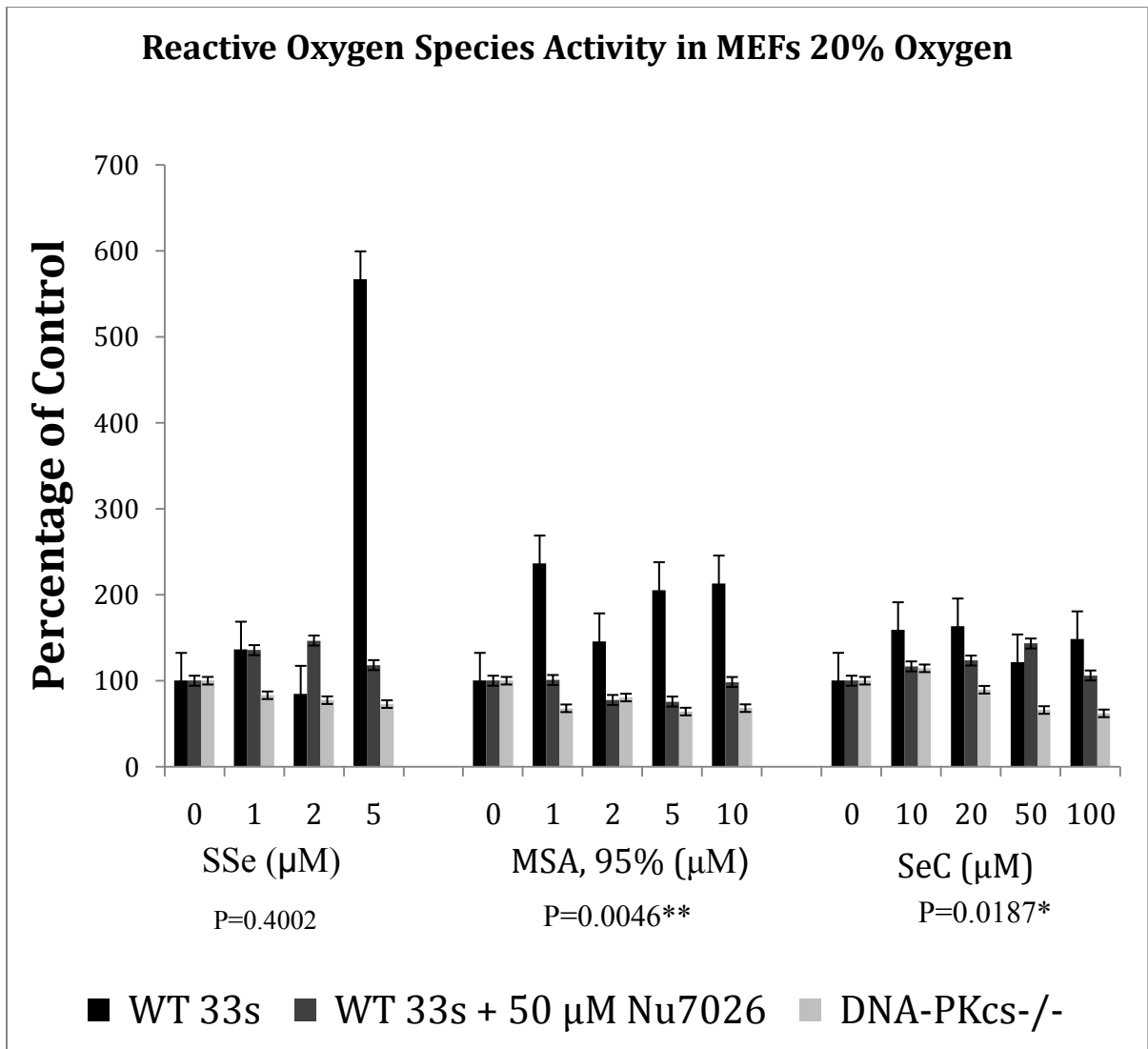


Figure 3.6 Effect of DNA-PK_{cs} inhibitor on ROS levels. Close up of mouse embryonic fibroblasts without DNA-PK_{cs} treated with selenium versus mouse embryonic fibroblasts with DNA-PK_{cs} treated with 50 μM NU 7026 followed by selenium treatment. WT 33s is included as the control (n=3; repeated measures ANOVA p for SSe=0.4002; p for MSA=0.0046; p for SeC=0.0187.) In each graph, the error bars represent standard error of the mean.

Figure 3.6 illustrates the effects of a DNA-PK_{cs} inhibitor on two types of cells. In wild type cells treated with selenium, as seen in Figure 3.5, and again in Figure 3.6, the percentage increase of ROS is significantly larger (200-600% percent) in wild type mouse embryonic fibroblasts than the percentage increase in either wild type

fibroblasts treated with NU 7026, or DNA-PK_{cs} knockout fibroblasts. However, treatment with a DNA-PK_{cs} inhibitor considerably decreases the selenium-induced ROS expression. Comparing the two dashed lines in figure 3.6, there is a decreased ROS response in wild type fibroblasts treated with the chemical DNA-PK_{cs} inhibitor, NU 7026. Therefore, the inhibitor is successful in mimicking the ROS response seen in the fibroblasts with the DNA-PK_{cs} gene knocked out.

With SeC treatment (See Figure 3.6), the trends and the error bars overlap, possibly because the dose chosen was too conservative to illicit a ROS response; SeC is an organic form of selenium, the salt, with the highest ROS response, is inorganic. In order to see a similar ROS response in MEFs treated with the SeC form of selenium, higher concentrations maybe needed. The necessity of the protein components of the NHEJ pathway to illicit a ROS response lead to the next experiment which established that mechanism responsible for the selenium-induced ROS response was the phosphorylation events surrounding the activation of DNA-PK_{cs} after selenium treatment.

3.3 Immunofluorescence

3.3.1 MRC-5 Cells

Figure 3.8 shows MRC-5 cells treated with or without 10 μ M KU 55933 for 24 h, an ATM inhibitor, and selenium for 24 h then probed for pDNA-PK_{cs} S2056, pDNA-PK_{cs} T2647 and γ H2A.X. Phosphorylation of DNA-PK_{cs} is decreased when KU 55933, an ATM inhibitor was present. ATM kinase inhibition results in decreased inhibition of phosphorylation of DNA-PK_{cs} at serine 2056 and threonine 2647, although there is greater decrease in serine phosphorylation following selenium treatment. Pretreatment with the inhibitor, but not selenium treatment, had the highest percentage of DNA-PK_{cs} phosphorylation at serine 2056. However, percentage of phosphorylation of threonine 2647 was highest when with no selenium or ATM inhibitor treatment. The trends are similar for MRC-5 cells pretreated with Aph for 24 h; except for threonine 2647 phosphorylation continues to decrease with increasing doses of selenium (See Figure 3.9).

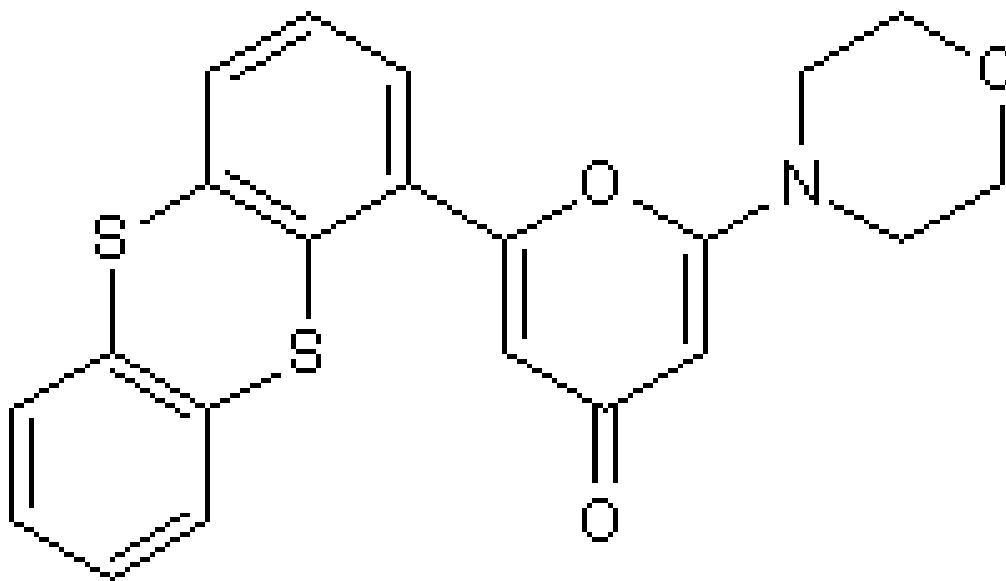


Figure 3.7: The structure of KU 55933 2-(4-Morpholinyl)-6-(1-thianthrenyl)-4H-pyran-4-one, a potent, selective and competitive ATM kinase inhibitor.

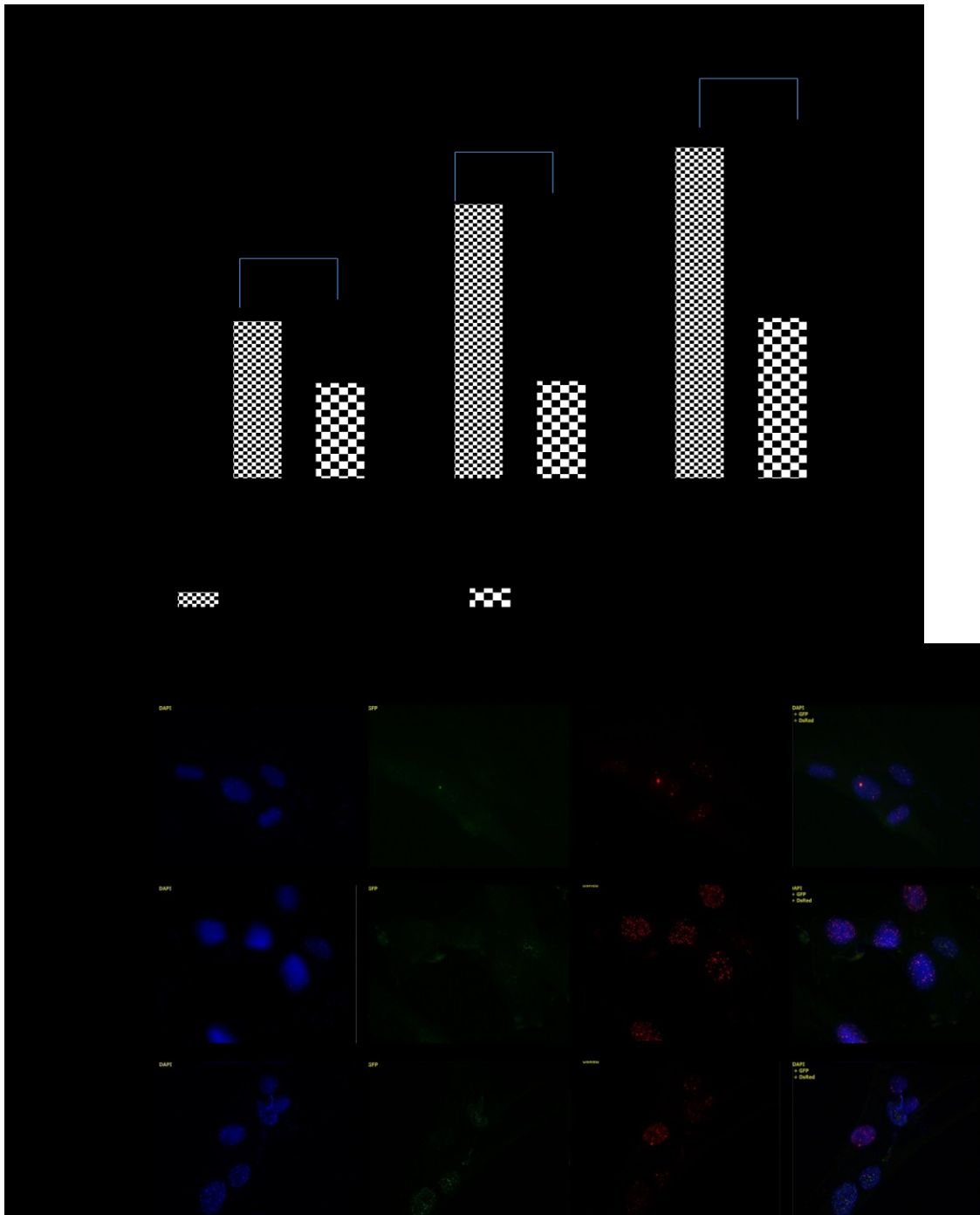


Figure 3.8: Selenite induces DNA-PK_{cs} phosphorylation at Threonine 2647 at the sites of DNA damage and this trend is decreased with ATM inhibition. MRC-5 cells treated with sodium selenite increases the phosphorylation of DNA-PK_{cs} at T2647. MRC-5 cells treated with 10 μ M KU 55933 for 24 h, an ATM inhibitor, and selenium for 24 h show decreased T2647 phosphorylation. A student's t-test was done ($p < 0.05$, $n=3$) that showed there is statistical significance between phosphorylation of T2647 with or without KU 55933 at 0, 1, and 2 μ M sodium selenite concentrations. The error bars on the graph represent the standard error of the mean.

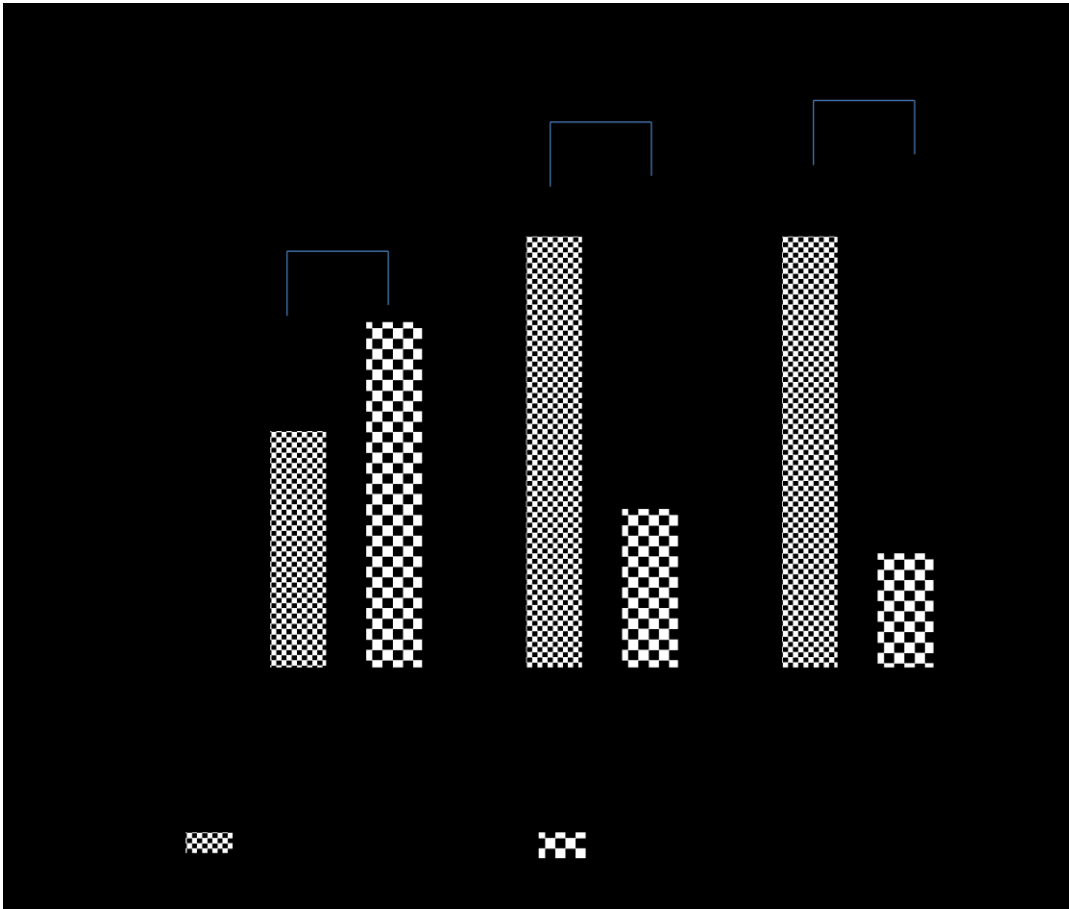


Figure 3.9: Selenite induces DNA-PK_{cs} phosphorylation at Serine 2056 at the sites of DNA damage and this trend is decreased with ATM inhibition. MRC-5 cells treated with 10 μM KU 55933 for 24 h, an ATM inhibitor, and selenium for 24 h show decreased S2056 phosphorylation compared to cells treated with only selenium. A student's t-test was done ($p < 0.05$, $n=3$) that showed there is statistical significance between phosphorylation of S2056 with or without KU 55933 at 0, 1, and 2 μM sodium selenite concentrations. The error bars on the graph represent the standard error of the mean.

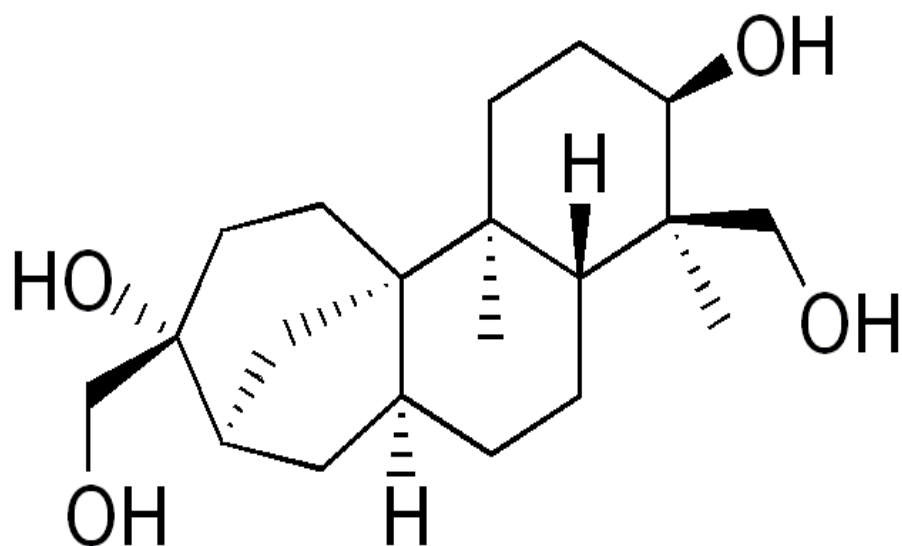


Figure 3.10: Chemical Structure of Aphidicolin Aphidicolin is an antibiotic that inhibits DNA polymerase α , stalling cells in the S-phase (See Figure 4.1).

Sodium selenite significantly enhances phosphorylation of S2056 and T2647 of DNA-PK_{cs} at sites of DNA damage, as measured by H2A phosphorylation. The cells treated with Aph, synchronized in S-phase, particularly the S2056 residue of DNA-PK_{cs}, show a decreasing phosphorylation (See Figure 3.11). Cells with no selenium treatment, but synchronized with Aph, show only partial phosphorylation at serine 2056 and not all cells in the field of view are phosphorylated when observing under a fluorescent microscope. However, with Aph synchronization and selenium treatment, there is greater phosphorylation and by using a fluorescent microscope it is clear most cells in the field of view are phosphorylated.

Treatment of synchronized MRC-5 cells can induce phosphorylation of DNA-PK_{cs} at threonine 2647, without concurrent phosphorylation of H2A. After fluorescent observation and statistical quantification, Aph synchronized MRC-5 cells without selenium treatment show complete overlap in DNA-PK_{cs} threonine 2647 phosphorylation and H2A phosphorylation in all cells in the treatment.

There is statistical significance between the phosphorylation intensity of threonine 2647 and serine 2056 of DNA-PK_{cs} in selenite treated MRC-5 cells depending whether they are in the S-phase. For both foci, there is a decrease in the phosphorylation at both DNA-PK_{cs} foci after synchronization in the S-phase.

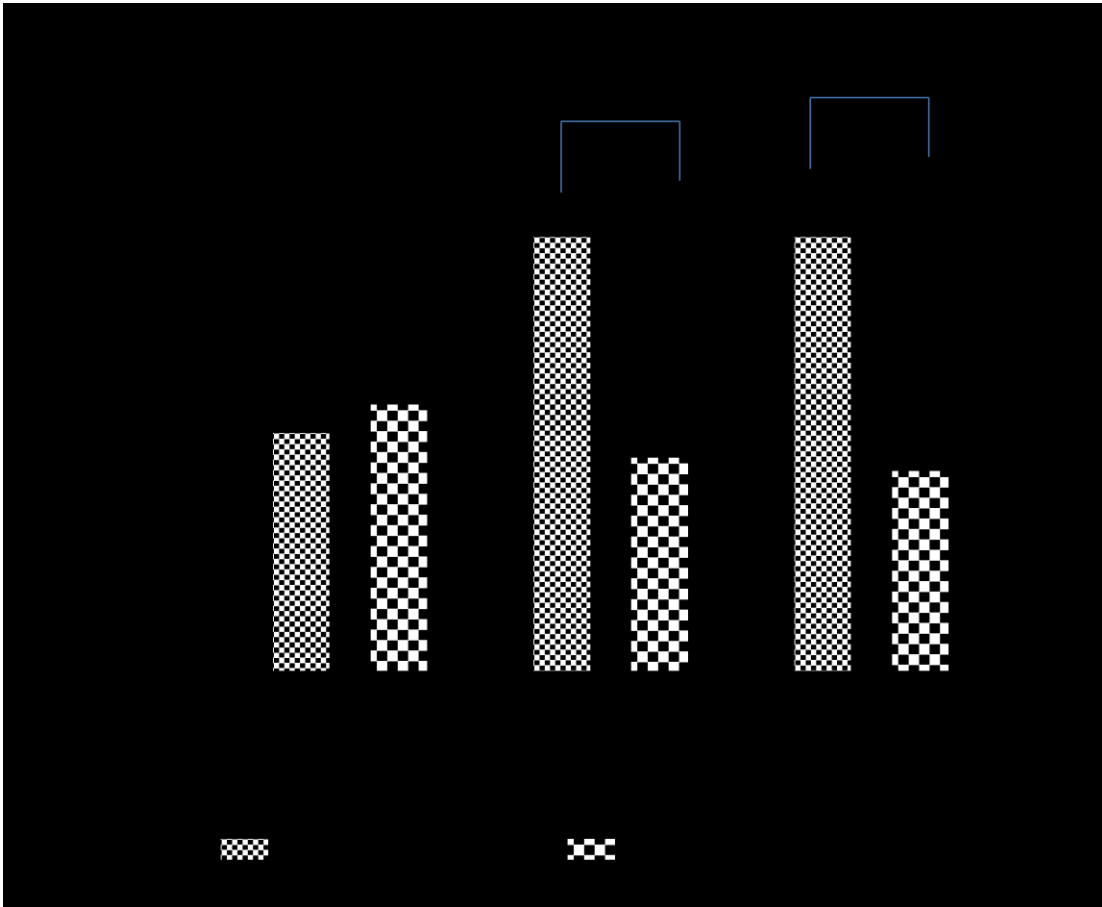


Figure 3.11 A: Selenite induces DNA-PK_{cs} phosphorylation at Serine 2056 at sites of DNA breaks in the S-phase. Cells were synchronized in the S-phase with aphidicolin. The total levels of DNA-PK_{cs} are the same in each treatment (data not shown). A student's t-test was done ($p < 0.05$, $n=3$) that showed there is statistical significance between phosphorylation of S2056 with or without Aph at 1 and 2 μM sodium selenite concentration. The error bars on the graph represent the standard error of the mean.

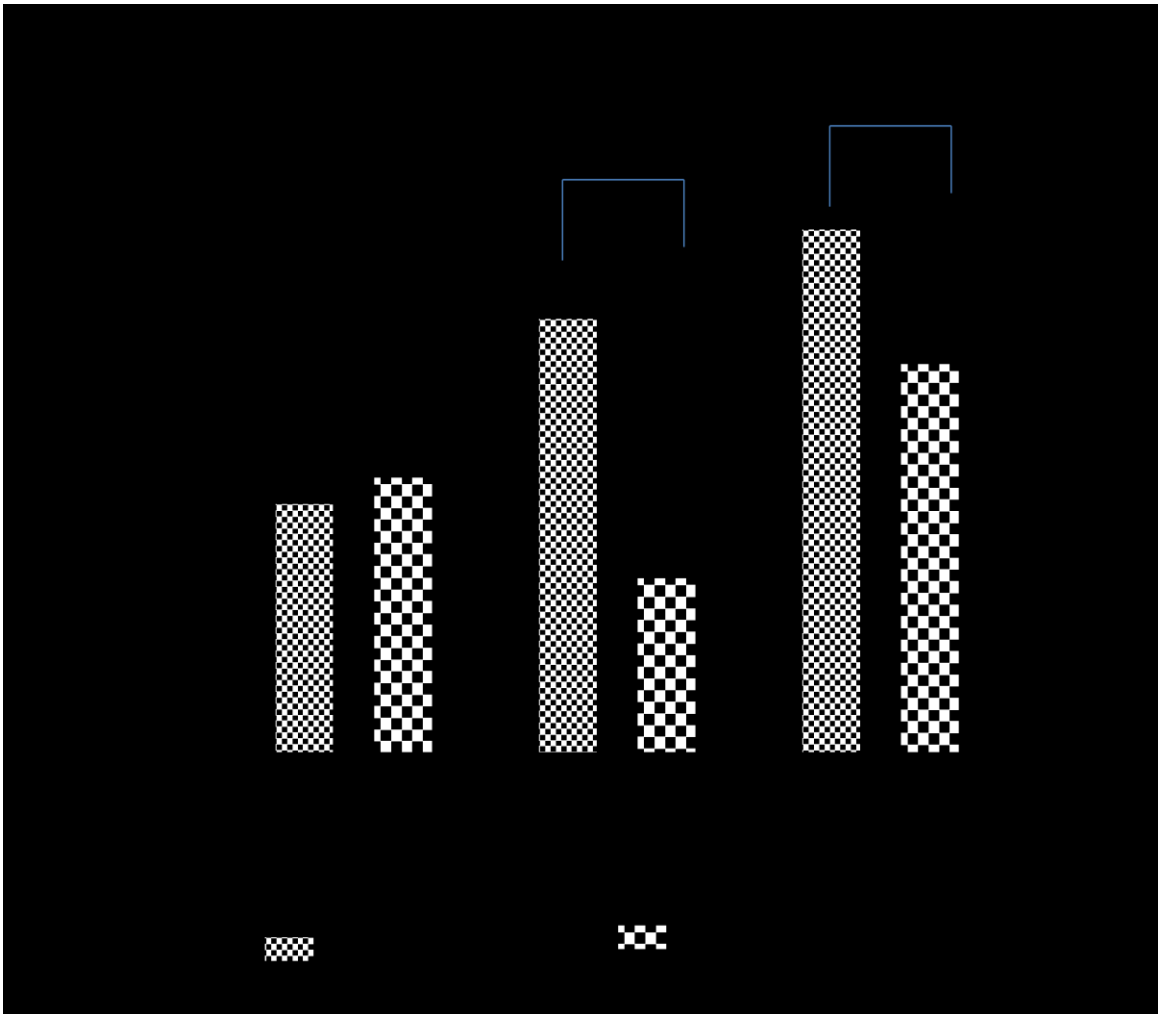


Figure 3.11 B: Selenite induces DNA-PK_{cs} phosphorylation at Threonine 2647 at sites of DNA breaks in the S-phase. Cells synchronized in the S-phase with aphidicolin. The total levels of DNA-PK_{cs} are the same in each treatment (data not shown). A student's t-test was done ($p < 0.05$, $n=3$) that showed there is statistical significance between phosphorylation of T2647 with or without Aph at 1 and 2 μM sodium selenite concentration. The error bars on the graph represent the standard error of the mean.

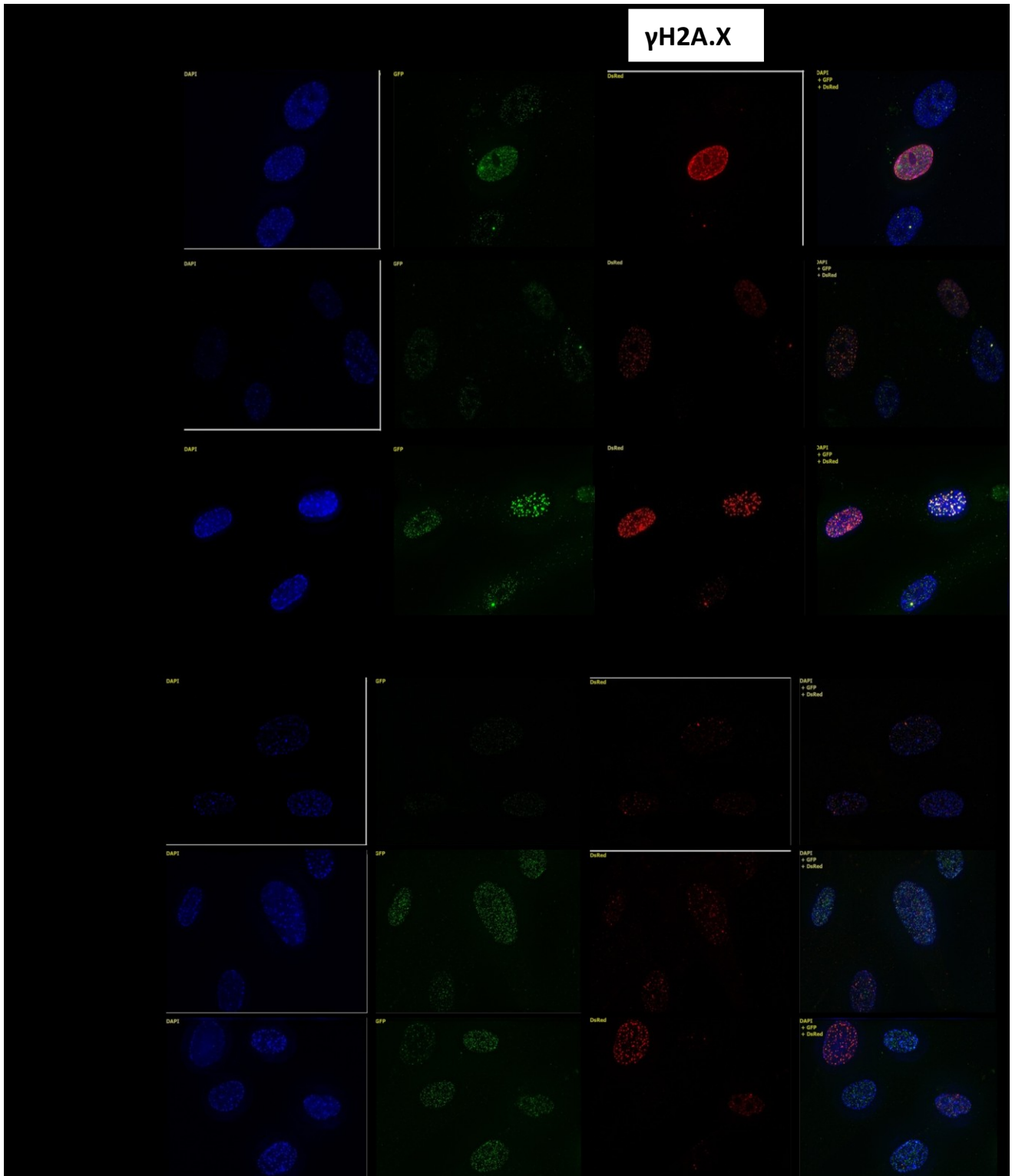


Figure 3.11 C: Selenite induces DNA-PK_{cs} phosphorylation at Serine 2056 and Threonine 2647 at sites of DNA breaks in the S-phase. MRC-5 cells treated with Aph to arrest cells in the S-phase followed by selenium treatment then probed for pDNA-PK_{cs} S2056, pDNA-PK_{cs} T2647 and γ H2A.X. Selenium treatment can induce phosphorylation of DNA-PK_{cs} at serine 2056 and threonine 2647.

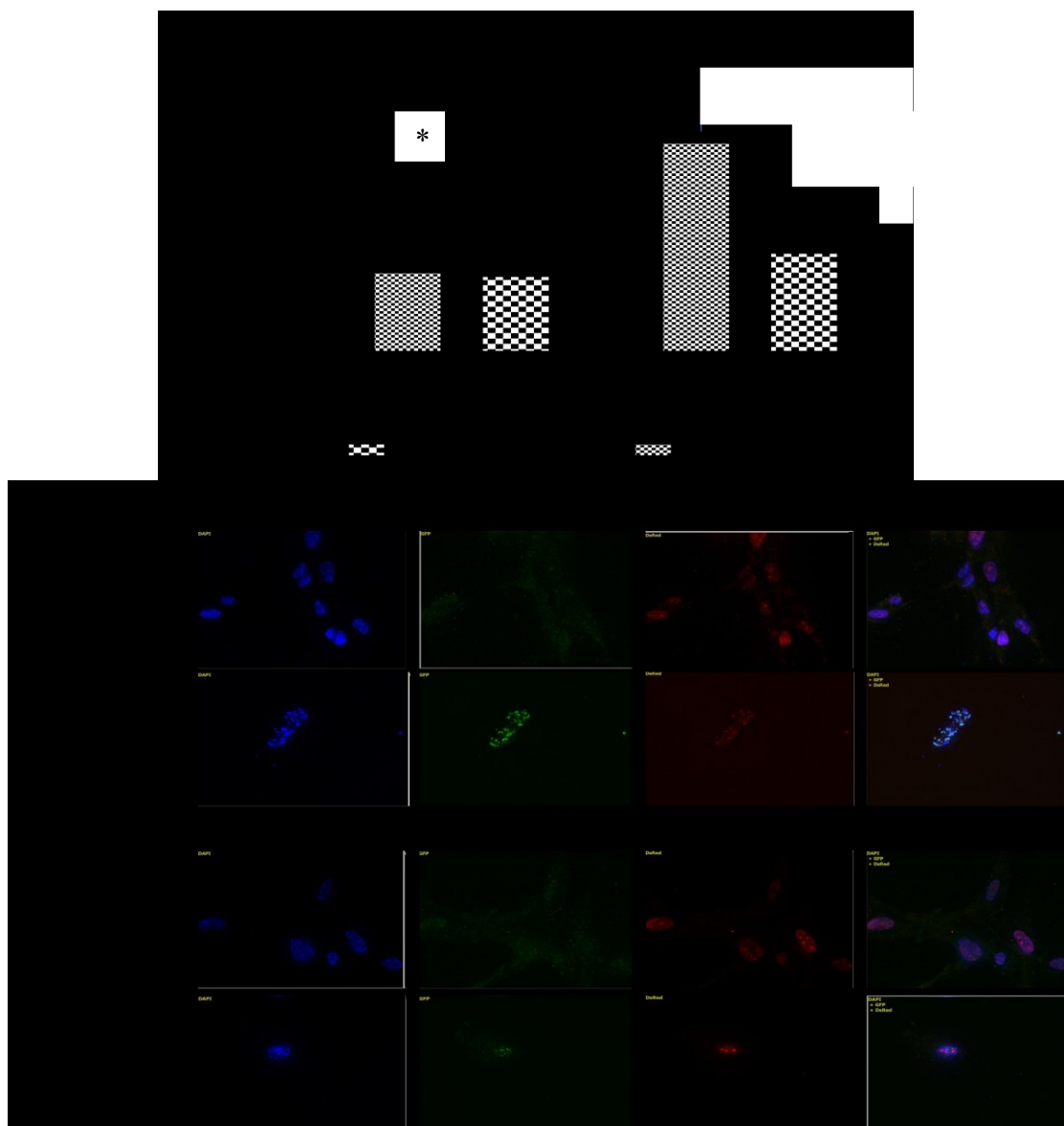


Figure 3.12: Neocarzstatin treatment effect on DNA-PK_{cs} phosphorylation. MRC-5 Cells treated with Neocarzstatin then probed for pDNA-PK_{cs} S2056, pDNA-PK_{cs} T2647, γ H2A.X, and Total DNA-PK_{cs} (not shown). Total levels of DNA-PK_{cs} were the same in each treatment. A student's t-test was done ($p < 0.05$, $n=3$) that showed there is statistical significance between phosphorylation of S2056 with NCS treatment and without. These results show a similar phosphorylation pattern to selenium treatment.

Normal MRC-5 cells are vulnerable to Neocarzstatin treatment in a dose dependent manner. However, higher doses of Neocarzstatin cause cell death, and therefore less DNA-PK_{cs} phosphorylation.

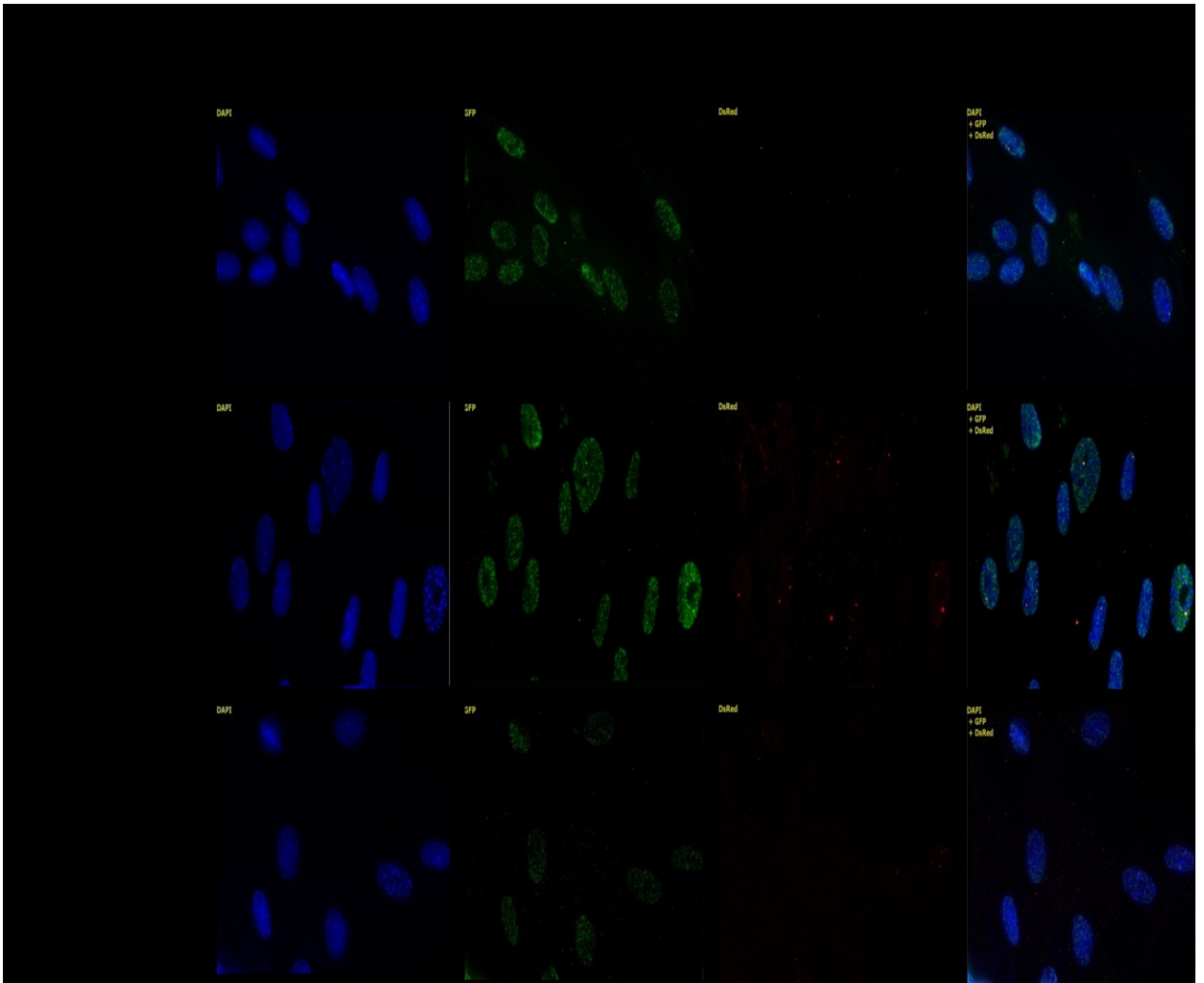


Figure 3.13 A: DNA-PK_{cs} inhibition effects on ATM phosphorylation. MRC-5 cells treated with 50 μM NU 7026, a chemical DNA-PK_{cs} inhibitor, and selenium then probed for total ATM and pATMS1981.

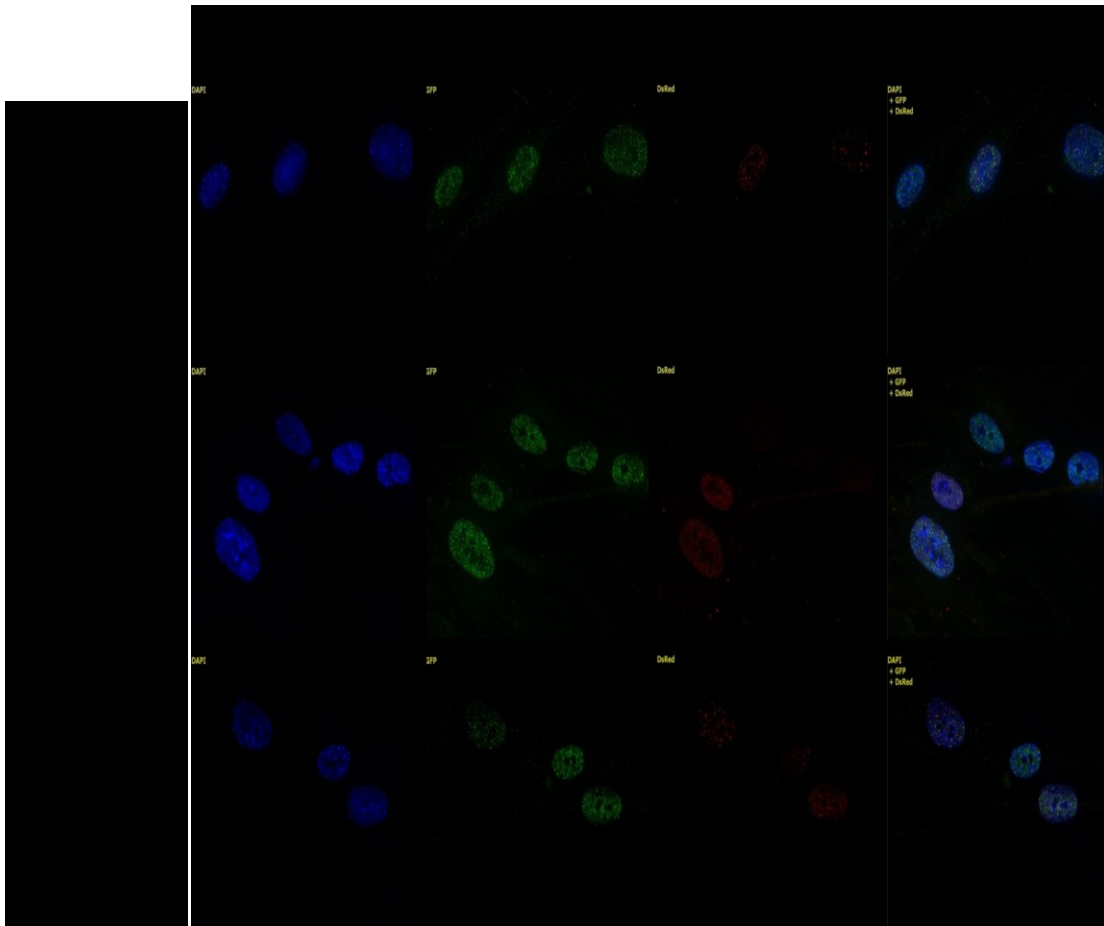


Figure 3.13 B: DNA-PK_{cs} inhibition effects on ATM phosphorylation in the S-phase. MRC-5 cells treated with Aph, 50 μM NU 7026, a chemical DNA-PK_{cs} inhibitor, and selenium then probed for total ATM and pATMS1981.

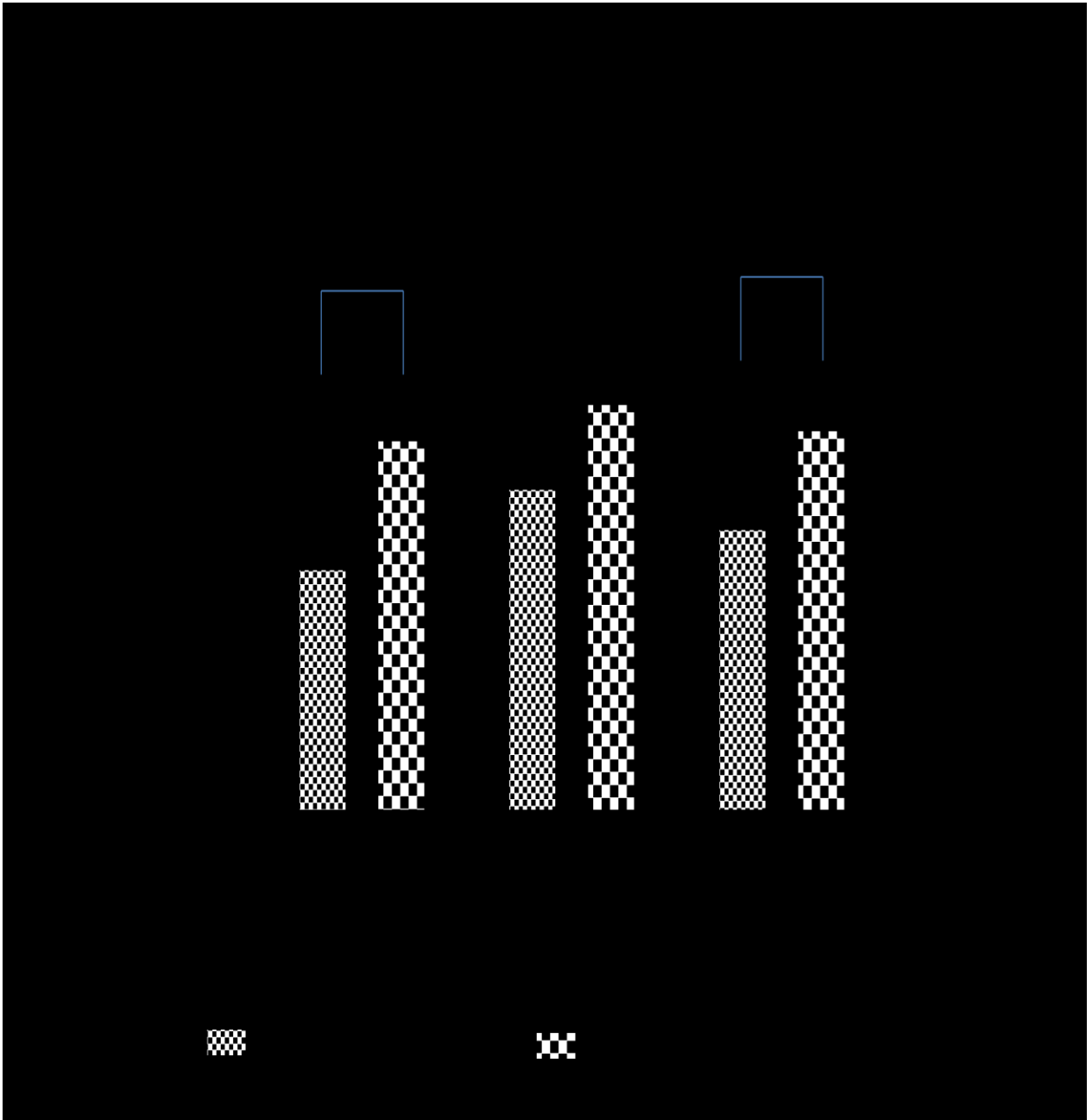


Figure 3.14: Effect of ATM phosphorylation in NU 7026 treated MRC-5 cells. Sodium selenite can induce the phosphorylation of ATM at serine 1981 independent of DNA-PK_{cs} kinase ability. A student's t-test was done ($p < 0.05$, $n = 3$) that showed there is statistical significance between phosphorylation of S1981 of ATM with or without Aph in NU 7026 pre-treated MRC-5 cells.

The total amount of ATM present in MRC-5 cells is independent of selenium and NU 7026 treatment. Measuring the total amount of protein was done in all experiments as a control regardless of protein type. The purpose is to show the differences in phosphorylation levels were not due to a difference in total protein concentration. As shown above, selenium treatment can induce phosphorylation of ATM at serine 1981, signaling its activation. The phosphorylation of ATM is due to selenium treatment, not due to total amount of ATM present.

The levels of total ATM are not dependent on selenium treatment; however selenium treatment induces phosphorylation in cells arrested in S-phase in cells treated with NU 7026. With no selenium treatment, only one cell in the field of view has phosphorylated ATM, while selenium treatment increases the number of cells in each field of view with ATM phosphorylation. Interestingly, ATM can be phosphorylated, signaling its activity, in the absence of DNA-PK_{cs} kinase activity. ATM phosphorylation at serine 1981 in cells pretreated with NU 7026 is an upstream event independent of DNA-PK_{cs} kinase activity.

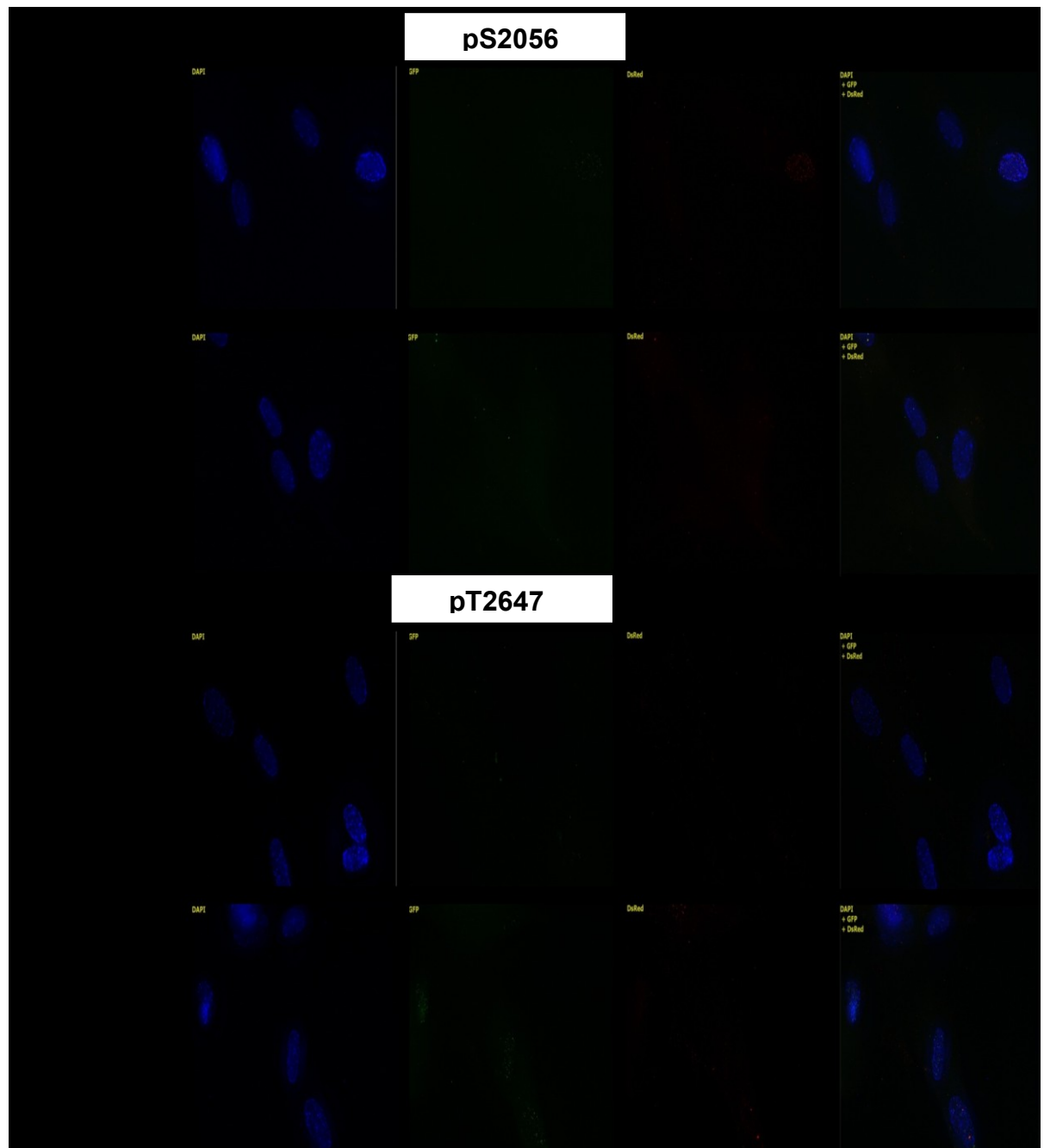


Figure 3.15: Effect of DNA-PK_{cs} inhibition on MRC-5 Cells. A) MRC-5 cells treated with 50 μ M NU 7026, a chemical DNA-PK_{cs} inhibitor, and selenium then probed for pDNA-PK_{cs} S2056, pDNA-PK_{cs} T2647 and pATMS1981.

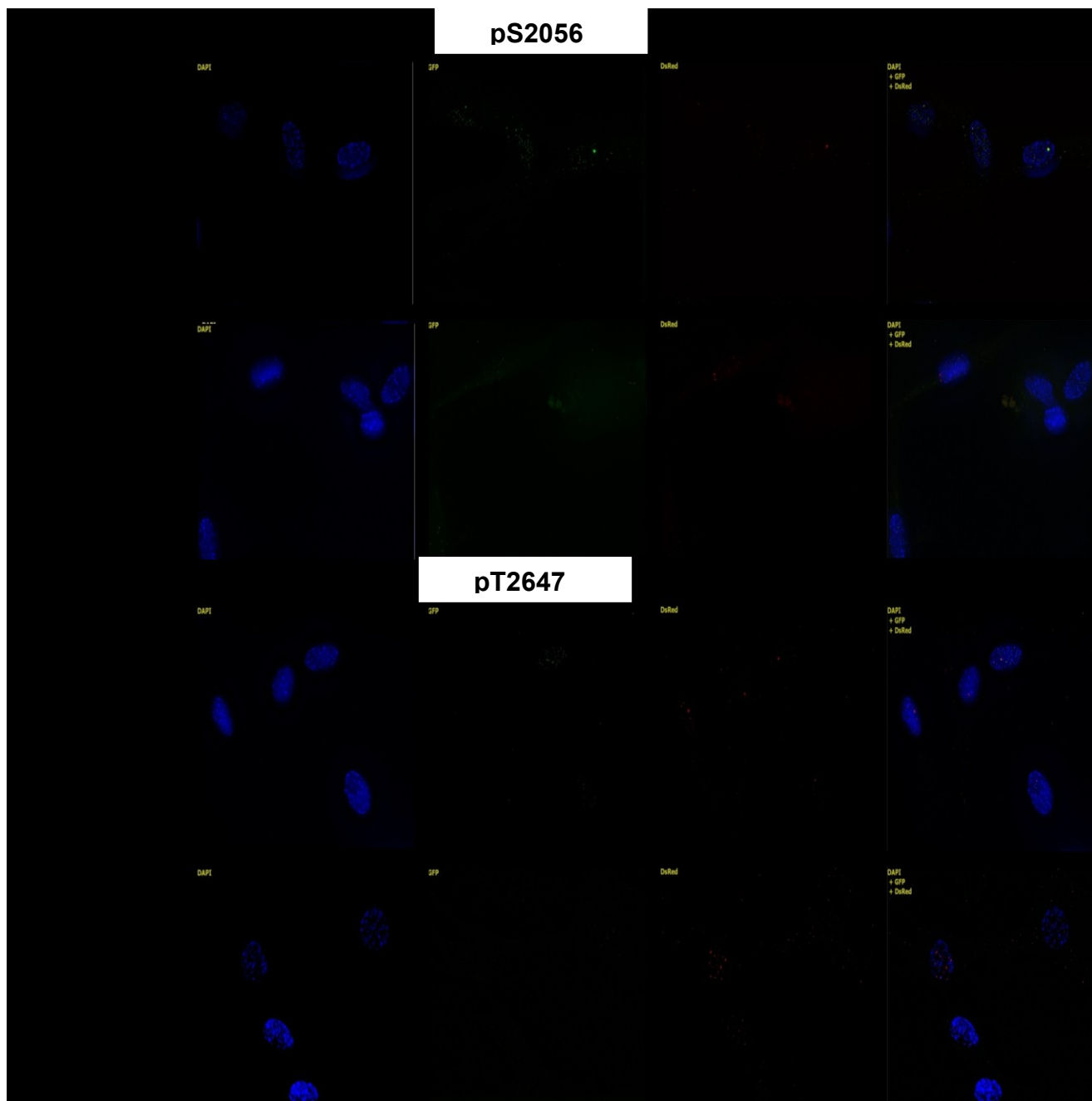


Figure 3.15: Effect of DNA- PK_{cs} inhibition on MRC-5 Cells. B) MRC-5 cells treated with Aph, 50 μ M NU 7026, a chemical DNA-PK_{cs} inhibitor, and selenium then probed for pDNA-PK_{cs} S2056, pDNA-PK_{cs} T2647 and pATMS1981.

Enzymatic inhibition of DNA-PK_{cs} decreases DNA-PK_{cs} autophosphorylation at serine 2056 and ATM autophosphorylation at serine 1981. Selenium treatment does induce slight phosphorylation of serine 2056 in DNA-PK_{cs} and of serine 1981 in ATM in MRC-5 cells treated with NU 7026. Using the same parameters but probing for DNA-PK_{cs} pT2647, selenium can induce phosphorylation of threonine 2647, most likely through an ATM dependent pathway.

In the synchronized experiment, enzymatic inhibition of DNA-PK_{cs}, decreases DNA-PK_{cs} autophosphorylation at serine 2056. ATM/ATR dependent phosphorylation of DNA-PK_{cs} at T2647 is eliminated after synchronization with Aph and treatment of NU 7026 and cannot be induced with selenium treatment unlike the unsynchronized experiment. Treatment of MRC-5 cells with NU 7026 and synchronized with Aph does not activate ATM, however, treatment of selenium can induce activation of ATM, determined by the phosphorylation of ATM at serine 1981.

3.3.2 ATM shRNA U-2 OS Cells

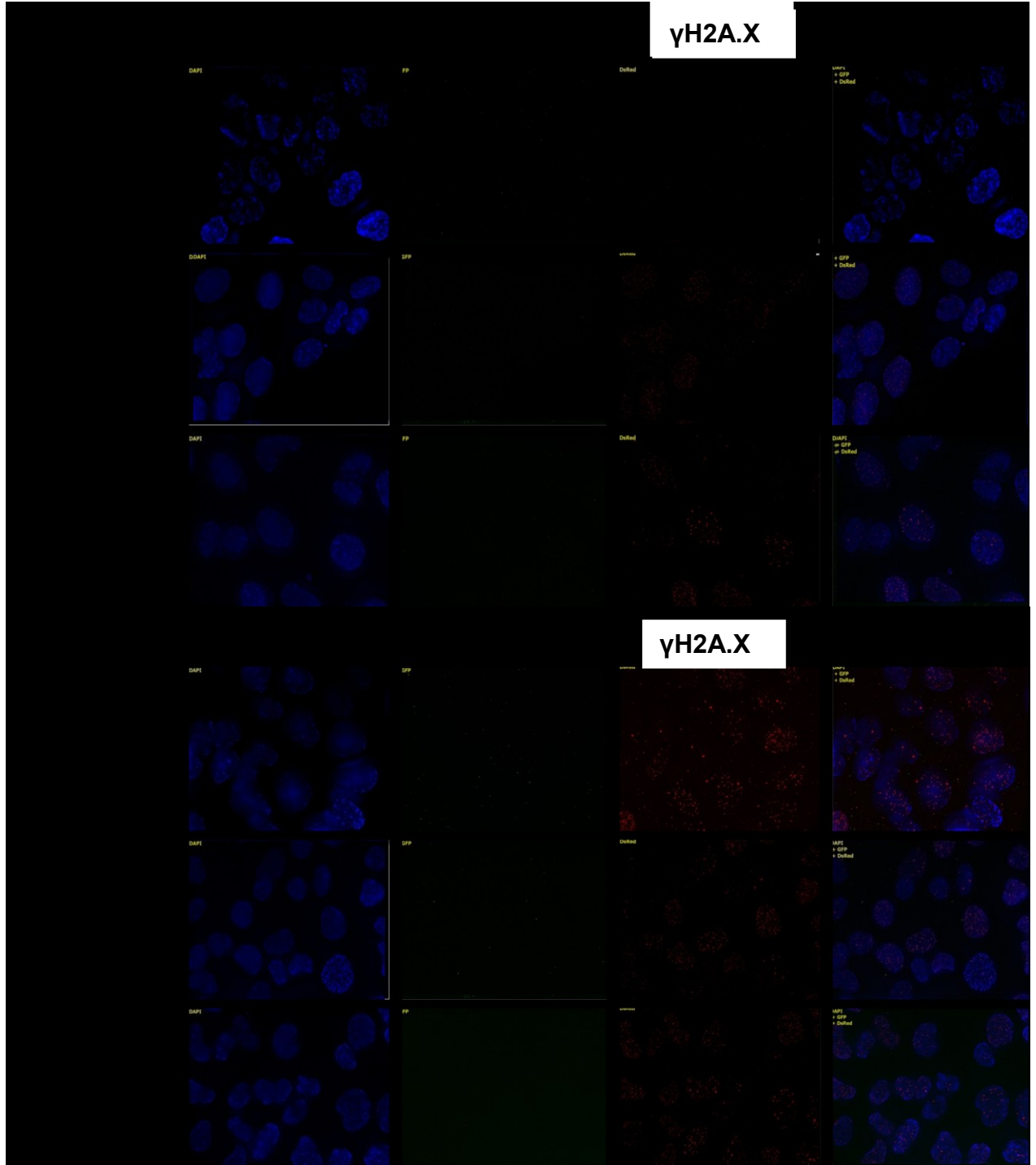


Figure 3.16 ATM shRNA U-2 OS cells treated with sodium selenite. A) Probed for pDNA-PK_{cs} S2056, pDNA-PK_{cs} T2647 and γ H2A.X. There is a DNA damage response, as evidenced by H2A phosphorylation, however with these doses of selenium, there is no phosphorylation of DNA-PK_{cs} at either foci.

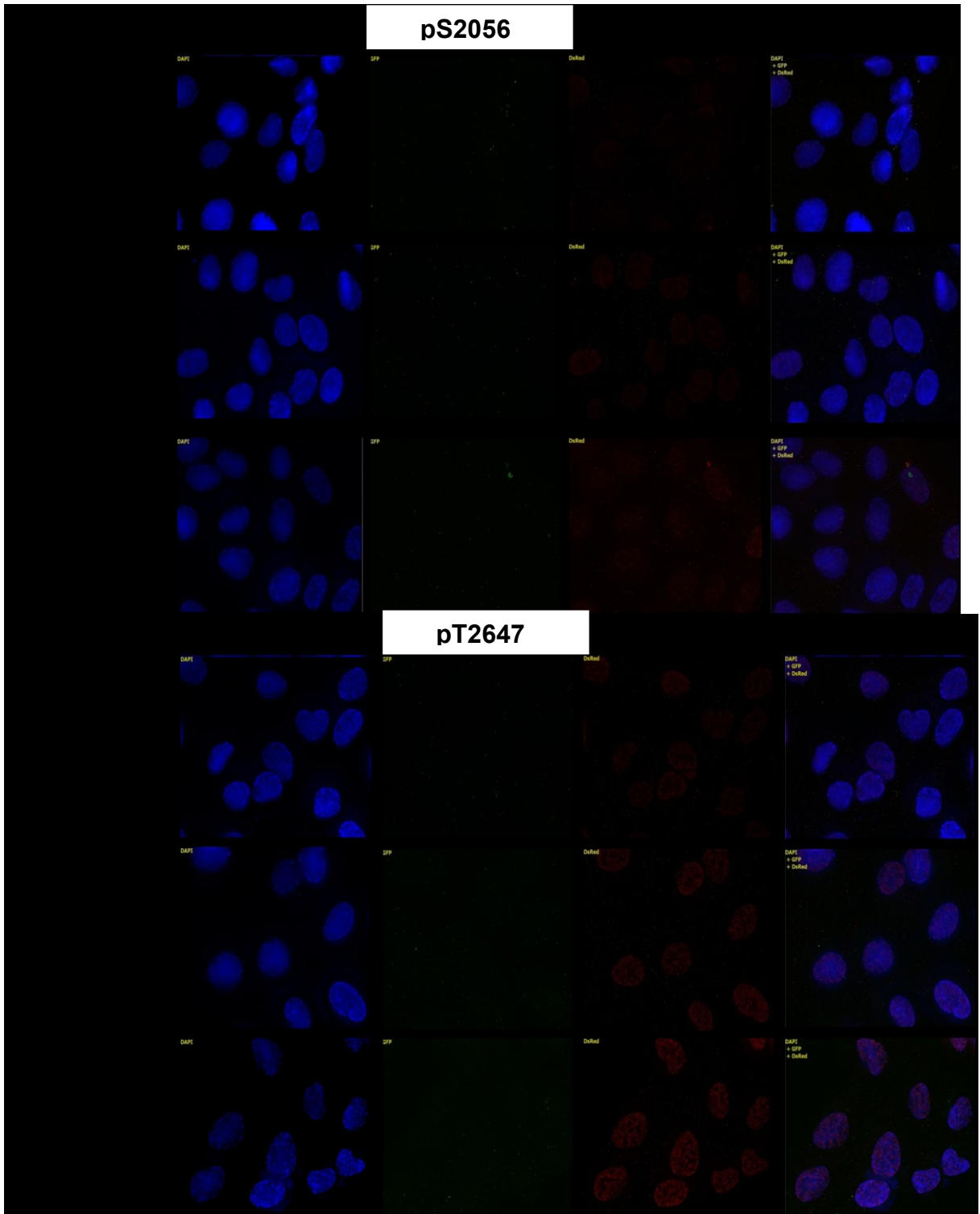


Figure 3.16 B) ATM shRNA U-2 OS treated with selenium then probed for pDNA-PK_{cs} S2056, pDNA-PK_{cs} T2647 and total DNA-PK_{cs}. The total level of DNA is consistent throughout the samples; however there is no phosphorylation of DNA-PK_{cs} at either foci.

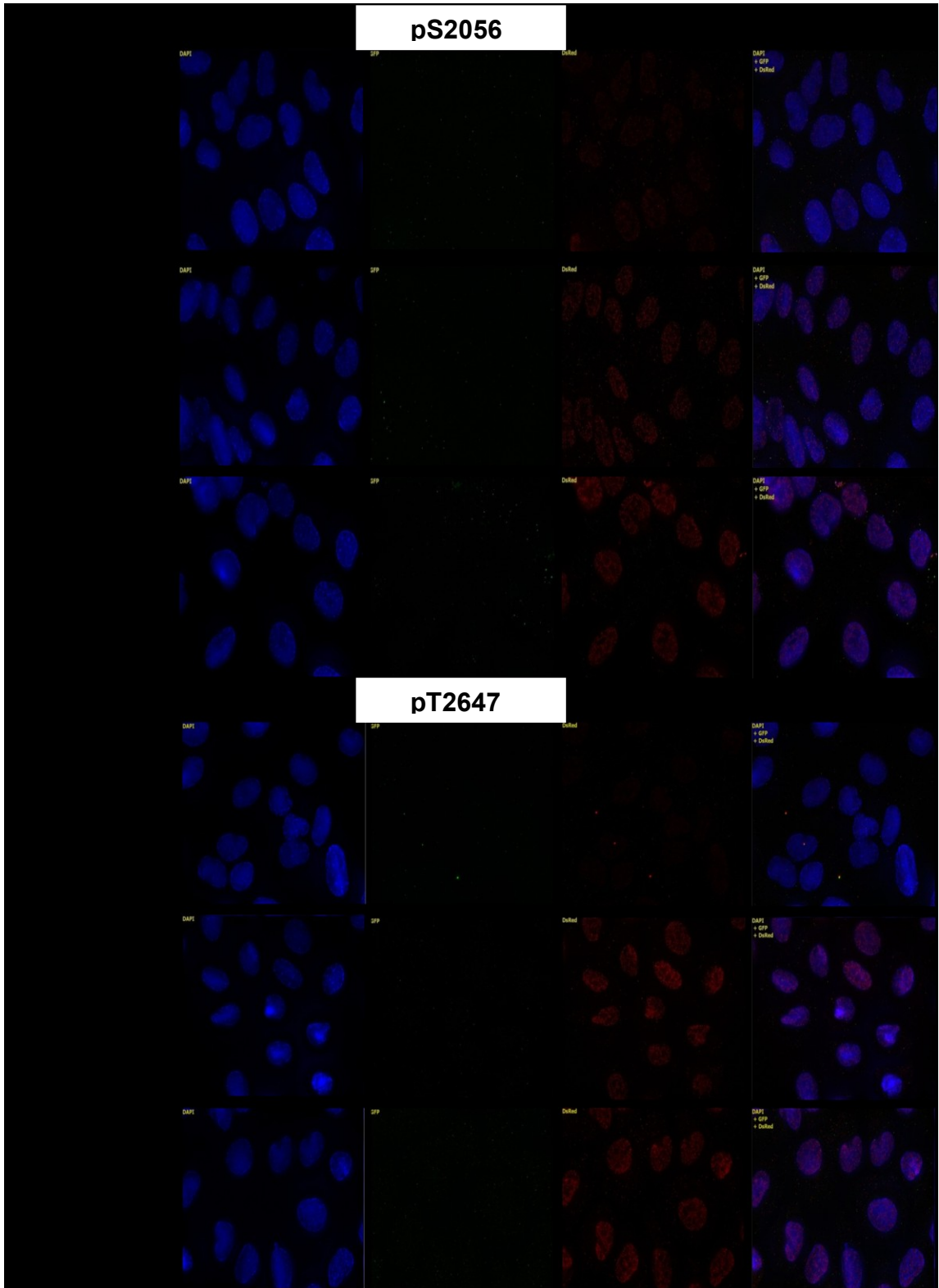


Figure 3.16 C) ATM shRNA U-2 OS cells treated with NCS then probed for pDNA-PK_{cs} S2056, pDNA-PK_{cs} T2647 and total DNA-PK_{cs}. There is DNA-PK_{cs} is presenting in each sample, although NCS cannot induce significant phosphorylation of DNA-PK_{cs} at either foci, unlike its effect on normal MRC-5 cells.

Unlike normal MRC-5 cells, there is no selenium-induced phosphorylation of DNA-PK_{cs} at serine 2056 or threonine 2647 in cancer cells with ATM knocked down. γ H2A.X signal is consistent in each treatment; it is not dependent on selenium treatment. Also, γ H2A.X signal is present even with ATM knocked down, showing that another protein must share the responsibility of phosphorylating H2A.

Unlike normal cells, there is no DNA-PK_{cs} phosphorylation of DNA-PK_{cs} at either serine 2056 or threonine 2647 under any treatment condition or in the control.

The NCS expression in shRNA U-2 OS is faint; however the response may increase if the concentration of NCS was increased. For cancer cells, the dose is most likely higher because normal cells are more vulnerable to chemical treatment. The total level of DNA-PK_{cs} expressed the same in every condition, with each cell having similar amounts expressed. Like ATM shRNA U-2 OS cells treated with selenium, there is no induction of phosphorylation at serine 2056 or threonine 2647 of DNA-PK_{cs}. Also total levels of DNA-PK_{cs} are the same in each treatment, with each cell expressing similarly high levels of DNA-PK_{cs}.

3.3.3 U2- OS cells

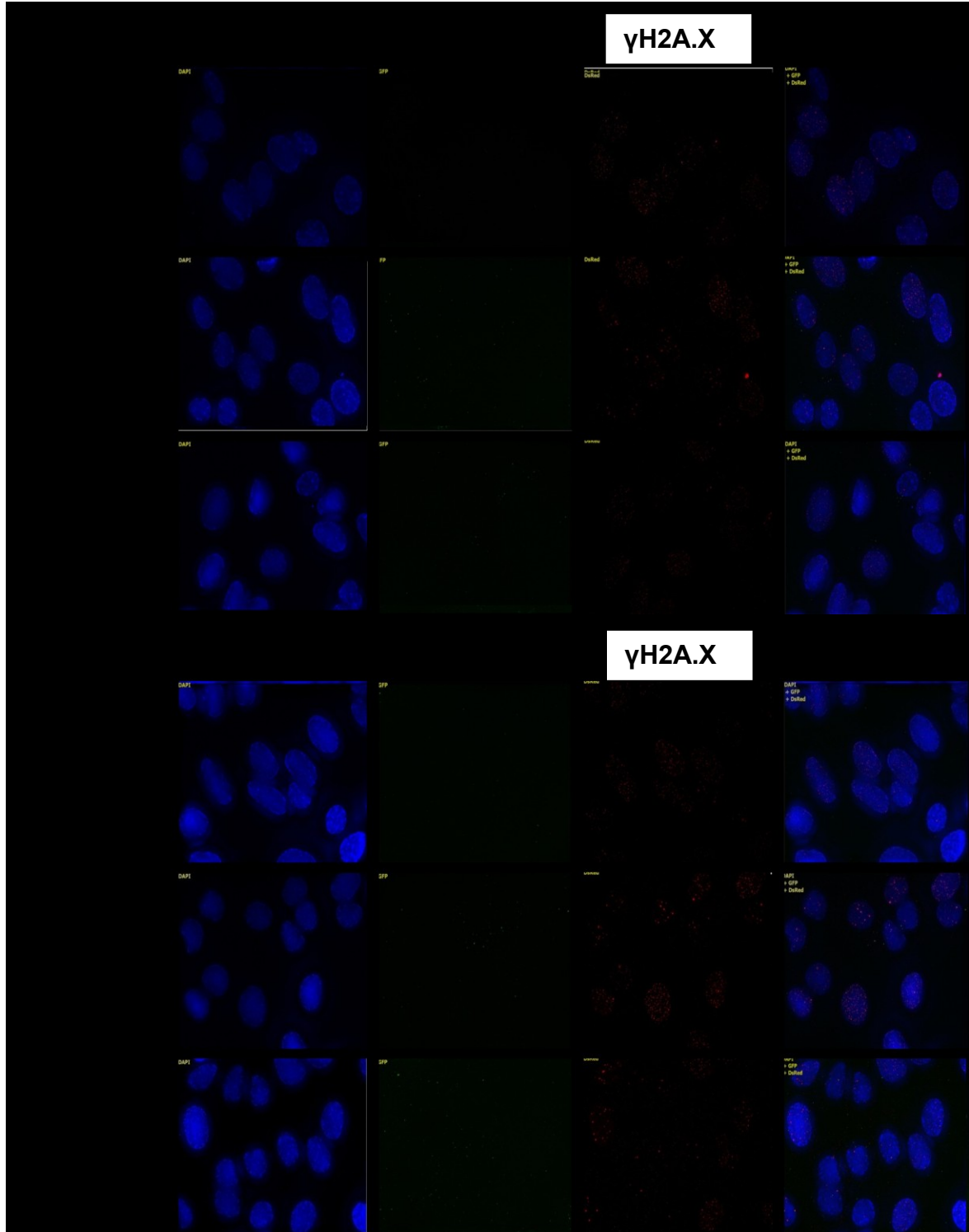


Figure 3.17 shRNA U-2 OS control cells treated with sodium selenite. A) After selenite treatment for 24 hours, the cells were probed for pDNA-PK_{cs} S2056, pDNA-PK_{cs} T2647 and γ H2A.X. The levels of phosphorylated H2A are less than U-2 OS cells with ATM knocked down.

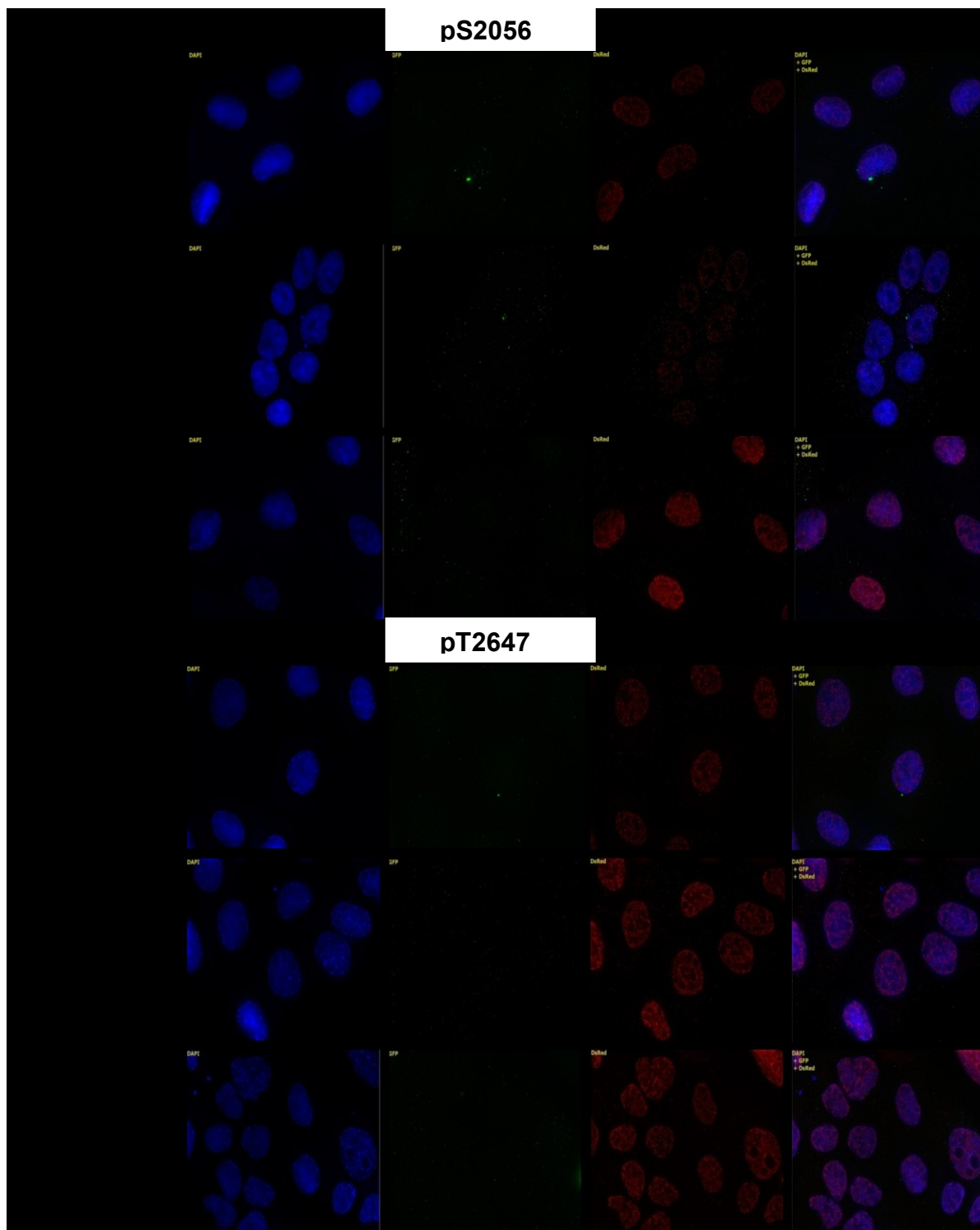


Figure 3.17 B) shRNA U-2 OS control cells treated with selenium then probed for pDNA-PK_{cs} S2056, pDNA-PK_{cs} T2647 and total DNA-PK_{cs}. The total levels of DNA-PK_{cs} are similar throughout the experiments.

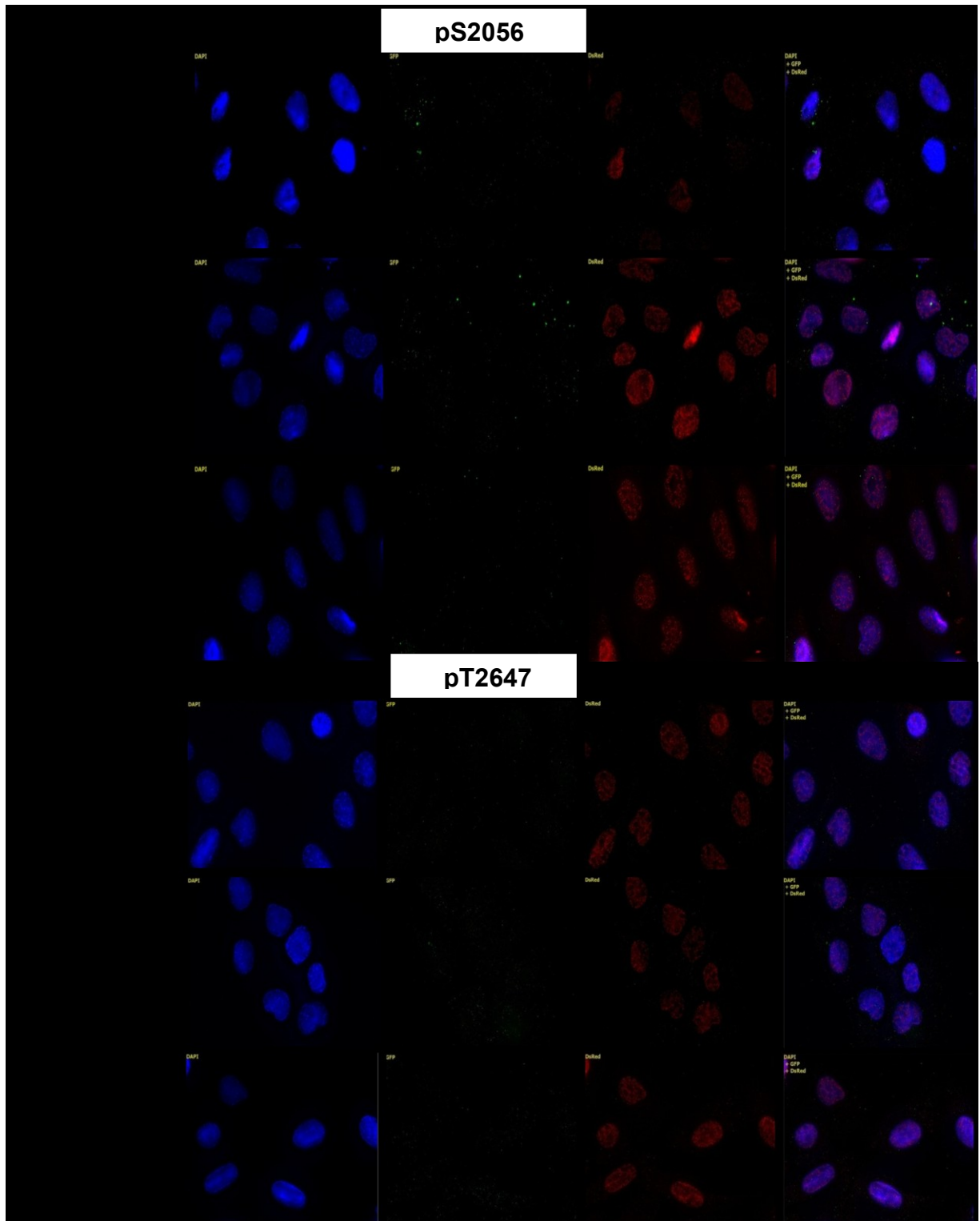


Figure 3.17 C) shRNA U-2 OS control cells treated with NCS then probed for pDNA-PK_{cs} S2056, pDNA-PK_{cs} T2647 and total DNA-PK_{cs}. With ATM present, there is an enhanced response to NCS treatment; illustrating the phosphorylation of DNA-PK_{cs} at both foci following NCS response is dependent on proper function of ATM.

Similar to ATM shRNA U-2 OS cells, there is very little selenium-induced phosphorylation of DNA-PK_{cs} at serine 2056 or threonine 2647. γ H2A.X signal is consistent with the pattern of ATM shRNA U-2 OS cells. Unlike the ATM shRNA U-2 OS cells, the control U-2 OS cells treated with selenium show very faint selenium-induced phosphorylation response; however this response is not significant or noticeable when compared to the normal MRC-5 cells. With ATM intact in the U-2 OS cells, there is a small dose dependent selenium-induced phosphorylation event at the two DNA-PK_{cs} phosphorylation sites.

The levels of total DNA-PK_{cs} in the control cells are similar to the levels in the ATM shRNA cells, and are independent of selenium treatment. Total DNA-PK_{cs} is the same in each treatment, regardless of NCS dose. Unlike the U-2 OS cells with ATM expression knocked down, serine 2056 and threonine 2647 of DNA-PK_{cs} in U-2 OS control cells has a more robust phosphorylation response to a dose dependent NCS treatment. Both cancer cell lines tolerate NCS treatment better than the normal MRC-5 cell line, and the U-2 OS control cells have phosphorylation of DNA-PK_{cs}, following NCS treatment although both cell lines have equal DNA PK_{cs} expression.

Chapter 4: Discussion

4.1 Conclusions

4.1.1 The protein components of the NHEJ pathway are necessary for a selenium-induced DNA Damage Response

The NHEJ is the predominant pathway that repairs potentially oncogenic DNA DSBs, although it is error prone (Mao et al., 2008). We showed here, through a knockout study, that competent Ku70, Ku80 and DNA-PK_{cs} proteins are necessary in mouse embryonic fibroblasts in order to generate a selenium-induced ROS response. Therefore, the proposed role of ROS in mitigating tumorigenesis might be dependent on a unique interaction with the protein components of the NHEJ pathway. The ability to scavenge free radicals is thought to decrease risk of cancer by decreasing free radical damage to tissue. However, here, we give support to an opposite hypothesis; the mild oxidative stress caused by selenium treatment preferentially activates DNA damage response and repair genes in normal cells, but not in cancer cells. Since the protein components of the NHEJ pathway are necessary for selenium-induced ROS production, their modification could be a missing link in selenium chemoprevention.

4.1.2 DNA-PK_{cs} is phosphorylated at T2647 and S2056 in response to DNA Damage

The ATM/ATR dependent phosphorylation site of DNA-PK_{cs}, threonine 2647, and the autocatalytic phosphorylation site of DNA-PK_{cs}, serine 2056, are both susceptible to selenium treatment. Both sites are up-regulated in response to sodium selenite treatment; meaning these cells have higher activity of proteins that are

important in maintaining the integrity of their DNA. An interesting analogy to consider is that the mild, tolerable oxidative stress generated by selenite is like lifting heavy weights. The oxidative stress up-regulates the cell's DNA damage response and the heavy lifting causing small tears in the muscle, both of which, when repaired are beneficial to the organism. In the case of the cell, the DNA has increased protection and immediate repair, and in the case of the muscle it heals by laying down extra muscle fiber which ultimately increases the fitness of the individual.

4.1.3 DNA-PK_{cs} phosphorylation is attenuated in the S-phase in MRC-5 cells

When normal MRC-5 cells are arrested in the S-phase using aphidicolin treatment, there is less phosphorylation of either residue of DNA-PK_{cs} compared to when the cells are in a mixed population. The other type of DNA DSB repair is homologous recombination which can take place when the cell has a homologous chromosome available as a template. The homologous template, gotten from a sister chromatid, is only available after the cell has replicated (Reference Figure 4.1). Therefore, our results are congruent with other's results that NHEJ is least active in S-phase, where primarily homologous recombination is used. Homologous recombination is less error prone because the damaged DNA invades its sister chromatid at the site of the DSB and forms a holiday junction, ensuring the correct sequence is replicated. This is in stark contrast to the NHEJ pathway because there is only minimal end-processing followed by a direct ligation of the DSB at the site of the lesion.

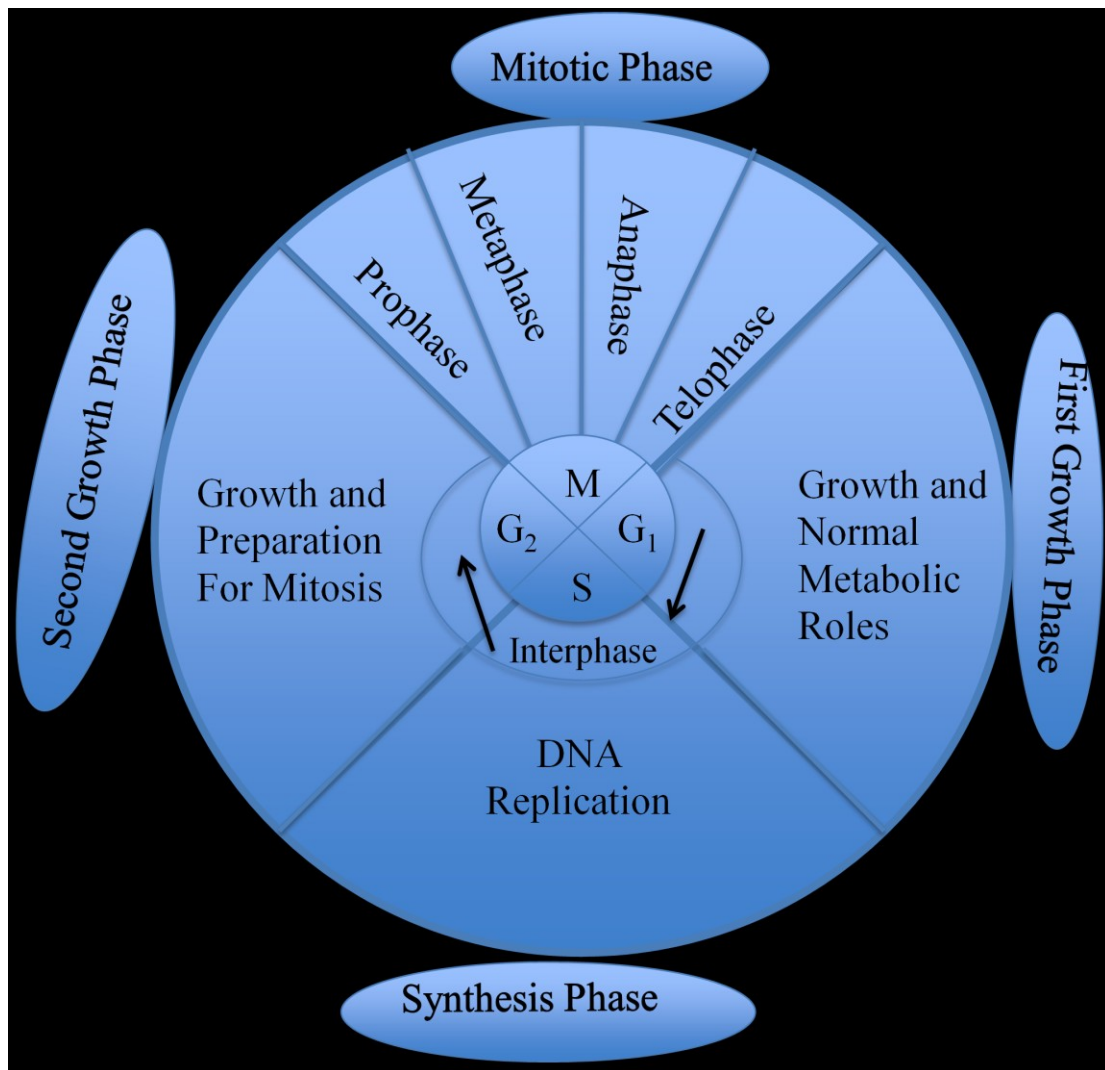


Figure 4.1 The Cell Cycle The cell cycle is important when studying DNA damage response because the activation of some DNA damage repair proteins are specific to cell phase. Selenium, the focus of this thesis work, has been shown to arrest cells in the G₁ phase (Zeng et al., 2009), which is a proposed mechanism of how selenium could reduce tumor cell invasion in other tissues. Also, some following experiments use chemicals that can interfere with cell cycle progression, such as aphidicolin, which inhibits DNA polymerase, arresting the cells in the synthesis phase.

4.1.4 DNA-PK_{cs} phosphorylation decreases in response to ATM inhibition in MRC-5 cells

ATM enzymatic inhibition down regulated DNA-PK_{cs} phosphorylation. Therefore, ATM activation and kinase activity is an upstream event and necessary for proper phosphorylation of DNA-PK_{cs} at threonine 2647 and at serine 2056. Even though serine 2056 is an autocatalytic site, where DNA-PK_{cs} itself phosphorylates the site, it still must require proper upstream signaling from ATM. When ATM is inhibited there is less phosphorylation than when ATM is not chemically inhibited. Furthermore, selenium is able to modify this phosphorylation event when ATM has kinase ability and when ATM does not have kinase ability. When ATM is fully functioning as a kinase, the phosphorylation of both sites on DNA-PK_{cs} is increased, while when ATM is enzymatically inhibited, the increase phosphorylation is not significant or consistent.

4.1.5 DNA-PK_{cs} inhibition increases T2647 phosphorylation and decreases S2056 phosphorylation after selenium treatment

When normal MRC-5 cells are treated with NU 7026, a chemical DNA-PK_{cs} inhibitor, the kinase ability of DNA-PK_{cs} is comprised. This is what we expected because the function of NU 7026 is to inhibit the kinase activity of DNA-PK_{cs} and serine 2056 is an autocatalytic phosphorylation site. Thus, DNA-PK_{cs} still can be phosphorylated at other residues by other kinases, such as why we see an increase in phosphorylation of threonine 2647 on DNA-PK_{cs}. T2647 is independent of DNA-PK_{cs} kinase activity and only needs proper function of ATM and/or ATR in order to be phosphorylated properly. Also, these results show DNA-PK_{cs} is downstream of ATM; with DNA-PK_{cs} kinase ability reduced, ATM activity is similar to control

conditions, when no kinase inhibitors are used. Therefore, DNA-PK_{cs} activation is downstream of ATM activation in the DNA damage response induced by selenium.

4.1.6 Selenium does not induce a DNA Damage Response in either cancer cell line

Selenium can preferentially target normal cells because they might be more sensitive to lower doses of chemicals. Therefore, in the case of the selenium, normal cells may have increased sensitivity to ROS signaling generated by supranutritional doses of selenium. In the two cancer cell lines used in this experiment, neither cell line exhibited significant DNA-PK_{cs} phosphorylation with any combination of chemicals (Figures 3.16 and 3.17). It is possible that we were unable to target the specific pathway we were in normal cells because cancer cells are able to replicate when they have DNA damage. Cancer cells can bypass checkpoints because of mutations in their DNA damage repair genes. This conclusion would support our hypothesis because if selenium can target and up-regulate DNA damage repair it would be unable to do so in cancer cells harboring mutations in their DNA damage repair genes.

4.2 Possible Weaknesses

One potential weakness of this experiment would be the specificity of the enzymatic inhibitors. Although these same inhibitors are used in high impact journals, their chemical structure and targets are somewhat analogous. Due to the similarity in structure and function of DNA-PK_{cs} and ATM, it could be that our inhibitors have cross-effects. There might be partial kinase inhibition of the kinase

the drug was not designed for; the chemical structure is similar, only differing by KU 55933, the ATM inhibitor, having added sulfur groups. A future experiment to confirm the efficacy of the inhibitors would involve a knock-down or a double negative.

Another possible weakness of this study involves the dosages and types of selenium used in all experiments, and the limitations involved in doing *in vitro* tissue culture work. Ideally, this study would test a greater variety of time points, types of selenium, doses of selenium, and methods of quantification. Also, the conclusions drawn from this work are based solely on *in vitro* experiments; which, when studying human nutrition, is not ideal. However, these experiments provide the first evidence of the cross-talk between two kinases of the PIKK protein family in the cellular response to selenium compounds.

4.3 Perspectives

Here we have identified a novel role of selenium as an inducer of the DNA-PK_{cs} pathway. Selenite can induce a DNA damage response, which is a known tumorigenesis barrier (Bartkova, Horejsi et al. 2005), in normal MRC-5 cells, but not in cancerous U-2 OS cells. Specifically, selenium can induce phosphorylation of serine 2056 and threonine 2647 of DNA-Pk_{cs} at sites of DNA breaks. This is significant because activation of these sites is important for signaling pathways after formation of double-stranded DNA breaks in normal MRC-5 cells, but not in cancerous U-2 OS cells.

We put forward that the mechanism by which selenium enhances the DNA damage response in normal MRC-5 cells, but not in cancerous U-2 OS cells is a redox response, presumably by the internal generation of ROS by selenium's innate metabolism at a supranutritional dose. Selenium has been widely studied for its antioxidant function, as a scavenger of ROS at the nutritional dose, but here we illustrate selenium could act as a generator of ROS at a supranutritional dose. This generation of ROS, we propose, is a signal for precancerous cells to enhance their DNA damage response, while cancer cells are immune to this signaling. It would be interesting to further this experiment and elucidate why normal cells are affected by the selenium-induced mild oxidative stress, while cancer cells can escape the signaling pathways generated by the mild induction of oxidative stress.

In conclusion, the results presented here show selenium may counter-act tumorigenesis through DNA damage response, specifically by a mechanism involving ATM-dependent DNA-PK_{cs} phosphorylation. Taken together, our results provide the first evidence of ATM and DNA-PK_{cs} cross-talk as an early tumorigenesis barrier in response to selenium exposure in noncancerous cells, and places ATM upstream of DNA-PK_{cs} in the selenium-induced DNA damage response process.

Appendices

Appendix I: Chemicals and reagents

10% SDS	Teknova S0184
10XTBS	Bio-Rad 170-6435
10XTBS	Bio-Rad 170-6435
Aphidicolin	Calbiochem 178273
DMEM, 1X	Cellgro 10-017-CV
DMSO	Sigma D5897
Ethanol (absolute)	Merck 100986
Fetal Bovine Serum	Atlanta S11550
Glycine	Sigma
Hydroxyurea	MP Biomedicals AAAL01120-03
KU 55933	Tocris
MEM	Cellgro
Methaneseleninic acid, 95%	Sigma 28274-57-9
Methanol	Fisher A452-4
Non-Fat Dry Milk	Bio-Rad 170-6404
NP40	Calbiochem 492016
NU 7026	Tocris
Penicillin-Streptomycin	Cellgro 30-002-CI
Ros Dye	Invitrogen
Se-Methyl-selno-L-cysteine	Sigma 26046-90-2
Sodium selenite	Sigma 10102-18-8
Tris-HCl	Quality Biological 351-007-101
Trypsin EDTA, 1X	Cellgro 25-052-C
Tween20	Calbiochem 655204

Appendix II: Buffer, solution and gel

10X PBS	80 g NaCl, 10 g KCl, 72 g Na ₂ HPO ₄ , 12 g KH ₂ PO ₄ , 1 L dd H ₂ O
Cell culture medium	500 mL DMEM medium, 10% Fetal Bovine Serum for DMEM 15% Fetal Bovine Serum for MEM, 5 mL Penicillin-Streptomycin, 5 mL Non essential amino acids, 5 mL essential amino acids, 5 mL MEM vitamins,
TBS-T	100 mL 10XTBS, 900 mL ddH ₂ O, 10 mL 10% Tween20

Appendix III: Commercial kits

BCA™ Protein Assay Kit	Thermo JI124811
Senescence Detection Kit	Qiagen 301107

Appendix IV: Equipments and facilities used

Balance	Denver Instrument S-403
Biological Safety Cabinet	Thermo 109578
CO ₂ incubator	Thermo 3595
FLUOstar OPTIMA	BMG 413-3128
Isotemp Air Bath	Fisher 11-715-1250
Isotemp Water Bath	Fisher 15-462-01
Legen RT centrifuge	Thermo 75004377
Optic Microscope	Motic AE21
Rocker	VWR 12620-906
Roto-Shake Genie	Scientific Industries S1-1100
Vortex-Genie	Scientific Industries 2-401968
Zeiss Z1 Microscope	Zeiss

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