Detection of γH2AX foci in animal tissue

Report

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I. Acknowledgment

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II. Contents

III. List of Abbreviations Used

ATM ..... ataxia telangiectasia mutated

BJ ..... human fibroblast cells

BSA ..... bovine serum albumin

DDR ..... DNA damage response

DNA ..... deoxyribonucleic acid

DNA-PK ..... DNA-dependent proteinkinase

DSB ..... DNA double strand break

Gen ..... genistein

Gy ..... Gray

HCT116 ..... colon carcinoma cells

IR ..... ionizing radiation

mtDNA ..... mitochondrial DNA

PBS ..... phosphate buffered saline

PBS-TT ..... PBS containing Triton X and Tween20

PI ..... propidium iodide

PI3K ..... phosphatidylinositol-3-OH-kinase-like family

ROS ..... reactive oxygen species

RT ..... room temperature

Topo1mt ..... mitochondrial topoisomerase I

VP16 ..... etoposide

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1. Abstract

H2AX is a histone which becomes rapidly phosphorylated after DNA double strand breakage. Just one DNA double strand break (DSB) leads to hundreds of copies of phosphorylated H2AX (γH2AX), which form foci covering many megabases of chromatin and encompassing the DSB location. Subsequently, DNA damage response proteins are recruited which in turn activate DNA repair processes. Thus, H2AX represents a key factor in the repair process of damaged DNA.

Measurement of γH2AX foci by immunofluorescene has become a popular method for detecting DSBs as the foci are easy to identify with antibodies and H2AX phosphorylation is a highly sensitive marker of DSB formation. Therefore, the first goal of this study was to learn the methodical skills to detect this important biomarker for DNA damage.

In a first approach, various cell lines were compared regarding their sensitivity toward ionizing radiation. Based on former findings that the soy constituent genistein acts as topoisomerase II poison, we investigated now, whether this compound is also able to enhance the number of γH2AX foci in cancer cells. We show that genistein causes a concentration-dependent increase in γH2AX foci after one hour of incubation in colon carcinoma cells.

In the second part of this study, we concentrated on detecting γH2AX foci in animal tissue. For this purpose, tissues of mice without or after irradiation were processed and stained for immunofluorescence. In a next step, tissues of Topo1mt deficient and wildtype mice were investigated regarding their basic level of γH2AX. There are more γH2AX foci in the crypts of the intestine of mice deficient for Topo1mt compared to wildtype mice, while in the testes of mice missing Topo1mt no difference in γH2AX foci formation could be detected. Yet, there are further studies needed to evaluate these preliminary results and to investigate whether knockout of Topo1mt might cause different effects in various tissues and if these results are dependent on other parameters like age or sex.

2. Introduction

DNA double strand breaks (DSBs) are serious lesions that can initiate genomic instability, ultimately leading to cancer (McKinnon and Caldecott, 2007). Thus, DNA integrity is closely monitored by processes that detect and repair those DSBs. A key component in DNA repair is the histone H2AX, which was first reported in 1980 by West and Bonner. H2AX is a member of the histone H2A family, one of the five families of histones that package and organize eukaryotic DNA into chromatin (Figure 1) (Bonner et al., 2008).

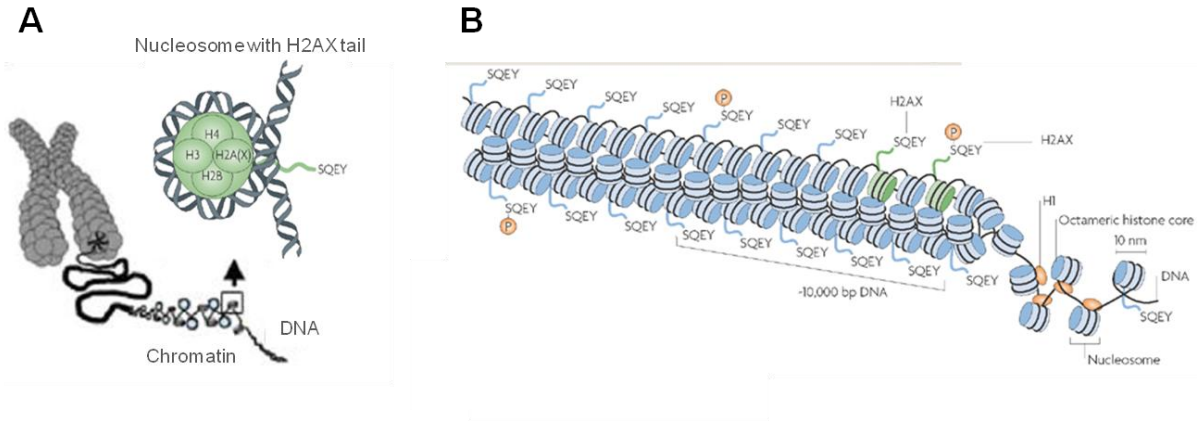

Figure 1: Organization of DNA and γH2AX foci. **(A)** H2AX is a component of the octomer of histones packaging DNA into a nucleosome, while many nucleosomes form the chromatin. The tail of histone H2AX, the SQEY motif, is strictly evolutionarily conserved and occupies the serine residue where H2AX becomes phosphorylated to form γH2AX (Dickey et al., 2009). **(B)** The nucleosomes form a fibre containing H2AX molecules in every fifth nucleosome on average in mammals. Approximately 10% of the H2AX molecules are phosphorylated at any one time in a focus (Bonner et al., 2008).

In response to DNA damage, H2AX is rapidly phosphorylated on serine 139 by PI3K-like kinases such as ataxia telangiectasia mutated (ATM), ATM-Rad3-related (ATR) and DNA-dependent protein kinase (DNA-PK) (Sedelnikova et al., 2003). About 30 minutes after DSBs occur, large numbers of γH2AX molecules accumulate to foci encompassing many megabases of chromatin adjacent to DSB sites (Dickey et al., 2009). It is suggested that H2AX molecules in a small region near the DSB site are phosphorylated first, and are followed by molecules at increasing distances from the break site. Many DNA repair and checkpoint proteins accumulate on the growing γH2AX focus, which may serve to open up the chromatin structure and form a

platform for the accumulation of DNA damage response (DDR) and repair factors (Bonner et al., 2008). These proteins include 53BP1, MDC1, RAD51, BRCA1, and the MRE11/RAD50/NBS1 complex. 53BP1 is a central transducer of the DNA damage signal to p53 and other tumor suppressor proteins and plays an important role in the maintenance of genomic stability (Wang et al., 2002). γH2AX focus formation also results in the recruitment of proteins of the ubiquitin ligase cascade (RNF8-RNF168-UBC13) which in turn allows the accumulation of the BRCA1-BARD1 complex and 53BP1 to the DNA lesion site (van Attikum and Gasser, 2009; Dickey et al., 2009). Phosphorylation of H2AX and recruitment of DNA repair proteins are illustrated in Figure 2.

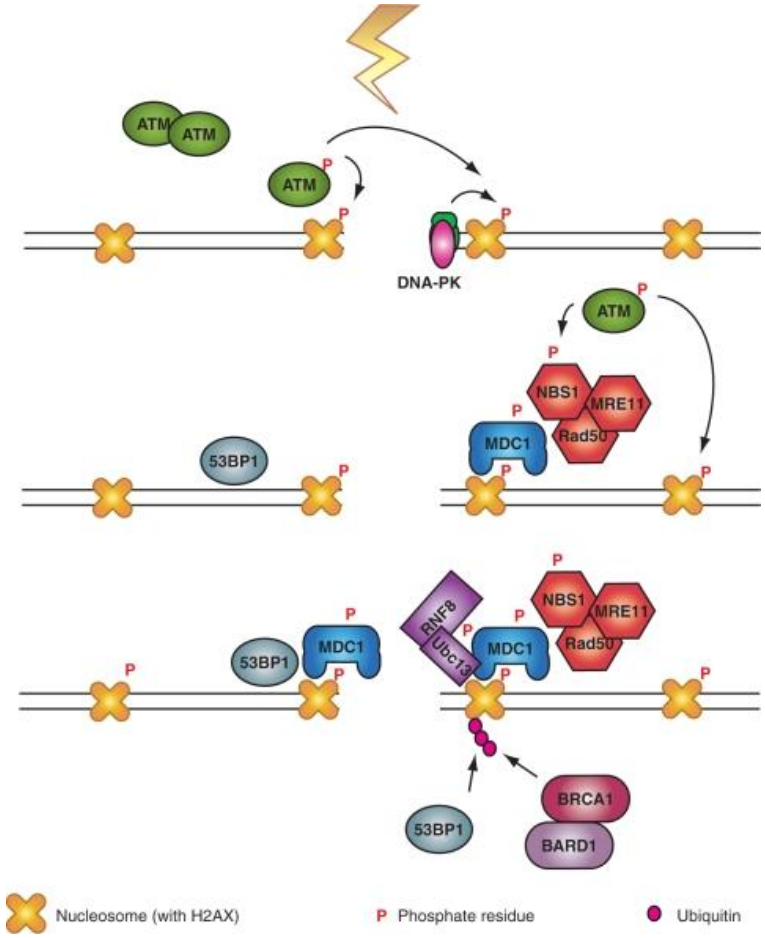

Figure 2: γH2AX plays a key role in DNA damage repair signaling. After initial phosphorylation of H2AX by kinases as ATM or DNA-PK, DNA repair proteins are recruited which further activate ATM. This generates a positive feedback loop that leads to further phosphorylation of H2AX and the chromatin modifications which allow 53BP1 to bind. The activation cascade culminates with the recruitment of RNF8 to phosphorylated MDC1 and the polyubiquitinylation of H2AX to recruit BRCA1/BARD1 (Kinner et al., 2008).

The amplification of γH2AX to foci makes it possible to detect individual DSBs with an antibody to γH2AX (Kinders et al., 2010). It has been demonstrated that one γH2AX focus is equivalent to one DSB (Bonner et al., 2008).

DSBs can be caused by a variety of factors. One possibility to induce DNA damage is by interfering with the actions of topoisomerases on DNA (Pommier, 2006). Topoisomerases are crucial enzymes that control and adjust the topologic states of DNA and therefore, they are involved in all fundamental DNA processes (McClendon and Osheroff, 2007). As these enzymes generate transient DNA strand breaks during their catalytic cycle, they have potential to fragment the genome every time they function. Usually the formation of enzyme-DNA cleavage complexes is tightly regulated. If the level of cleavage complexes falls too low, cells are unable to undergo chromosome segregation and ultimately die of mitotic failure (Wilstermann and Osheroff, 2003). If the level of cleavage complexes becomes too high, the actions of DNA replication or transcription machinery can convert these transient complexes to permanent DNA strand breaks. The resulting strand breaks, as well as the inhibition of essential DNA processes, initiate multiple repair pathways and may cause genomic instability. If the DNA strand breaks overwhelm the cell, cells may go into apoptosis. Although, topoisomerases are essential enzymes, they bear a genotoxic potential (McClendon and Osheroff et al., 2007). This makes these enzymes a meaningful therapeutic target of various natural and synthetic compounds and many of the most widely used anticancer drugs act by stabilizing topoisomerase II-DNA complexes (Fortune and Osheroff, 2000). These compounds convert the essential enzyme into a cellular poison by stabilizing the covalent complex of topoisomerase linked to DNA. Thus, permanent DNA strand breaks occur when replication or transcription complexes collide with the covalently attached enzyme (Wilstermann and Osheroff, 2003). Therefore, it seems to be inevitable to monitor DNA damaging properties when investigating topoisomerase poisons. We showed previously, that the soy constituent genistein poisons topoisomerase II *in vitro* and *in vivo* (Kalfalah et al., 2011; Baechler et al., *in preparation*). Thus, the first aim of this study was to learn the methodical skills of detecting γH2AX foci to investigate if genistein causes DSBs in colon cancer cells.

Although most of a cell´s DNA is found in its nucleus, there is another cell organell containing DNA. Mitochondria, known as cellular power plants, enclose around 5–10

copies of covalently closed duplex DNA (mtDNA). Because mtDNA molecules are closed circular and therefore topologically constrained, there must be a mechanism to relieve the torsional stress that arises during replication processes and it seems likely that topoisomerases may be involved. In sum, there are three topoisomerases found in mitochondria, but only mitochondrial DNA topoisomerase I (Topo1mt) is encoded by a specific gene for mitochondria (Zhang et al., 2001). So far, little is known about the function of this enzyme, but as it is present in all vertebrates, Topo1mt might have a role in regulating mtDNA topology (dalla Rosa et al., 2009; Douarre et al., 2012).

To investigate the function of Topo1mt several studies in the group of Y. Pommier have been performed after silencing of the Topo1mt gene. It has been shown that the lack of Topo1mt leads to enhanced levels of reactive oxygen species (ROS) within cells and as a consequence DDR pathways mediated by ATM and γH2AX are activated (Douarre et al., 2012). Thus, the level of H2AX phosphorylation is increased after inactivation of Topo1mt. Moreover, in the intestine of Topo1mt deficient mice the number of γH2AX foci was elevated compared to the tissue of wildtype mice (Douarre et al., 2012). So the question arises if there is a difference in phosphorylation of H2AX in various tissues between Topo1mt knockout mice compared to wildtype mice. The second aim of this study was to investigate the γH2AX level in tissues as intestine and testes of Topo1mt deficient mice versus wildtype mice.

3. Methods

3.1. Preparation of cell cultures for immunostaining

Cells are seeded on 4-well slides at 50 000 cells/well and are allowed to grow overnight in a tissue culture incubator (37°C, 5% CO_2). After cell treatment, cells are washed with PBS and fixed for 20 min with 2% paraformaldehyde in PBS (Electron Microscopy Sciences, Hatfield, PA). 70% ethanol chilled to -20°C is added for 5 min to permeabilize the cells. Samples may be stored at 4°C in 70% ethanol (Nakamura et al., 2006).

3.2. Preparation of frozen sections from animal tissue

After removal, the tissue is put in a plastic cryomold (Tissue-Tex disposable vinyl specimen molds, Miles, Inc. Elkhart, IN) and filled with OCT compound embedding medium (Miles, Inc Diagnostic Division, Elkhart, IN) until the whole tissue is covered and immediately stored on dry ice. Afterwards 8 µm thick sections are made of the OCT blocks using a cryostat. Slides were washed for 15 min with PBS to remove the OCT and afterwards immersed for 20 min in 2% paraformaldehyde in PBS to fix the cells. To permeabilize the cells, the samples are put in 70% ethanol at -20°C and may be stored at 4°C for several days (Nakamura et al., 2006).

3.3. Immunofluorescence staining

When slides were stored in 70% ethanol, samples are washed with PBS for 15 min. Afterwards, blocking solution (PBS-TT (PBS containing 0.5% Tween20 and 0.1% Triton-X100) with 5% BSA) is added for 30 min at room temperature (RT). Samples are rinsed with PBS for 5 min and incubated with primary antibody (anti-γH2AX, 1 : 500 dilution in PBS-TT containing 1% BSA) for 2 hours at RT. Slides are washed twice for 5 min with PBS and incubated for another hour at RT with the secondary antibody conjugated with Alexa Fluor 488 (1/500 dilution in PBS-TT containing 1% BSA). Samples are rinsed twice with PBS for 5 min and slides are mounted with Vectashield mounting medium with propidium iodide to counterstain the DNA (Vector Laboratories, Burlingame, CA). Slides were examined using a laser scanning confocal microscope (Zeiss LSM510) and images were collected and processed using the Zeiss AIM software (Nakamura et al., 2006).

4. Results and Discussion

4.1 Immunofluorescence staining of γH2AX *in vitro*

In a first approach, mammalian cells were used to learn the methodical skills of γH2AX immunofluorescence staining. Therefore, cells were exposed to different doses of ionizing radiation (IR), as the most damaging lesion introduced by IR into cells is the DSB. Cells respond rapidly to nascent breaks in order to locate them in the chromatin and repair the damage as quickly and accurately as possible. As a

consequence, γH2AX forms rapidly foci at the DSB sites after IR (Redon et al., 2002). *In vitro* studies showed that γH2AX formation peaks at around 30 min after IR proportionally to the dose, and then declines after several hours (Cucinotta et al., 2008; Rogakou et al., 1999).

Cells of the colon carcinoma cell line HCT116 were exposed to 2 Gy and 20 Gy, respectively and γH2AX formation was investigated after 30 min and compared to the basic level of non-treated cells. Figure 3 shows the results of this approach.

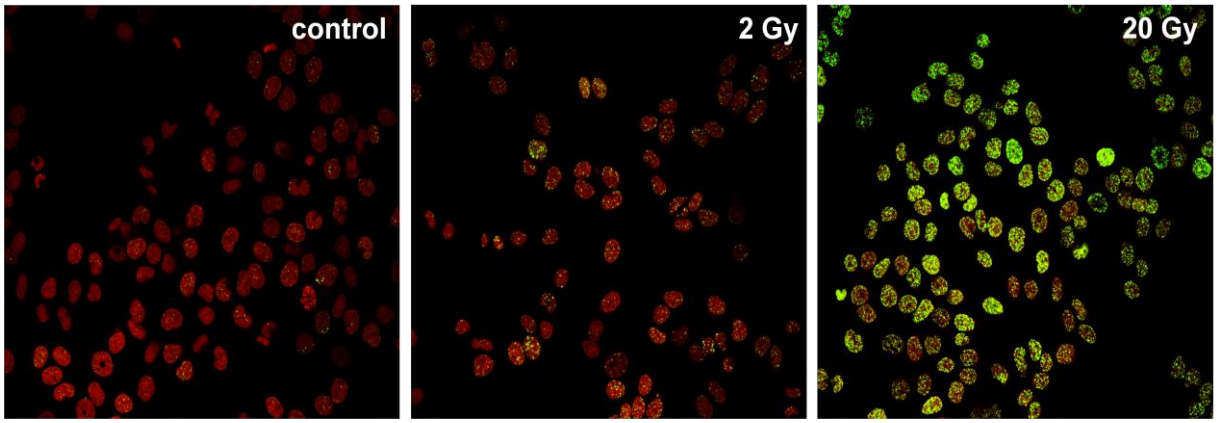

Figure 3: Immunocytochemical detection of γH2AX foci 30 min after exposure to different doses of ionizing radiation in HCT116 (colon carcinoma) cells. Cells were fixed 30 min following irradiation and stained for γH2AX (green), DNA was counterstained with propidium iodide (PI, red).

DNA was stained with propidium iodide (red) and γH2AX foci are marked green. The left panel of Figure 3 shows non-treated cells which exhibit just a few γH2AX foci. With increased ionization dose the number of γH2AX foci increases significantly. Some cells exposed to 20 Gy show even a pan-staining.

Since various cell lines may not have the same base level of γH2AX and may also differ in their sensitivity to IR, γH2AX foci formation was additionally investigated in another cell line. The human fibroblast cell line BJ established from normal human foreskin was chosen to compare a normal cell line to the cancer cell line HCT116. As cells originating from normal human tissue are more sensitive to IR than cancer cells, an exposure of 2 Gy was chosen (Figure 4).

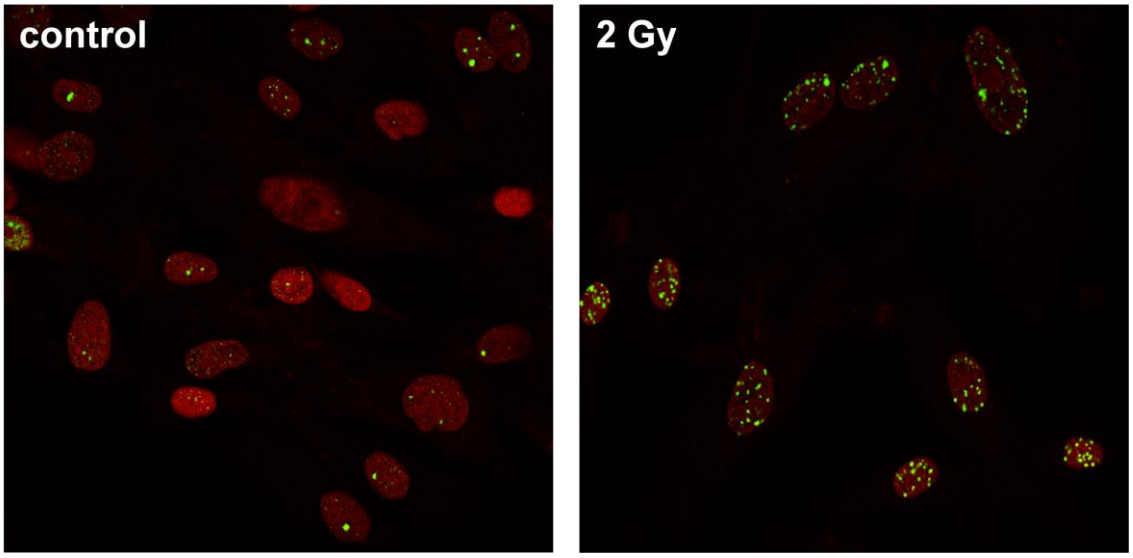

Figure 4: Detection of γH2AX foci (green) by immunofluorescence. BJ (human normal fibroblast) cells were fixed 30 min after exposure to 0 Gy and 2 Gy, respectively. DNA was counterstained with propidium iodide (PI, red).

The left panel of Figure 4 shows control cells, each of them containing 1-2 foci on average, while the panel on the right illustrates cells after exposure to 2 Gy of IR. Irradiated cells display a significant increase in γH2AX foci with more than 10 foci per nucleus compared to control cells. The basic level of γH2AX foci in BJ cells compared to HCT116 cells is lower, which is probably due to the fact that cancer cells have frequent alterations in the H2AX gene location which may cause a 3-fold increase in γH2AX. Moreover, there are often mutations and alterations in cell cycle control in cancer cells, also contributing to a higher γH2AX level (Kuo and Yang, 2008).

In the next approach, colon carcinoma cells were treated with the soy constituent genistein for 1 h in different concentrations. As genistein is known to poison topoisomerase II *in vitro* (Kalfalah et al., 2011), the question arises if this poisoning also leads to DNA damage.

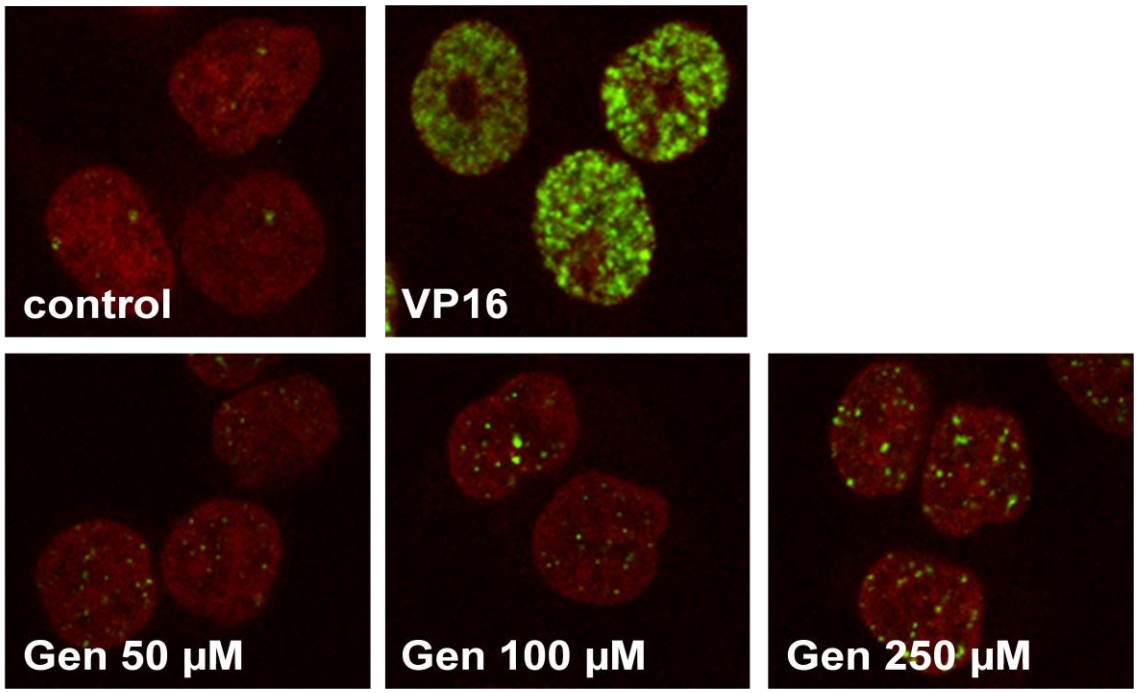

Figure 5: Immunofluorescence staining of γH2AX foci after treatment with the soy constituent genistein (Gen). HCT116 cells were fixed 1 h following drug treatment and stained for γH2AX (green) and DNA was counterstained with propidium iodide (PI, red).

The upper lane of Figure 5 shows the topoisomerase II poison VP16 in comparison to untreated cells after one hour of incubation. As expected, the topoisomerase II poison causes DNA damage and increases the level of γH2AX foci significantly, while just a few foci are detectable in the untreated nuclei. Samples treated with the soy constituent genistein show enhanced γH2AX foci formation compared to the negative control, even in the lowest tested concentration of 50 µM. Altogether, there is a concentration-dependent increase in γH2AX foci after genistein treatment. While in the negative control there are just 1-3 foci, the cells treated with the highest concentration of genistein show in average around 15 foci per nucleus. Thus, the soy consitutent genistein causes DNA damage in the concentration range of 50-250 µM after one hour of exposure.

4.2 γH2AX staining of mouse tissue

In the next approach, γH2AX foci were investigated in mice tissue originating from a B6 strain. To learn the methodical skills, especially the cutting of tissue before

staining, tissues of a mouse were processed which had been irradiated with 2 Gy to induce γH2AX foci. For comparison, tissues of an untreated mouse were processed in the same manner. Figure 6 shows images of the testes of B6 mice.

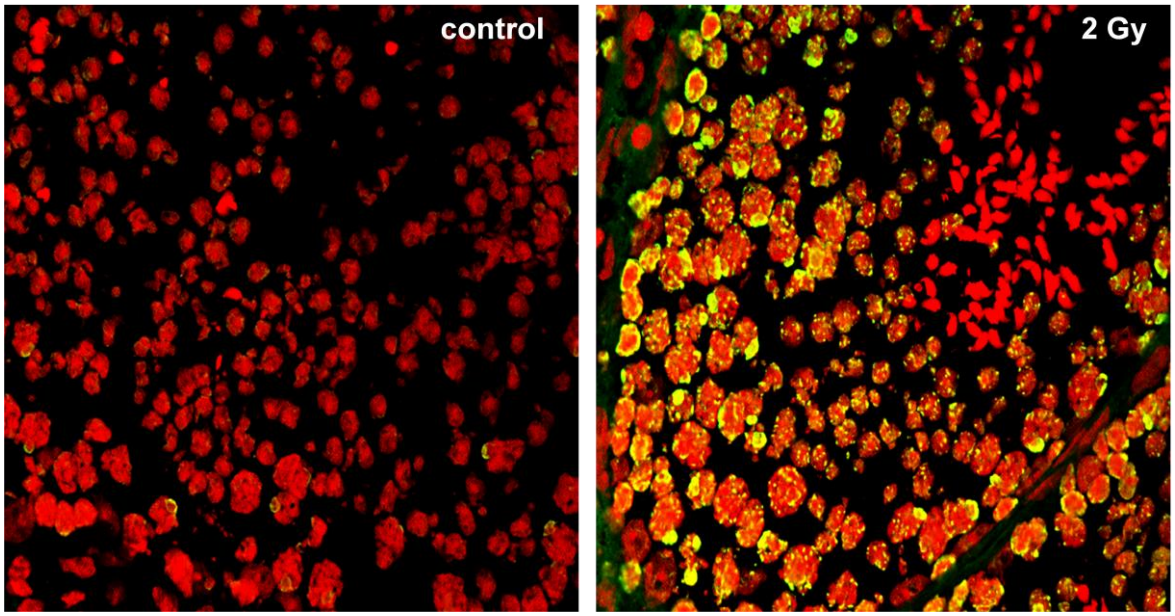

Figure 6: Detection of γH2AX foci in 8 µm frozen sections of the testis from 1 year old mice. The panel on the right shows the testis in a 40x magnification of a non-treated mouse, while the left panel exhibits the testis of a mouse exposed to 2 Gy. The mouse was dissected 30 min after irradiation and the tissue was stained for γH2AX (green). Propidium iodide (PI, red) was used to mark DNA.

Both panels of Figure 6 exhibit a dissection of a seminiferous tubule of testes. The non-treated sample on the left contains just 1-2, or even less, γH2AX foci per nucleus. However, the irradiated sample shows a strong green signal, meaning a lot of γH2AX foci are formed in the spermatocytes, which are surrounding the spermatids located in the center of the tubule. At least 7 foci appear in the spermatocytes of the irradiated tissue, while the sperm in the center does not show any foci. This is consistent with previous findings, that H2AX is rapidly phosphorylated after irradiation of a whole living organism with IR (Rogakou et al., 1999).

As another target tissue for H2AX phosphorylation after IR the intestine was chosen and Figure 7 shows the images taken of this tissue.

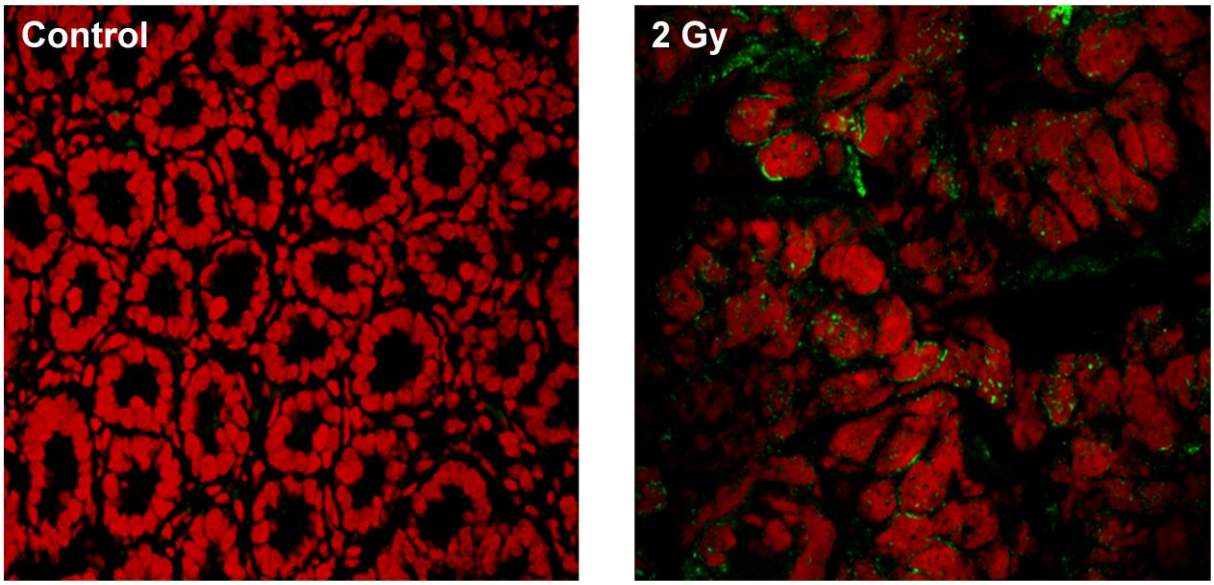

Figure 7: Immunocytochemical detection of γH2AX foci in 8 µm frozen sections of intestine from 1 year old B6 mice without (left panel) and after (right panel) irradiation with 2 Gy. Cells were fixed 30 min following irradiation and stained for γH2AX (green) and DNA was marked with propidium iodide (PI, red).

While the crypts of the intestine of the control mouse show no γH2AX formation, γH2AX foci appear in the crypts of the irradiated mouse. As the left panel show almost no green signal, it can be assumed that only rare DSBs occur in the intestine of B6 mice. In contrary, after irradiation almost every nuclei shows at least one γH2AX focus. This suggests, that γH2AX foci are built in the colon 30 min after irradiation of a B6 mouse. When compared to the testes, the intestine seems not as sensitive to irradiation as more foci are detected in the testes. As also other tissues were investigated after irradiation including liver, brain, heart and spleen, the here represented tissues are among the most sensitive towards irradiation with IR (furter data omited here).

It was shown previously that the lack of Topo1mt increases the level of ROS within cells. As a result, DDR pathways are activated and the level of H2AX phosphorylation is increased (Douarre et al., 2012). These findings were mainly made by investigations *in vitro*, yet in the intestine of Topo1mt deficient mice the number of γH2AX foci was also elevated compared to the tissue of wildtype mice (Douarre et

al., 2012). So the question arises if there is also a difference in H2AX phosphorylation in other tissues than the intestine. Yet, to confirm the results of the former study, the intestine was investigated first. Figure 8 illustrates the results of γH2AX staining in the intestine of B6 mice.

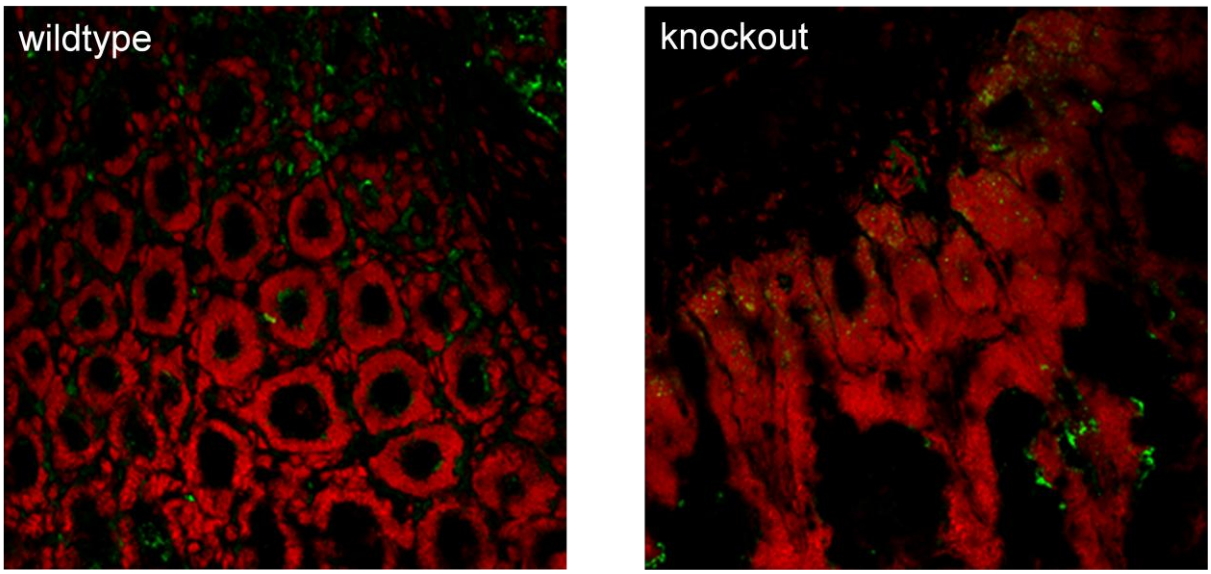

Figure 8: Detection of γH2AX foci in 8 µm thick sections of the intestine of 9 week old B6 mice. The left panel shows the intestine in a 40x magnification of a wildtype mouse, while the left panel exhibits crypts of a Topo1mt knockout mouse. After fixation, cells were stained for γH2AX (green) and DNA was counterstained with propidium iodide (PI, red).

First, the intestine of Topo1mt knockout mice was investigated regarding the basic level of γH2AX foci. The left panel of Figure 8 shows the crypts of the intestine of a wildtype B6 mouse in a 40x magnification. Only a few γH2AX foci are visible in the control intestine, while the crypts of the Topo1mt deficient mouse in the right panel show several γH2AX foci. This is in line with former results, as Topo1mt deficient mice have enhanced ROS level which are suggested to lead to activation of DDR pathways and therefore, to the activation of γH2AX (Douarre et al., 2012).

As the testes were more sensitive to IR, we investigated whether this tissue exhibits also differences in H2AX phosphorylation comparing wildtype to Topo1mt knockout mice (Figure 9).

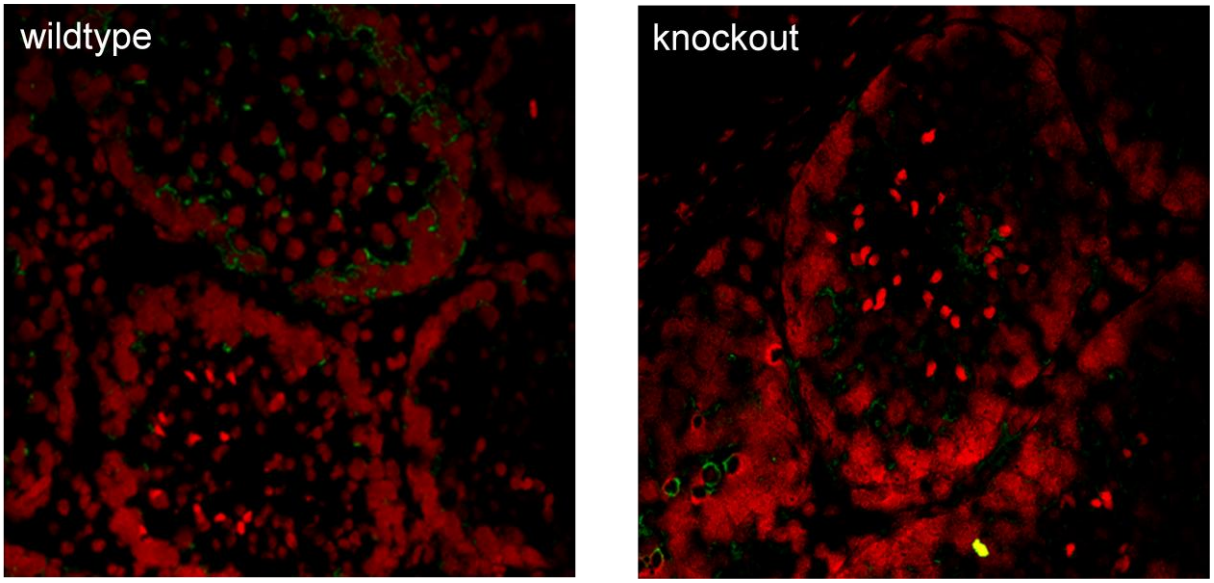

Figure 9: Immunofluorescence staining of γH2AX foci in 8 µm frozen sections of the testis from 9 week old mice. The panels show seminiferous tubuli in a 40x magnification. Samples were fixed and stained for γH2AX (green) and DNA was counterstained with propidium iodide (PI, red).

Figure 9 exhibits representative images of seminiferous tubuli of a testis of B6 mice. The testis on the left originating from a wildtype mouse shows just few γH2AX foci within the nuclei of the cells. In the seminiferous tubulus illustrated at the top of the left panel some green signal is visible which might be due to unspecific binding of the secondary antibody to the nuclear membrane. Yet, there is no increased specific γH2AX pattern observable, meaning the DSB rate is quite low. Moreover, the testis of the Topo1mt deficient mouse shows no enhanced green signal compared to the spermatocytes of the wildtype mouse. So far, there is no difference in the testes of Topo1mt deficient mice compared to wildtype mice regarding γH2AX phosphorylation. Yet, further studies are needed to investigate if there are differences in the basic level of γH2AX in other tissues.

5. Summary

When DNA damage forms double stranded breaks, it is always followed by the phosphorylation of the histone H2AX (Kuo and Yang, 2008). H2AX is a member of the histone H2A family, which is a component of the histone octomer packing DNA in nucleosomes (Bonner et al., 2008). Phosphorylation of H2AX to γH2AX is one of the first steps in recruiting and localizing DNA repair proteins. Quickly after DSBs occur, large numbers of γH2AX molecules form in the chromatin around the break site, creating a focus (Rogakou et al., 1999).

The use of γH2AX as a marker for DNA damage is of high interest as it is particularly precise and more sensitive to DSBs than other techniques (Dickey et al., 2009; Kuo and Yang, 2008). Monitoring DSBs by detecting γH2AX foci is an attractive method, as it is simple and sensitive. Another advantage of this method is that γH2AX formation can be detected in various tissues and in blood. So the main issue of this study was to learn the methodical skills for detecting γH2AX foci *in vitro* and *in vivo* and to apply the learned skills in further approaches.

In a first approach, we investigated if the topoisomerase II poison genistein causes DSBs in colon cancer cells. The soy constitutent genistein led to a slight but concentration-dependent increase of γH2AX foci in HCT116 cells after one hour of incubation, even in the lowest tested concentration of 50 µM.

In the next approach, different tissues of Topo1mt deficient mice were compared to tissues of wildtype mice regarding their basic level of γH2AX. A difference was observed in the intestine of the respective mice, where the number of γH2AX foci in the crypts of the knockout mouse was enhanced compared to the wildtype B6 mouse. This might be due to enhanced ROS levels which are generated due to the lack of the mitochondrial enzyme. Yet, there was no difference in the testes between Topo1mt deficient mice and wildtype mice. However, further investigations are needed to evaluate if there are differences among various tissues and to elucidate the function of this protein.

Taken together, γH2AX is a sensitive, precise and very useful tool to detect DSBs *in vitro* and *in vivo*.

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