Role of DNA damaging agents in the transformation of breast epithelial cells

A Thesis

submitted to

Indian Institute of Science Education and Research Pune
in partial fulfillment of the requirements for the
BS-MS Dual Degree Programme

by

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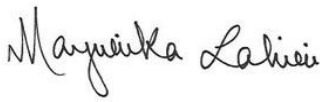
Certificate

This is to certify that this dissertation entitled '***Role of DNA damaging agents in the transformation of breast epithelial cells***' towards the partial fulfillment of the BS-MS dual degree program at the Indian Institute of Science Education and Research, Pune represents study/work carried out by ***Kezia J Ann*** at Indian Institute of Science Education and Research under the supervision of ***Dr. Mayurika Lahiri***, Associate Professor, Department of Biology, during the academic year 2017-2018.



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Declaration

I hereby declare that the matter embodied in the report entitled '***Role of DNA damaging agents in the transformation of breast epithelial cells***' are the results of the work carried out by me at the Department of Biology, Indian Institute of Science Education and Research, Pune, under the supervision of ***Dr. Mayurika Lahiri*** and the same has not been submitted elsewhere for any other degree.

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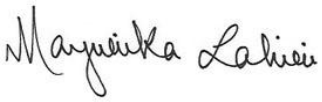

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Abstract

DNA is exposed to different damaging agents and prolonged exposure to these agents may lead to cancer. This study aims to understand the role of DNA damaging agents such as UV and Proflavine in transformation of breast epithelial cells. Previous studies form the lab showed that activation of DNA-PKcs upon MNU (N-Methyl-N-Nitrosourea) treatment leads to aberrant Golgi morphology. Alteration in Golgi morphology was observed upon UV induced DNA damage suggesting that this might be a characteristic feature of DNA damage. The other DNA damaging agent of interest, Proflavine is a DNA intercalator and is widely used as a topical antiseptic. Recent reports reveal that Proflavine can lead to frameshift mutations and has carcinogenic properties. Preliminary results from this study reveal that Proflavine can cause single stranded DNA breaks leading to DNA damage. Further experiments are required to identify the DNA damaging properties of Proflavine. Aberrant Golgi morphology and DNA–PKcs activation was also observed in Proflavine treated cells which further validate that damage induced altered Golgi phenotype is through the activation of DNA-PKcs.

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Introduction:

DNA damage and repair mechanisms in the cell

DNA is prone to different types of damaging agents that can lead to mutagenesis, senescence or even carcinogenesis in the cell. Oxidative damage caused by mitochondrial respiration, lipid peroxidation, endogenous alkylating agents such as S-adenosylmethionine are some among the endogenous DNA damaging agents (De Bont and van Larebeke 2004). Exogenous DNA damaging agents include both environmental and chemical DNA damaging agents. Ionizing radiation (IR) and ultraviolet (UV) radiation which causes double-stranded breaks (DSBs) and single-stranded breaks (SSBs) in DNA are environmental DNA damaging agents. Chemical DNA damaging agents include different chemotherapeutic agents such as methyl-methanesulfonate (MMS), cisplatin, nitrogen mustard which causes a variety of DNA lesions. Cigarette smoking is another DNA damaging agent that causes different adduct formation and oxidative damage in the lungs and other tissues (Ciccia and Elledge 2010).

Different DNA damage response (DDR) pathways are present in the cell, as a defense against these damages. The DNA damage response pathway consists of sensors, transducers, and effectors. DNA damage sensors include MRE11-RAD50-NBS1 (MRN) complex that detects DNA DSBs. Single-stranded DNA is detected by replication protein A (RPA) and RAD9-RAD1-HUS1 (9-1-1) complex (Sulli, Di Micco et al. 2012). The proteins that get activated after detecting the damage include ataxia telangiectasia mutated (ATM), ataxia telangiectasia and Rad3-related protein (ATR), DNA-dependent protein kinase (DNA-PK) and members of poly(ADP-ribose) polymerase (PARP) family (Ciccia and Elledge 2010). ATM has a significant role in DNA DSB repair pathway (Shiloh and Ziv 2013). Nijmegen Breakage Syndrome1 (NBS1) and Breast Cancer gene1 (BRCA1) are required for the recruitment and stabilization of ATM to the DNA damage sites (Kitagawa and Kastan 2005). Replication protein A (RPA) coated ssDNA is necessary for the recruitment of ATR and ATR interacting protein (ATRIP) upon DNA SSBs (Cimprich and Cortez 2008).TopBP1 activates ATR ATRIP complex, which has an ATR activation domain (Kumagai, Lee et al. 2006). Activation of ATM and ATR leads to the activation of checkpoint kinase 2 and 1 (Chk2 and Chk1) respectively (Zhou and

Elledge 2000). Following the activation of Chk1 and Chk2 different downstream regulators are activated that give can lead to cell cycle arrest (Cdc25C,Plk3), DNA repair (BRCA1,p53) or apoptosis (Pml1, E2F1)(Zhou and Elledge 2000, Bartek and Lukas 2003) (Figure1). Any alteration of this pathway such as mutations in the mitotic pathway, apoptosis, or in DNA damage responses will permit the survival of cells with genomic abnormalities (Kastan and Bartek 2004). Genomic instability and mutations are one among the different hallmarks of cancer cells. Self-sufficiency in growth signals, evasion of programmed cell death (apoptosis), limitless replicative potential, sustained angiogenesis, insensitivity to growth-inhibitory signals, and tissue invasion and metastasis are the other essential features that a normal cell acquires to become malignant (Hanahan and Weinberg 2011).

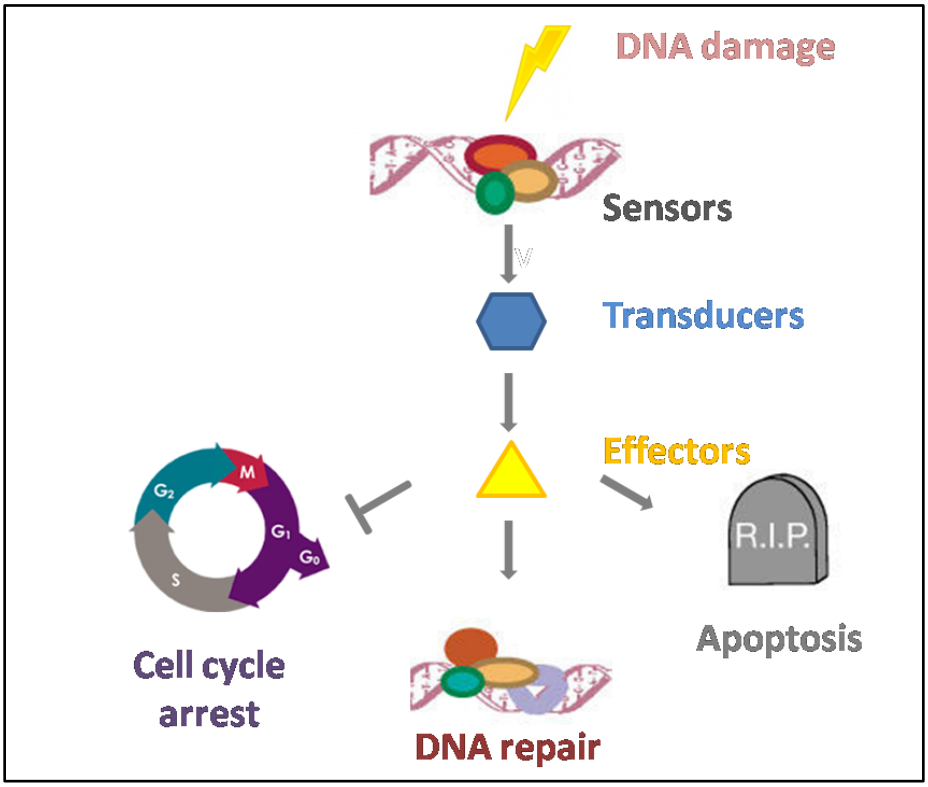

Figure 1: Schematic diagram representing the DNA damage response pathway. Adapted from (Zhou and Elledge 2000).

N-Methyl-N-Nitrosourea damage in cell

Farber-Katz *et al.* showed a dispersal of Golgi when cells were treated with different DNA damaging agents such as camptothecin (CPT), doxorubicin and ionizing radiations. They also reported that the dispersal of Golgi upon DNA damage occurred through the DNA-PK and GOLPH3 (Farber-Katz, Dippold et al. 2014). A similar result was observed in our lab where activation of DNA-PK leading to aberrant Golgi morphology in MCF10A cells treated with MNU (N-Methyl-N-Nitrosourea) an alkylating agent. Transformation phenotypes such as disruption in apicobasal polarity and EMT like phenotype was observed in MNU treated MCF10A cells grown as three-dimensional cultures. Upon inhibition of DNA-PK with DMNB, there was a reversal in the dispersal of Golgi (Figure 2). A partial reversal was found in polarity defects, and EMT like phenotype in DNA-PK inhibited MNU treated cells MCF10A cells. Suggesting that MNU induced transformation is mediated by DNA-PK activation (Anandi, Chakravarty et al. 2017).

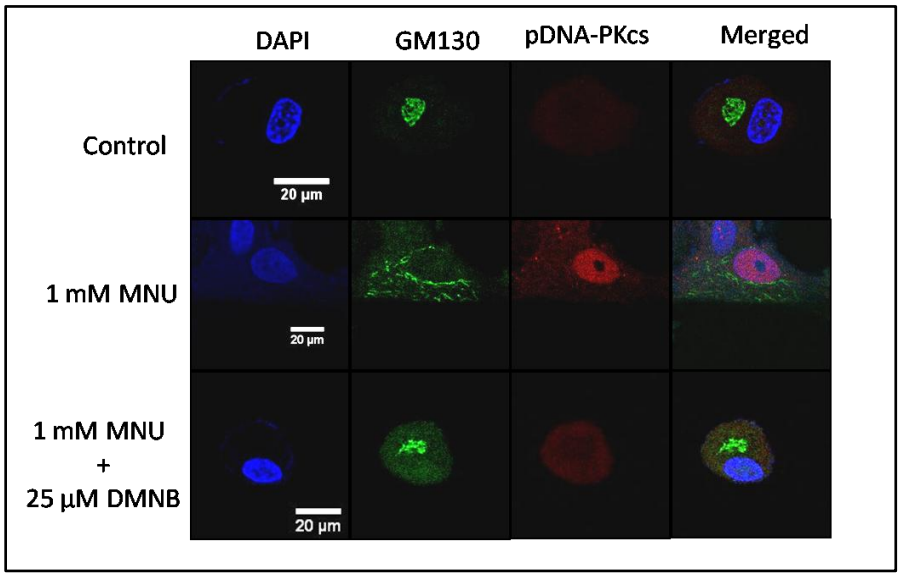

Figure 2: GM130 which is a cis-Golgi marker was stained to observe the Golgi morphology. Dispersal of Golgi and activation of DNA-PK was observed in cells treated with 1mM MNU. Inhibition of DNA-PK by DMNB shows a partial reversal of the phenotype (image courtesy: Libi Anandi)

So to investigate whether the phenotypes observed upon MNU damage are a characteristic feature of MNU damage or does all other DNA damage would show the same phenotype, we need to use different DNA damaging agents and validate the results.

Radiation damage caused by ultraviolet (UV) was used an alternate DNA damaging agent. A dose dependent study was previously done to identify the minimum dose of UV for the expiremnents.

Proflavine as a DNA damaging agent

Intercalating agents are molecules that can intercalate between DNA bases. This interaction results in structural changes in DNA such as unwinding of the helix, lengthening of DNA, inhibition of replication (Ferguson and Denny 2007).

Acridine (2,3-Benzoquinoline) (Figure 3A) is a planar molecule that is widely used as a dye. Acridine and its derivatives are known to intercalate DNA and have a wide pharmacological applications(Kumar, Kaur et al. 2012).

Proflavine (3,6-diaminoacridine) (Figure 3B), is also an acridine derivative, known to intercalate DNA with a minor G-C specificity (Feigon, Denny et al. 1984).Studies show that Proflavine can inhibit the synthesis of RNA and proteins (Watts and Davis 1966).The intercalation of Proflavine with DNA results in frameshift mutations. Proflavine induced frameshift mutations are either deletions or duplications (Ripley and Clark 1986). Experimental studies showed that Proflavine could also cause ultrastructural lesions such as cytoplasmic lesions, nuclear and nucleolar lesions (Simard 1966). Because of these properties, Proflavine is widely used as topical antiseptic agent.

Recent studies also reveal the antitumor activity of Proflavine. In vivo studies in hamster showed that Proflavine could inhibit protein synthesis in melanoma (Birkmayer and Balda 1971). Studies in human osteosarcoma cells revealed that Proflavine suppressed the growth of these cell through apoptosis and autophagy (Zhang, Niu et al. 2015). Cytotoxic and clastogenic properties are also shown by Proflavine derivatives

(Kozurkova, Sabolova et al. 2008, Benchabane, Di Giorgio et al. 2009, Polyanskaya, Kazhdan et al. 2010).

Proflavine is known to cause mutations and also inhibits cancer progression in cells. Reports from Gatasheh and team showed that Proflavine can lead to formation of ROS (Gatasheh, Kannan et al. 2017). In the current project, we aim to investigate whether Proflavine can cause DNA damage and lead to transformation using MCF10A , a non-tumorigenic breast epithelial cells, as a model system.

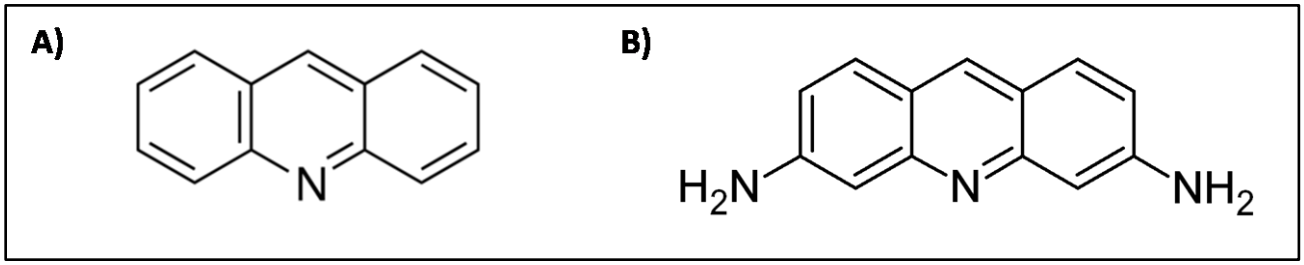

Figure 3: Structural representation of acridine and its derivative: A) Acridine, B) Proflavine.

Breast epithelial three-dimensional cultures

Mouse models, primary tumor tissues, monolayer (2D) cultures are widely used techniques in cancer research. Studies using primary tumor and mouse models give information on genetic events in cancer formation, but these models are not sufficient enough to study the biochemical and cell biological pathways involved in tumor formation (Debnath, Muthuswamy et al. 2003). Striking differences are observed in gene expression, cell signaling, and cell structure when cells are cultured in monolayer and 3D culture system (Bissell, Weaver et al. 1999, Sung, Su et al. 2013). Mammary epithelial cells, when grown as monolayer culture,fail to recapitulate the individual structural features of the glandular epithelium in vivo (Debnath and Brugge 2005).

The mammary gland comprises an extensive network of ductal system that connects the functional unit acini to the nipple (Figure 4A, B). The epithelial cells in mammary gland contain different cell types, inner luminal cells and outer basal cells (Shackleton, Vaillant et al. 2006) (Figure 4C). Luminal cells present in the inner side majorly consist of milk secreting cells and a subpopulation of hormone receptor expressing cells

(Sleeman, Kendrick et al. 2007). Adjacent to the luminal cells are myoepithelial cells which are responsible for the deposition of the basement membrane (BM) proteins (collagen IV and laminin V) and also for luminal cells polarity maintenance (McCave, Cass et al. 2010). Because of the contractile nature, myoepithelial cells also function in the secretion of milk from the mammary gland during lactation (Schmeichel and Bissell 2003).

MCF10A cells are nontumorigenic breast epithelial cells, derived from human fibrocystic mamary tissue (Soule, Maloney et al. 1990). MCF10A cells when grown in 3D culture system, recapitulate some features of mammary gland such as the formation of acini like spheroids, the apicobasal polarization of the spheroids, and deposition of BM components (Debnath, Muthuswamy et al. 2003) (Figure 4C).

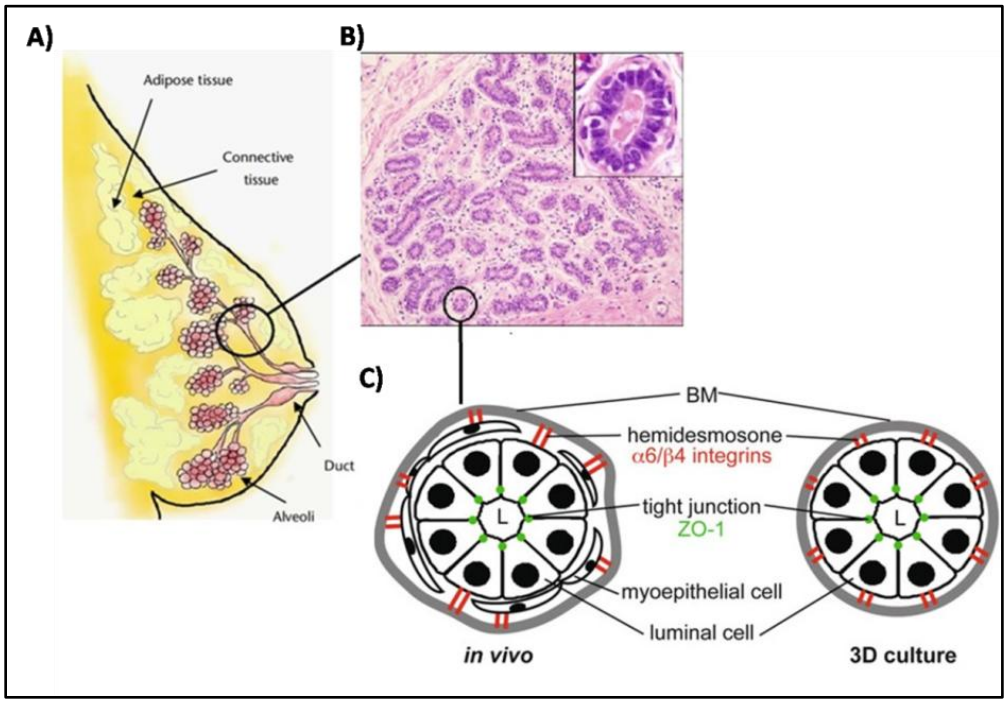

Figure 4: Anatomy of mammary gland: A) Schematic representation of mammary gland. B) Immunohistochemical staining of a breast lobule (blue indicates nucleus). C) The morphological architecture of acini formed in the mammary gland (left) and acini formed in 3D culture system (right). Luminal cells with BM and apicobasal polarity is present in the 3D culture system, but it lacks the myoepithelial cells. Black dots represent nucleus and L for the lumen. Adapted from (Vidi, Bissell et al. 2013).

MCF10A cells, when cultured on top of matrigel (ECM mixture isolated from Engelbreth-Holm-Swarm mouse sarcoma cells), proliferate and form acini like spheroids (Debnath, Muthuswamy et al. 2003). Apicobasal polarization occurs as an early event during the initial stages of 3D culture. From day 5-8 two distinct populations of cells, an outer polarized cells which are in contact with the matrix and an internal subset of poorly polarized cells that lack the contact with the matrix are formed. By day 8, the poorly polarized cells in the center undergo apoptosis resulting in the formation of the hollow lumen. The acinus remains hollow after that (Debnath, Muthuswamy et al. 2003, Debnath and Brugge 2005) (Figure 5).

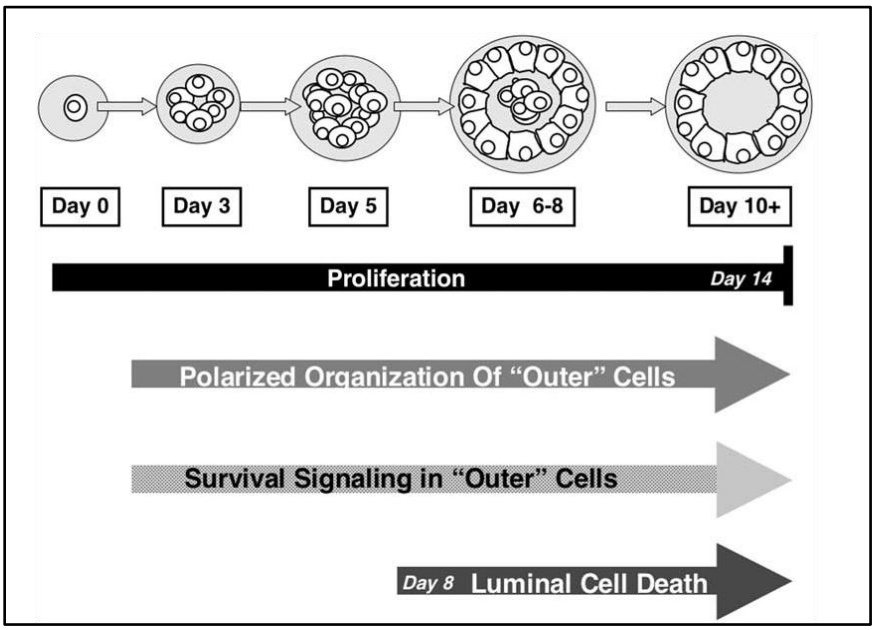

Figure 5: Schematic representation of events in MCF10A acinar morphogenesis Reproduced from (Debnath, Muthuswamy et al. 2003).

Aim I:

To determine the effect of UV damage in DNA-PK activation and altered Golgi morphology

Objective I:

i. To investigate whether Golgi dispersal upon UV damage is through the activation of DNA-PK
ii. To investigate whether inhibition of DNA-PK leads to the reversal of Golgi dispersal

Aim II:

To investigate the role of Proflavine in transformation of non-tumorigenic breast epithelial cells

Objective II:

i. To identify whether Proflavine causes DNA damage.
ii. To investigate whether Proflavine causes transformation of breast epithelial cells in 3D cultures.

Materials and Methods:

Cell line and culture conditions:

MCF10A cell line, a generous gift from Prof. Raymond C. Stevens (The Scripps Research Institute, California, USA) were maintained on 100mm tissue culture grade dishes (Corning) at 37°C in humidified 5% CO_2 incubator (Eppendorf). These cells were cultured in High Glucose DMEM without sodium pyruvate (Lonza) and contain 5% horse serum (Invitrogen), 100ng/ml cholera toxin (Sigma-Aldrich), 20ng/ml EGF (Sigma-Aldrich), 0.5g/ml hydrocortisone (Sigma-Aldrich), 10g/ml insulin (Sigma-Aldrich) and 100units/ml penicillin-streptomycin (Invitrogen). During sub culturing the cells were trypsinized using 0.05% trypsin-EDTA (Invitogen) and were resuspended in High Glucose DMEM without sodium pyruvate with 20% horse serum (Invitrogen) and 100units/ml penicillin-streptomycin (Invitrogen).

UV irradiation:

Ultraviolet C (UVC) irradiation (254nm) was done on cells after aspirating media. The media was replenished and the cells were maintained at 37°C in humidified 5% CO_2 incubator (Eppendorf) for recovery.

Proflavine treatment:

Proflavine hemisulfate salt hydrate (Sigma-Aldrich) was dissolved in filter sterilized distilled water. Cells were then treated with required concentration of Proflavine and incubated for 24 hours.

Immunofluorescence:

Cells were grown on 24 well plate (Corning) with cover slips. After the experiment, media was aspirated and 1X PBS wash was given to the cells. These cells were then fixed using 4% formaldehyde (Fisher Scientific) solution. It was then incubated for 20min in dark room. 1X PBS wash was given twice. Permeabilization of cells was done using 0.5% Triton-X-100 (USB Corporation) in 1X PBS and kept at 4^{O}C for 10 minutes. It was then aspirated and a PBS-glycine (100mM glycine in 1X PBS) wash and two 1X PBS washes were given. Blocking was done with 10% FBS in1X Immunofluorescence

(IF) buffer (1X PBS, 0.05% [w/v] sodium azide, 0.1% [w/v] BSA, 0.2% [v/v] Triton-X-100 and 0.05% Tween 20) and was incubated at room temperature (RT) for 1 hour. Primary antibody was prepared in blocking solution for staining the cells. The cells were incubated with the required primary antibody (Table1) for 16 hours at 4°C. 1X IF buffer washes was given thrice before incubating with Alexa Flour® secondary antibody prepared in 10% FBS in 1X IF buffer. After 1 hour incubation of secondary antibody at RT, a 1X IF buffer wash and two 1X PBS washes were given for 10 mins. 0.5µg/ml of Hoechst 333258 (Invitrogen) in 1X PBS was then added and was incubated for exact 5 minutes at RT to stain the nucleus. Slow Fade® Gold antifade reagent (Molecular Probes) was added to mount the cells after two 1X PBS washes. Slides were then stored at 4°C. The imaging of the cells were done using Zeiss LSM710 confocal microscope with 63X oil objective.

Table 1: Antibodies used for immunofluorescence assay

Primary Antibody	*Dilution*
GM130, anti-Mouse (BD)	1:100
pDNA-PKcs (Thr 2069), anti-Mouse (Abcam)	1:100
pDNA-PKcs (Ser 2056), anti-Rabbit (Abcam)	1:100
pRPA (Thr21), anti-Rabbit (Abcam)	1:200
γH2AX-FITC (Ser139) (Millipore)	1:100
TopBP1, anti-Rabbit (Abcam)	1:200

Cell viability assay:

4,000 cells/well were seeded on a 96 well flat bottom tissue culture treated (Eppendorf). After 16 hours of incubation different concentration of Proflavine was added to each well as triplicates and incubated at 37°C in humidified 5% CO_2 incubator (Eppendorf). After 24 hours incubation, media containing drug was removed and replenished with media containing 0.5mg/ml MTT solution and incubated for 4 hours. To dissolve the formazan crystals formed, media-MTT mixture was removed and was incubated with DMSO for 5 min on a nutating shaker at RT. The absorbance at 570nm was recorded using Varioskan® Flash Mutimode Plate Reader (Thermo Scientific).

Single cell gel electrophoresis (comet assay):

MCF10A cells were seeded with a density of 1x 10^5 cells and allowed to grow for 14-16 hours. Cells were then trypsinized and collected using 0.05% trypsin-EDTA (Invitrogen) after 24 hours of Proflavine treatment. Cell suspension was collected and centrifuged at 900rpm for 10 min. With few modifications, alkaline and neutral comet assay was performed as per standard protocols (Olive and Banath 2006).For alkaline comet assay, lysing solution contains 2.5M NaCl, 10mM Trizma base, and 100mM anhydrous EDTA. 1x electrophoresis buffer contains 1mM anhydrous EDTA and 0.3N NaOH. Neutral lysing buffer for neutral comet assay contains 0.5M anhydrous EDTA with pH 8. 1x TBE (Trizma base, Boric acid, and anhydrous EDTA) is used as electrophoresis buffer for neutral comet assay. For staining 2x ethidium bromides was added and incubated for 5min. Excess stain was removed by washing with distilled water. Imaging was done immediately using Apotome microscope.

Immunoblotting:

Proflavine and UV treated cell lysates were collected using 2x sample buffer containing 1M Trizma base (pH 6.8), 1% Bromophenol blue, 0.1% SDS, Glycerol, and DTT. Lysates were the resolved using 10% sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and was then transferred to polyvinylidene difluoride (PVDF) membrane (Millipore). One hour blocking was done using 4% (w/v) Block Ace (AbDSerotec) for phospho proteins and 5% (w/v) skimmed milk (SACO Foods,USA) for non-phosho proteins. It was then washed with distilled water and incubated with primary antibody (Table2). After three 1xTBS-T washes for 15 min, blots were incubated with peroxidase-conjugated secondary antibody in for 1 hour at RT. The blots were then developed using Immobilon^{TM} western chemiluminescent HRP substrate (Millipore).

Table 2: Antibodies used for immunoblotting

Primary Antibody	*Dilution*
pChk1 Ser345 (CST)	1:2500
Chk1 G4 (Santacruz)	1:1000
pChk2 Thr68 (CST)	1:2500
Chk2 (CST)	1:1000
GAPDH (Sigma)	1:40000

Results:

I.i.a) 10J/m^2 UV damage causes aberrant Golgi morphology

MCF10A cells were grown and irradiated with 10J/m^2 UV to determine the minimum dose of UV required for Golgi dispersal. Immunostaining was done 24 hours post damage for GM130 which is a cis-Golgi matrix protein as a Golgi marker for checking aberrant Golgi morphology. A significant dispersal of Golgi and reduced cell death was observed in cells irradiated with 10J/m^{2}UV followed by 24 hours recovery period (Figure 6). This suggests that 10J/m^2 can be used as a minimum dose of UV for the further experiments.

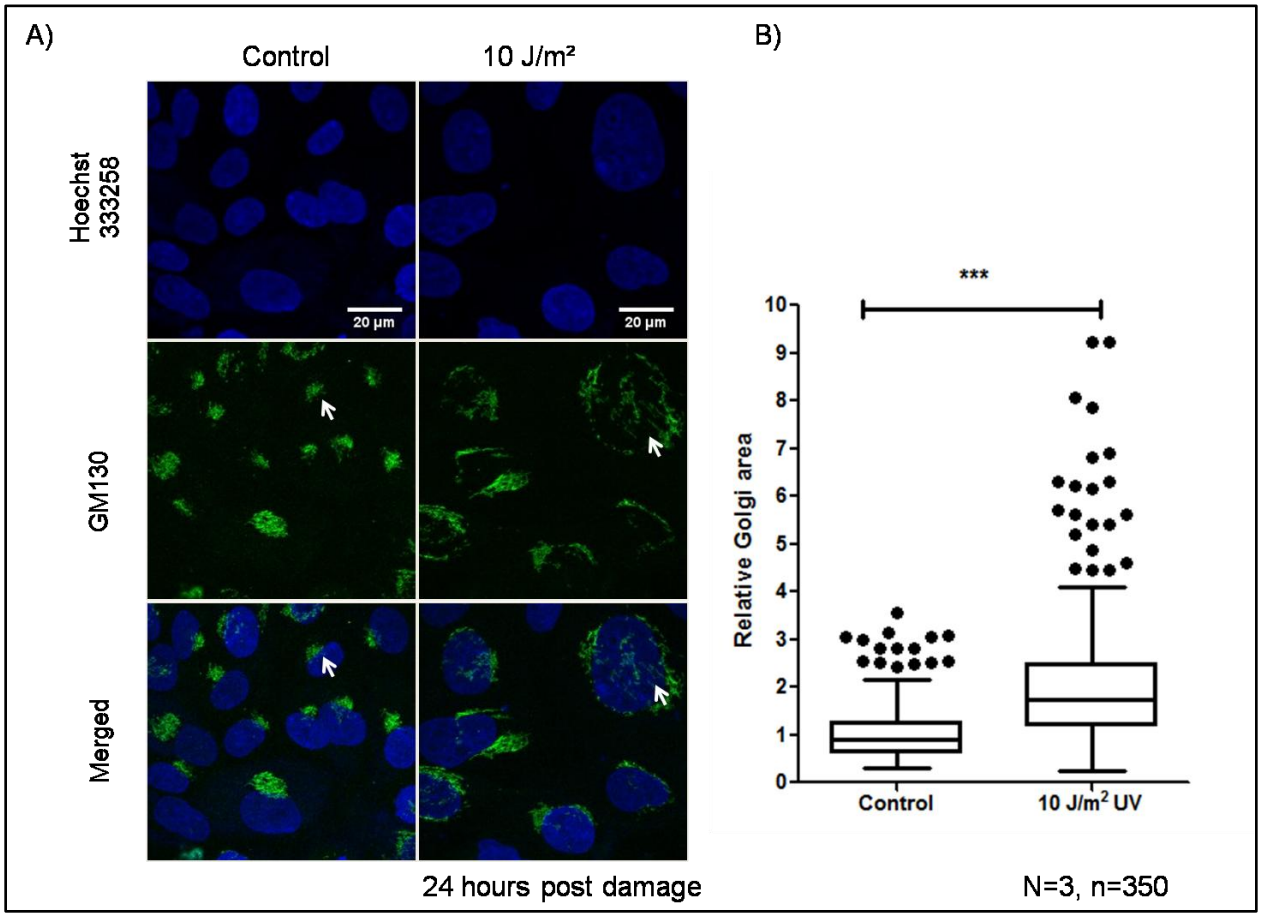

Figure 6: MCF10A cells were grown and irradiated with 10J/m^2 UV. Cells were fixed and stained for GM130 24 hours post damage. Cells were then imaged under confocal microscope using 63X oil objective. A) Dispersal of Golgi was observed upon 10J/m^2

UV 24 hours post damage. B) Images were quantified as relative Golgi area. n=350, number of nuclei used to calculate Golgi dispersal in three independent experiments.

I.i.b) Time dependent Golgi dispersal upon UV damage

To identify the minimum time required for Golgi dispersal, $10J/m^2$ UV treated cells were incubated for different time points (2, 6, 12, 18, 24 hours) post damage. A significant difference in Golgi area was observed in UV treated cells from 6 hours to 24 hours post damage. Whereas no significant Golgi dispersal was observed in cells that were irradiated with UV followed by 2 hours recovery (Figure 7).

I.i.c) UV damage causes time dependent activation of DNA-PK

To understand whether Golgi dispersal following UV damage has any relation with DNA-PK activation, a time-dependent assay was done to identify DNA-PK activation. $10J/m^2$ UV treated cells were incubated for different time points (2, 6, 12, 18, 24 hours) post damage and was fixed and stained for pDNA-PKcs (Thr2609) the catalytic subunit of DNA-PK. Activation of DNA-PK was observed after 2 hours (Figure 8).This suggests that the dispersal of Golgi observed might be because of the activation of DNA-PK.A reduction in activation was observed after 12 hours.

The reduction in activity observed can be due to the activation of different phosphorylation site of DNA-PKcs upon damage or it can be because of the activation of checkpoint proteins which leads to the damage repair.

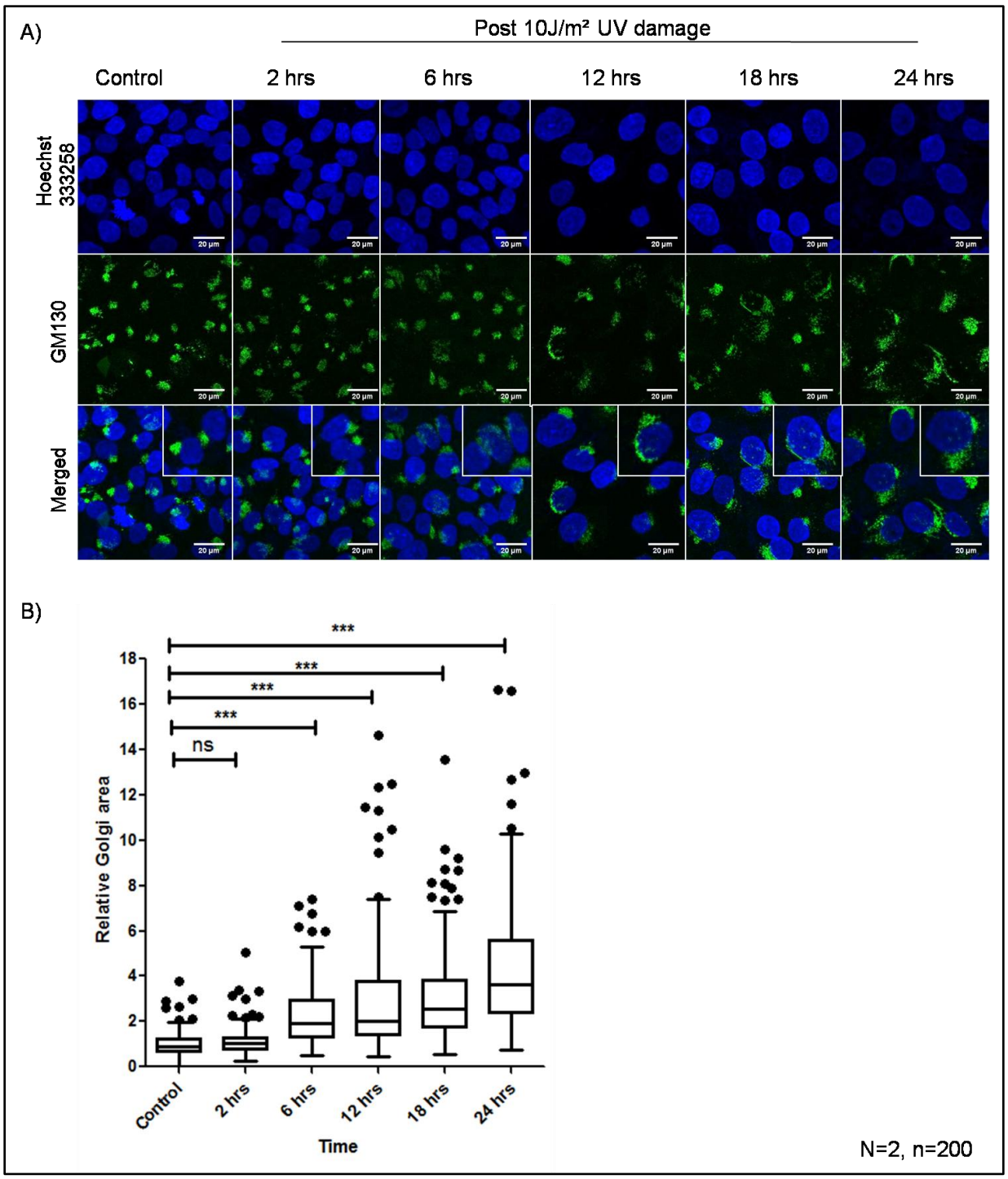

Figure 7: Time-dependent assay to identify the Golgi dispersal time upon 10J/m² UV. A) MCF10A cells were fixed and stained for GM130 for different time points. B) Images were quantified as relative Golgi area using One-way ANOVA and Bonferroni's multiple comparison test as the post test, ***p<0.0001. n=200 indicate number of nuclei used to calculate Golgi dispersal in two independent experiments.

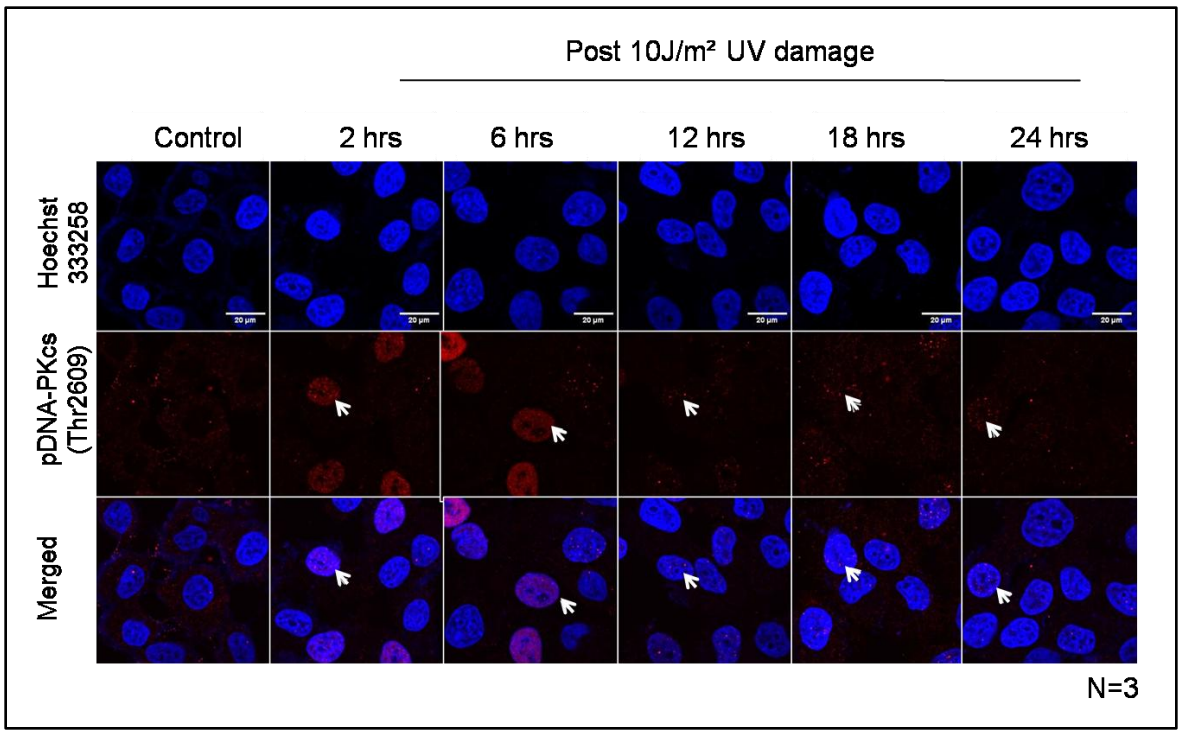

Figure 8: Time-dependent assay to identify the activation of DNA-PK time upon 10J/m² UV. MCF10A cells were fixed and stained for pDNA-PKcs for different time points. N indicates number of biological replicates.

I.i.d) Activation of different phosphorylation site of DNA-PKcs upon UV damage

Threonine2609 and Serine2056 are known autophosphorylation sites of DNA-PK (Chan, Chen et al. 2002, Chen, Chan et al. 2005). It is known that phosphorylation of DNA-PK at Thr2069 site promotes end resection, whereas the phoshorylation of DNA-PK at Ser2056 limits end resection (Cui, Yu et al. 2005). So the reduction that was observed may be due to the change in phosphorylation site of DNA-PKcs due to its differential function.

To identify whether there is activation of different phosphorylation site of DNA-PKcs, UV treated cells were fixed and stained for Thr2609 and Ser2056 site of DNA-PKcs. Activation of DNA-PKcs Thr2069 was observed from 2hrs and a reduction after 6hrs whereas, no consistency in results was observed in Ser2056 site in repeated experiments (Figure 9).

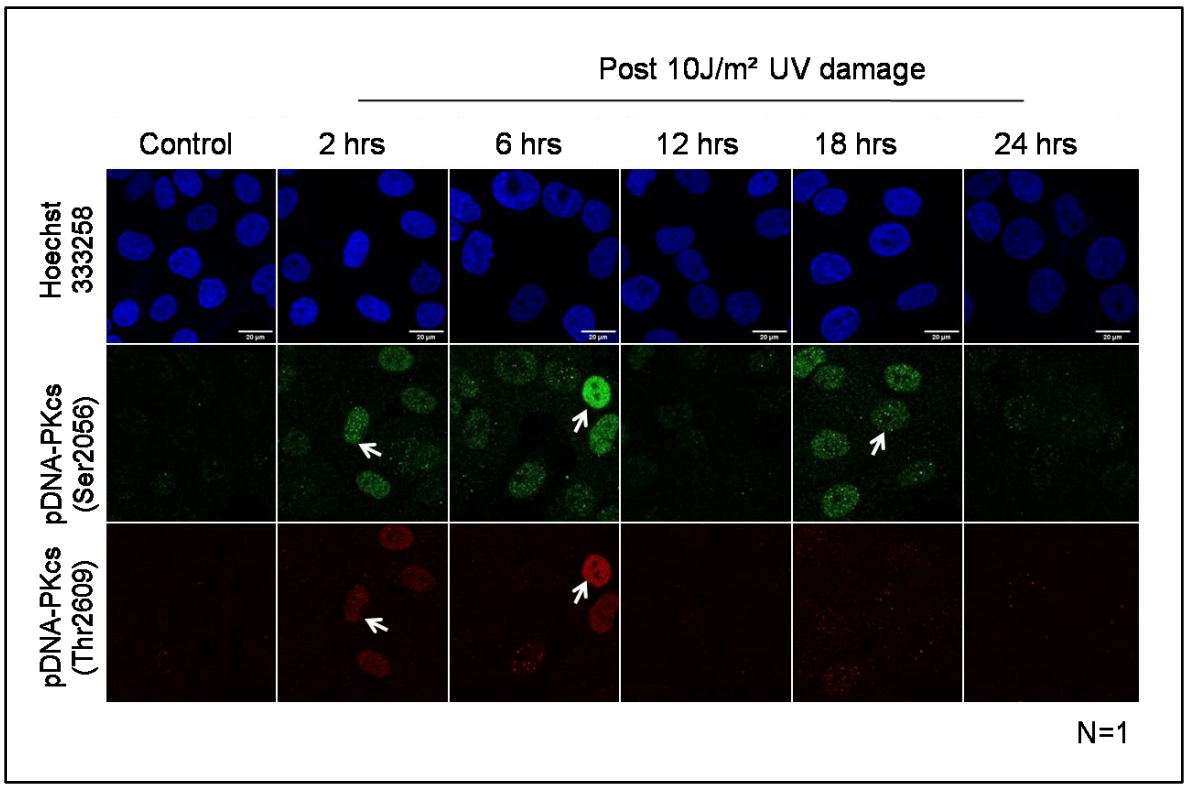

Figure 9: Time dependent assay to identify the change in phosphorylation site of DNA-PK upon UV irradiation.

I.i.e) Activation of damage response proteins upon UV damage

RPA is a ssDNA binding protein, which has a significant role in DNA replication and repair. DNA-PK is known to activate damage induced RPA phosphorylation (Block, Yu et al. 2004).

To identify whether the reduction in DNA-PK observed upon UV damage is due to the DNA repair, 10J/m^2 UV irradiated cells were incubated for different time points (2, 6, 12, 18, 24 hours) post damage. Cells were then fixed and probed for pRPA (Thr21), the phosphorylated RPA32 (Figure 10). Activation of pRPA was observed which was similar to that of DNA-PKcs, suggesting the activation of DNA damage repair pathway upon UV damage.

To further confirm the above result, UV irradiated cells were probed for TopBP1, a DNA damage response protein which is involved in activation of DNA damage checkpoint pathway (Xu and Leffak 2010). Activation of TopBP1 was observed in later time points

(Figure 11), which indicates that there is activation of DNA damage response pathway in later time points.

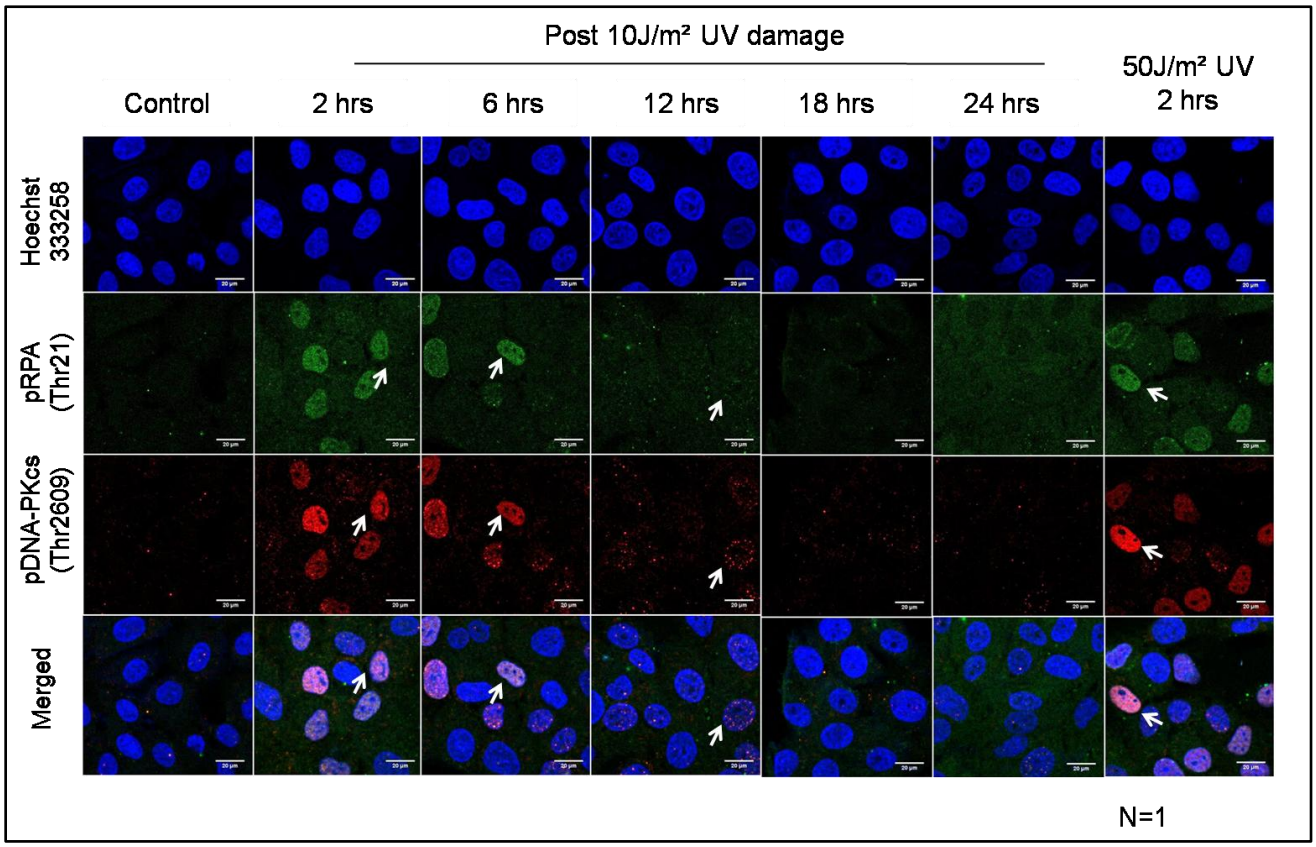

Figure 10: Time-dependent assay to identify the activation of pRPA upon 10J/m² UV damage. MCF10A cells were fixed and stained for pRPA for different time points. Co-localization of pRPA with pDNA-PKcs was observed. N indicates number of biological replicate.

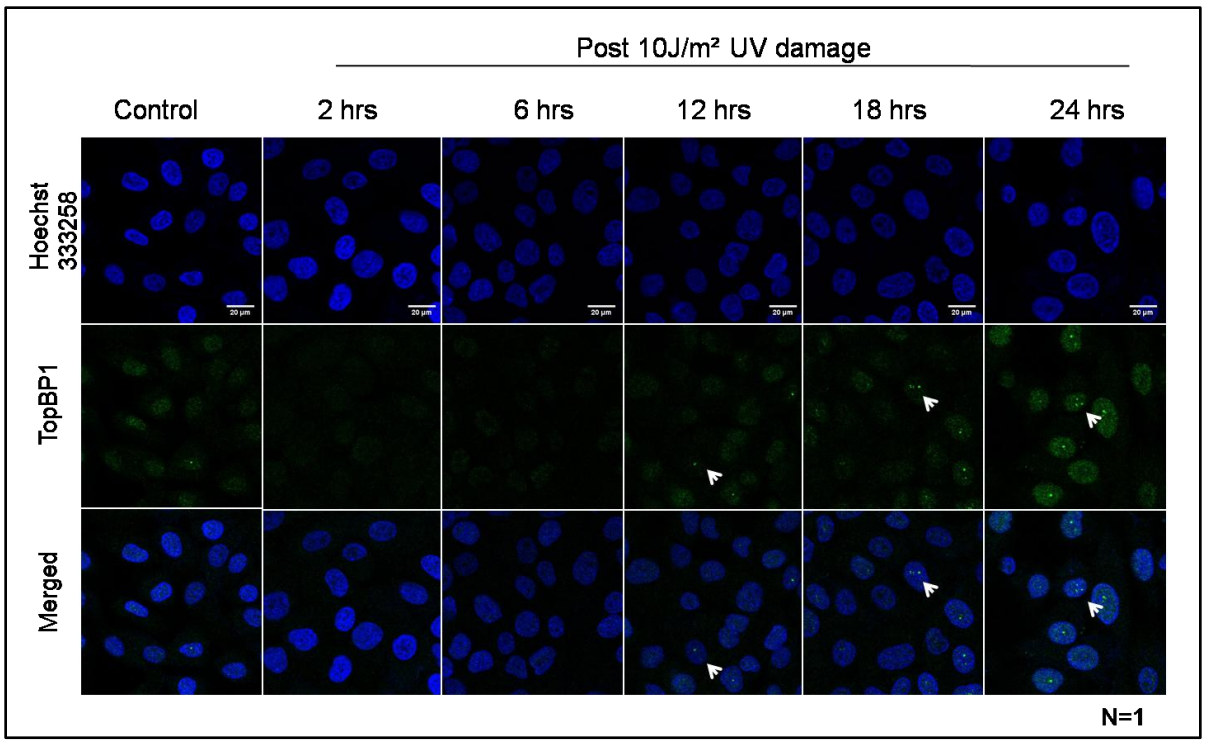

Figure 11: Activation of TopBP1 was observed in late time points upon UV induced DNA damage.

I.i.f) Activation of checkpoint proteins upon UV damage

Chk1 is known to be activated at Ser345 and Ser317 by ATR and Chk2 activation at Thr68 by ATM upon DNA damage leading to the activation of different downstream proteins that bring about cell cycle arrest, activation of damage repair or apoptosis (Patil, Pabla et al. 2013, Zannini, Delia et al. 2014). To identify whether the activation of damage response pathway result in the activation of checkpoint pathways that leading to damage repair. Immunoblotting was done to check the levels of Chk1 (Figure 12A) and Chk2 (Figure 12B). Activation of Chk1 (Figure 12A) and Chk2 (Figure 12B) were observed upon UV damage. Adjustments in loading are required to further conclusions regarding the pattern of activation.

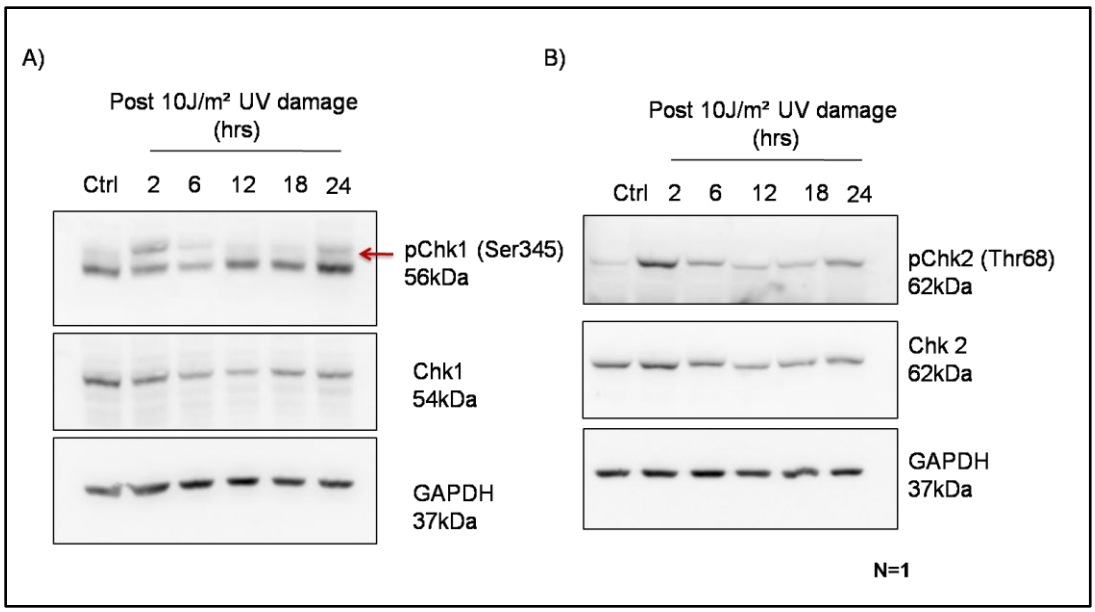

Figure 12: Immonoblotting of UV treated cells collected at different time points post damage and probed for pChk1, pChk2, total Chk1, total Chk2 and GAPDH as loading control. Chk1 and Chk2 activation was observed in treated cells compared to control. N indicates the number of biological replicates.

II.i.a) Determining sub-lethal concentration of Proflavine

To determine the sub-lethal concentration of Proflavine, MTT- based cytotoxicity assay was performed.MCF10A cells treated with different concentrations of Proflavine were incubated for 24 hours. A reduction in cell viability was observed upon increased concentration of Proflavine in cells. Doses of 0.4µM and 0.6µM were selected since it was showing cell viability above 80% (Figure 13).

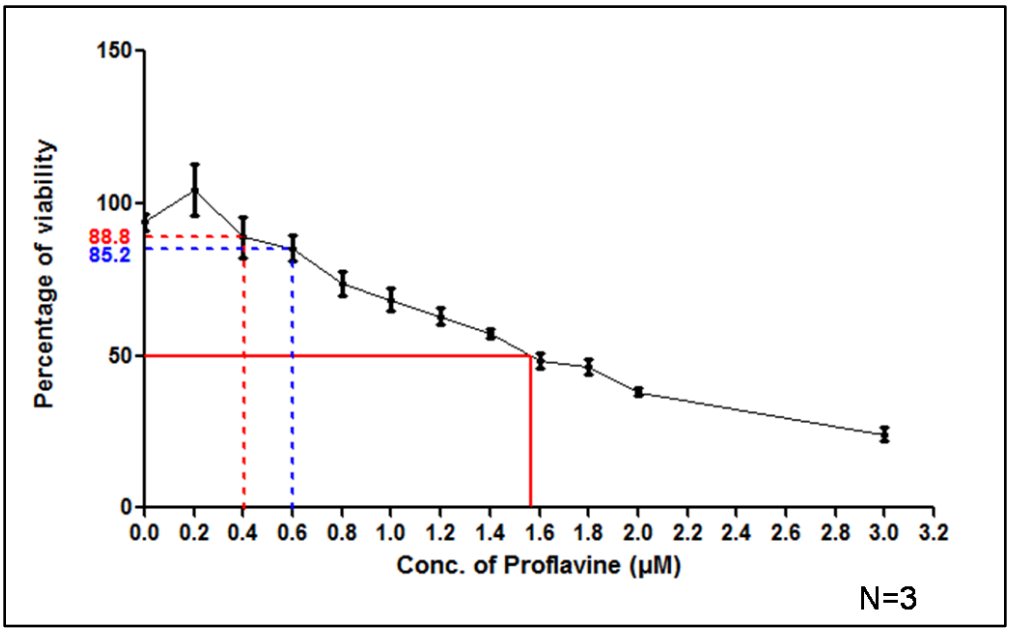

Figure 13: Cell viability graph of MCF10A cells treated with different concentrations of Proflavine ranging from 0.2µM to 3µM for 24 hours. N indicates number of biological replicates.

II.i.b) Proflavine causes single stranded DNA damage

In order to identify the DNA damage caused by Proflavine, MCF10A cells were treated with the selected doses of Proflavine. Alkaline comet assay was done to visualize single stranded caused due Proflavine exposure respectively. % of tail DNA and % tail length were calculated to measure the extent of damage caused by Proflavine (Figure 14B and C). Higher amount of single stranded DNA damage were present in Proflavine treated cells compared to control (Figure 14A). This indicates that Proflavine treatment can cause single stranded DNA damage in the cell.

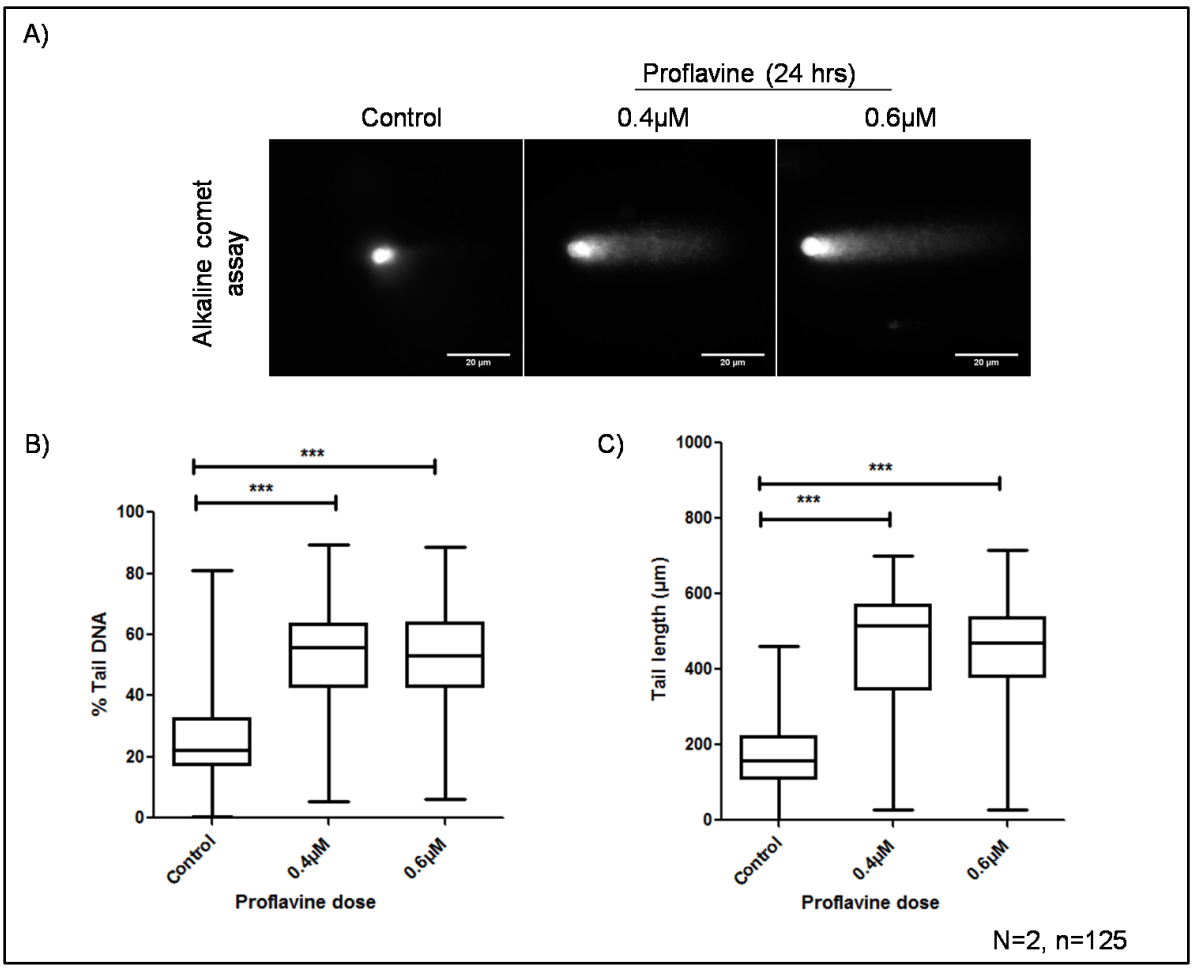

Figure 14: Alkaline comet assay to identify SSB cause due to Proflavine. A) Ethidium Bromide staining of DNA. B) % tail DNA indicates the DNA content in tail. C) Tail length indicated the length of comet tail. % tail DNA and tail length were quantified using One-way ANOVA and Bonferroni's multiple comparison test as the post test, ***p<0.0001. n=125, number of comet used for calculations from two independent experiments.

To further validate the result, cells treated with Proflavine were fixed and probed for pRPA (Thr21), the phosphorylated RPA32 (Figure 15). RPA activation was observed in Proflavine treated cells suggesting that Proflavine treatment causes single stranded DNA damage.

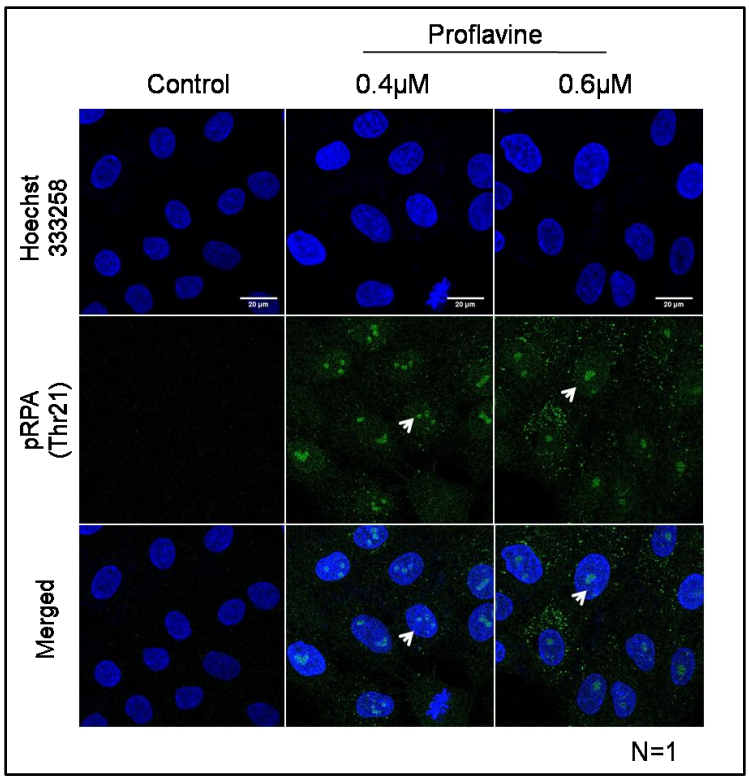

Figure 15: Activation of pRPA upon 0.4µM and 0.6µM Proflavine treatment. N indicates the biological replicates.

II.i.c) Proflavine treatment causes double stranded DNA damage

γH2AX, phosphorylated form of histone variant H2AX (serine139) is a known marker for DNA double stranded break (Rogakou, Pilch et al. 1998). Upon DNA damage ATM, ATR and DNA-PK is known to phosphorylate H2AX, which then can lead to cell cycle arrest (Podhorecka, Skladanowski et al. 2010).To identify whether Proflavine treatment can cause double stranded DNA damage, cells treated with Proflavine were fixed and probed for γH2AX (Ser139) (Figure 16). γH2AX activation was observed upon Proflavine treatment which indicate that Proflavine treatment leads to activation of damage response proteins.

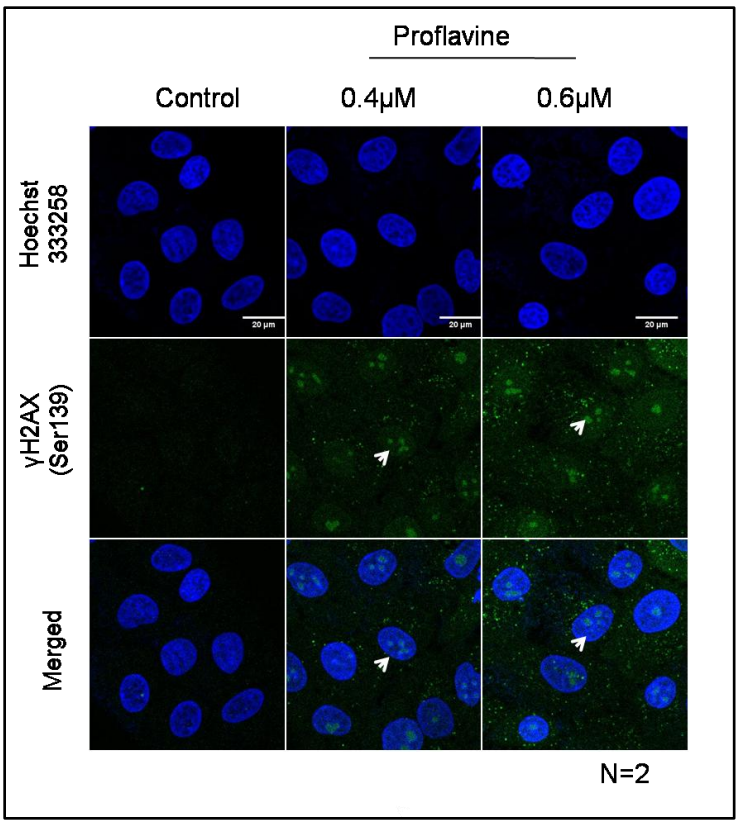

Figure 16: Activation of γH2AX upon 0.4μM and 0.6μM Proflavine treatment. N indicates the biological replicates.

II.i.d) Activation of checkpoint proteins upon Proflavine treatment

DNA damage leads to activation of different damage repair proteins in the cell. In order to identify the downstream DNA damage response pathway that gets activated upon Proflavine treatment, lysates of cells treated with and without Proflavine were collected. The lysates were then probed for pChk1 (Ser345), and pChk2 (Thr68) the site that get phosphorylated upon DNA damage. Activation of Chk1 (Figure 17A) and Chk2 (Figure 17B) was observed in treated cells compared to control.

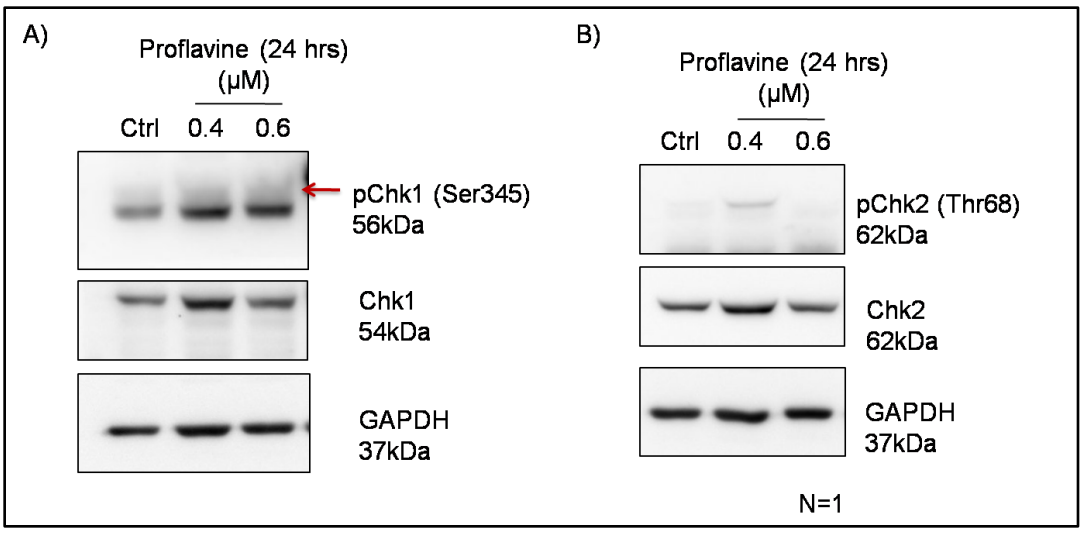

Figure 17: Immonoblotting of Proflavine treated cells probed for pChk1, pChk2, total Chk1, total Chk2 and GAPDH as loading control. Chk1 activation was observed in treated cells compared to control. N indicates the number of biological replicates.

II.i.e) Proflavine treatment causes aberrant Golgi phenotype and DNA-PKcs activation

Studies have reported that DNA damage can lead to altered Golgi morphology which is through the activation of DNA-PKcs (Farber-Katz, Dippold et al. 2014, Anandi, Chakravarty et al. 2017). Preliminary results upon UV induced DNA damage also showed an aberrant Golgi morphology. Since preliminary results suggest that Proflavine treatment leads to DNA damage. In order to identify whether Proflavine treatment also leads to aberrant Golgi morphology, cells treated with Proflavine was fixed and stained for GM130 the cis-Golgi marker. Aberrant Golgi morphology was observed in cells treated with Proflavine (Figure 18A and B).

Studies have shown that altered Golgi phenotype observed is due to the activation of DNA-PKcs (Farber-Katz, Dippold et al. 2014, Anandi, Chakravarty et al. 2017) and altered Golgi phenotype was observed in Proflavine treated cells. To indentify whether the aberrant Golgi phenotype that is observed upon Proflavine treatment is due to the activation of DNA-PKcs, treated cells were fixed and stained for the known autophosphorylation sites of DNA-PKcs (Figure 18C).

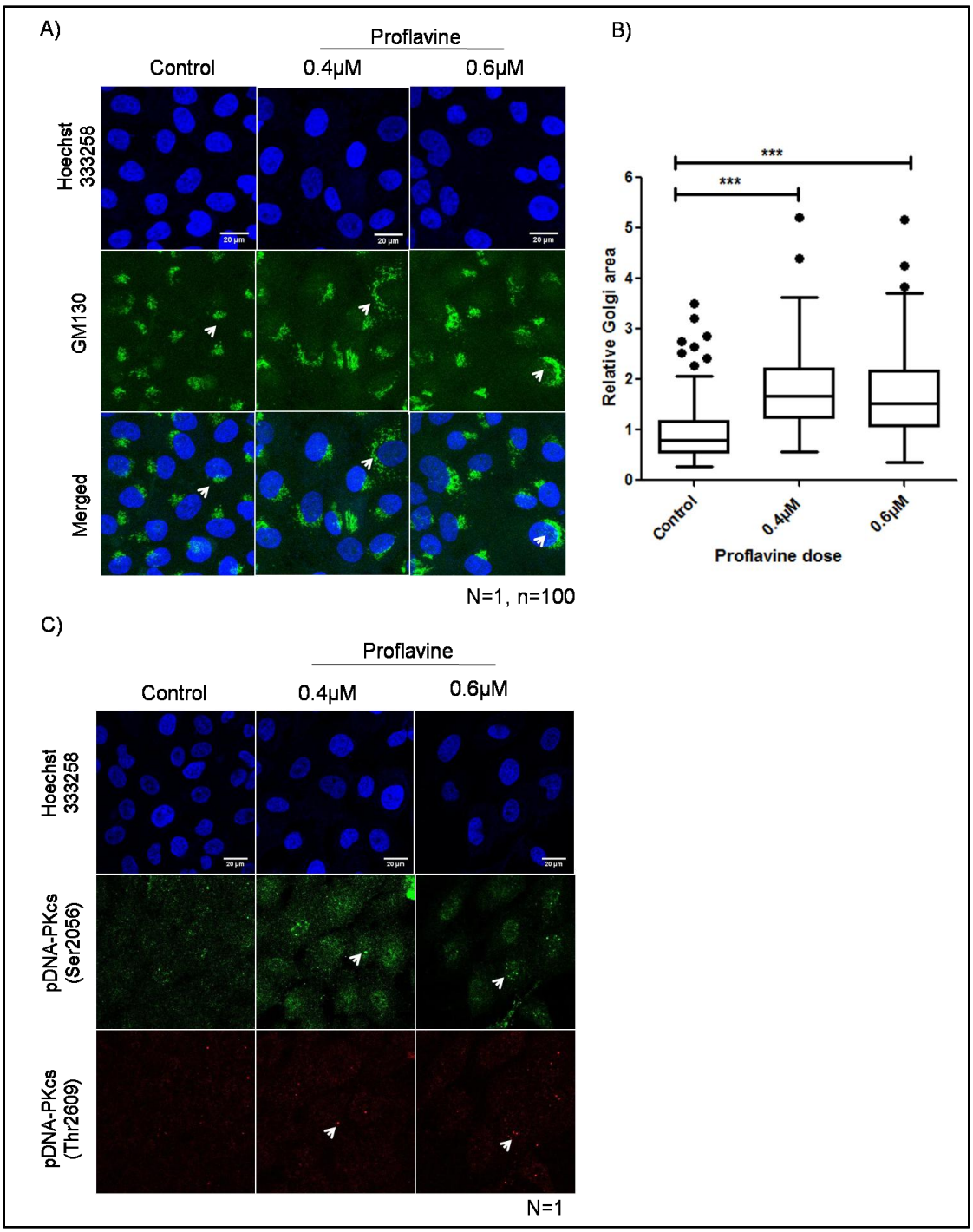

Figure 18: Cells treated with different concentration of Proflavine was incubated for 24 hrs. A) Dispersal of Golgi was observed Proflavine treatment. B) Images were quantified as relative Golgi area using One-way ANOVA and Bonferroni's multiple comparison test as the post test, ***p<0.0001. n=100 indicate number of nuclei used to calculate Golgi dispersal in an experiment. C) Activation DNA-PKcs at Ser2056 and Thr2609 sites were observed upon Proflavine treatment.

Discussion:

When a cell undergoes damage, it can lead to single stranded breaks (SSBs) or double stranded breaks (DSBs). SSBs when not repaired can be converted to DSBs and can be repaired. Unrepaired DSBs can be lethal to the cell or it can lead to mutations in genes which in effect may lead to cancer progression (Nowsheen and Yang 2012).

DNA-dependent protein kinase (DNA-PK) has a major role in the DSBs repair mechanism. It also has important role in triggering apoptosis upon severe DNA damage or critically shortened telomeres (Burma and Chen 2004). Different studies have shown the functions of DNA-PK other than DNA repair processes, redefining the paradigm of DNA-PK activation and signaling. Studies have also shown the implication for this protein kinase in tumor progression and therapeutic response (Goodwin and Knudsen 2014, Anandi, Chakravarty et al. 2017). Cytoplasmic response upon DNA damage was reported by Suzette E. Farber-Katz *et al.* where they observed a dispersal of Golgi through DNA-PK activation (Farber-Katz, Dippold et al. 2014). Similar results were observed in UV treated cells suggesting that UV induced damage can lead to dispersal of Golgi and activation of DNA-PKcs. It was also observed that DNA-PKcs activation precedes aberrant Golgi morphology in UV damage cells. Activation of DNA damage response proteins in the late time points and activation of checkpoint proteins suggest the activation DNA damage response pathway and checkpoint pathway. Activation of these pathways indicates that there is DNA damage repair, which might be the reason for the reduction in pDNA-PKcs levels in late time points.

No inhibitory studies of DNA-PKcs were done to check whether it leads to a reversal in Golgi phenotype to conclude that the altered Golgi phenotype that is observed upon UV damage is through the activation of DNA-PKcs. Since no experiments were also done to check for the activation of other sensor proteins such as ATM/ATR, it cannot be concluded that the altered Golgi phenotype that is observed upon UV induced damage is solely through the activation of DNA-PKcs.

Proflavine is widely used as a topical antiseptic. Studies have shown that proflavine can lead to mutation in bacterial cells as well as mammalian cells (Ripley and Clark 1986,

DeMarini, Brock et al. 1988). The preliminary results upon proflavne treatment show that it can lead to DNA damage. Preliminary results from this study also show that Proflavine treatment leads to aberrant Golgi phenotype and activation of DNA-PKcs. In order to identify whether DNA-PKcs is highly active in early time points in Proflavine treated cells, a time dependent study has to be done to check the levels of DNA-PKcs after Proflavine treatment.

DNA-PK activation is known to cause transformation of non-tumorigenic cells (Anandi, Chakravarty et al. 2017) and higher levels of DNA-PK is observed in most of the cancer cells (Goodwin and Knudsen 2014). This leads to the concern regarding the usage of the drug even though it shows anti-tumor activity, since activation of DNA-PKcs ws observed upon Proflavine treatment. So studies regarding the effect of Proflavine in non-tumorigenic cells are essential.

Future Plans:

Section 1:

- To investigate whether the dispersal of Golgi upon UV damage is through the activation of DNA-PK.
- To investigate whether inhibition of DNA-PK in UV damaged cells leads to reversal of Golgi.

Section 2:

- To investigate the level of DNA-PKcs activation in early time points after Proflavine treatment
- To investigate whether Proflavine causes transformation of breast epithelial cells in 3D cultures.

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