EFFECT OF DSB DNA REPAIR MECHANISMS ON CHROMATIN MOBILITY

BY

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LIST OF ABBREVIATIONS

Ataxia telangiectasia and Rad3 related (ATR).

Base excision repair (BER).

Bleomycin (BLM).

Chromatin diffusion speed (*D*).

Diffractive optical element (DOE).

DNA dependent protein kinase catalytic subunit (DNA PKcs).

DNA damage response (DDR).

Double-strand breaks (DSBs).

Enhanced chemiluminescent (ECL).

Homologous recombination (HR).

Immunofluorescence (IF).

Irradiation (IR).

Liquid-liquid phase separation (LLPS).

Mean squared displacement (MSD).

Mismatch repair (MMR).

Mitomycin C (MMC).

Non-homologous end joining (NHEJ).

Non-targeting siRNA (siNT).

Nucleotide excision repair (NER).

Nucleotide transporter (NTP).

Poly (ADP-ribose) polymerase (PARP).

Photoactivatable green fluorescent protein (PAGFP).

Replication protein A (RPA).

Single-strand annealing (SSA).

Standard deviation (S.D.).

TP53 binding protein (53BP1).

Ultraviolet rays (UV)

ABSTRACT

Nuclear DNA of eukaryotes is compactly packaged with histone proteins to form chromatin. Chromatin organization and the constant motion of the DNA may regulate genomic functions like gene expressions, DNA synthesis, and DNA repair. Two major pathways are used for repair of DNA double-strand breaks (DSB): non-homologous end joining (NHEJ) and homologous recombination (HR). The reciprocal dependencies of repair by HR and NHEJ and chromatin mobility are largely unknown in higher eukaryotes. We are characterizing these dependencies using osteosarcoma cells coexpressing histone H2A tagged with photoactivable GFP (PAGFP-H2A) and a DNA damage sensor (mCherry-53BP1). Cells are treated with DSB-causing agents like mitomycin C and bleomycin after a specific inhibition of either DSB repair pathway by a RAD51 inhibitor (HR) or a DNA-PK inhibitor (NHEJ). In addition, we use siRNA-based inhibition of NHEJ as a complementary approach to manipulate DSB repair pathway usage. Motions of chromatin microdomains (PAGFP-H2A) are captured using structured illumination with a diffractive optical element. The system produces a 7x7 array of photoactivated chromatin spots. Motions of the 49 spots are captured with time-lapse imaging and particle tracking and related to DNA damage presence (mCh-53BP1). Here, we present validation of the approach and preliminary results on chromatin dynamics in HR vs. NHEJ repair. We find that the chromatin motions when NHEJ is inhibited was not different but surprisingly, when HR is inhibited, the global chromatin mobility increases in the absence of DNA damage compared to untreated cells. We also find that the chromatin mobility is faster at the break sites compared to non-break sites. For further investigation on the effect of these DSB repair pathways and their effects on chromatin dynamics, cell cycle guided analysis will be required. Indeed, HR repair is mostly restricted to the S phase of the cell cycle. To this end, we have implemented a novel method to select cells in S phase for live cell cycle analysis. This approach will be used for the continuation of this project.

INTRODUCTION

CHROMATIN STRUCTURE, ORGANIZATION, AND FUNCTION

The DNA in the nucleus is highly condensed and present in the form of a protein-DNA complex known as chromatin (Widom 1998; Boyle et al. 2001). The major proteins present in chromatin are called histones. A short DNA stretch of 147 bp is wrapped in 1¾th turns around an octamer of histone proteins called nucleosome. The histone octamer has two H2A-H2B heterodimers and one tetramer complex of H3 and H4 heterodimers. H1 linker histone present at the end of the octamer helps in the organization of the linker DNA which connects two consecutive nucleosomes. Such a state of chromatin structure is known as a nucleosome filament. The DNA and histone proteins interact with each other with salt bridges, electrostatic interactions, hydrogen bonds, and nonpolar interactions. The core histone proteins have domains protruding from the core referred to as histone tails. The H2A and H2B have similar domains at their C termini whereas the other core histone proteins have ~10-40 aa-long and highly positively charged N-terminal regions (Widom 1998). These tails are highly dynamic and mobile and are greatly conserved all over evolution and are often the sites of post-translational modifications important in chromatin function (Widom 1998). These nucleosomes serve as the lowest level of chromatin organization and are the fundamental units of chromatin structure. Chromatin fibers and nucleosomes are highly dynamic in nature as a consequence of nucleosome arrangement, nucleosome positioning for protein-DNA interaction, and post-translational modifications of histones (Boyle et al. 2001; Dion and Gasser 2013). H1 histones found as a part of the linker are freely exchanged for the local unwrapping of a turn (~ 6 nucleosomes) (Widom 1998). Dynamic histone exchanges in nucleosomes account for the suggested “site exposure model” needed for protein-DNA binding as it is one of the essential steps in activities like transcription and DNA repair (Widom 1998). The 30 nm fiber (often depicted in textbooks in a higher compaction state) has been controversial as the chromatin’s dynamicity disproves the rigidity that the 30 nm fiber model implies. Instead, the chromosomes in the nucleus exist in their territories in a distinct nuclear space, hence called chromatin territories (Cremer and Cremer 2010) (**Fig. 1**). Recent studies have shown that the chromatin exists in a less rigid state than previously envisioned (Gibson et al. 2019). The chromatin can undergo liquid-liquid phase separation (LLPS) (Gibson et al. 2019), which may promote chromatin organization. Histone H1 promotes such phase separation and increases the concentration of the nucleosomes present in the LLPS droplets (Gibson et al. 2019). Intrinsic properties of chromatin including the dynamic arrangement of histones, other DNA-associated proteins, and LLPS of nuclear sub-domains are thought to promote chromatin mobility. This dynamic aspect of the chromatin may aid in the processes of DNA replication, gene expression, and DNA repair.

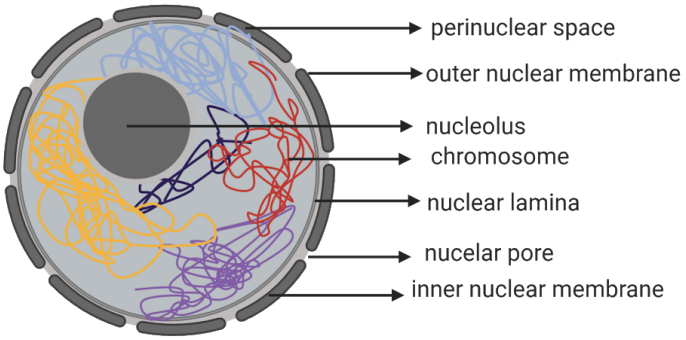


Figure 1. **Representation of nuclear organization.** Individual chromosomes are shown with different colors to illustrate the presence of defined chromosome territories.

DNA DAMAGE

Genome maintenance is highly important to safeguard the existence and continuation of life. The damage to DNA can be caused by exogenous and endogenous factors such as irradiation (IR), ultraviolet rays (UV), reactive oxygen species, chemical agents like cisplatin, mitomycin C, bleomycin, etc (Chatterjee and Walker 2017; Ciccia and Elledge 2010). DNA lesions are recognized and resolved by DNA repair mechanisms tailored to the nature of the lesion. DNA lesions can affect one of the two or both strands. The repair mechanisms for single strand break include base excision repair (BER), nucleotide excision repair (NER), and mismatch repair (MMR) (Abbotts and Wilson 2017). The double-strand breaks (DSBs) are repaired by double-strand break repair mechanisms, mostly non-homologous end joining (NHEJ) and homologous recombination (HR) (Chatterjee and Walker 2017), described in detail in the next section. All these repair pathways include multiple proteins that have specific functions such as DNA damage recognition proteins, endonucleases/exonucleases, polymerases for DNA synthesis, ligases for the sealing of DNA breaks and nicks. Primary proteins to respond at the site of the damage form a signalling cascade known as DNA damage response (DDR). DDR comprises several proteins such as BRCA1, 53BP1, ATM, ATR, PARP, and phosphorylated γH2AX (minor H2A variant) etc, that majorly influence the choice of the repair pathway (Giglia-Mari, Zotter, and Vermeulen 2011). Phosphorylation of γH2AX plays a critical role in DDR signalling and initiating DSB repair (Mah, El-Osta, and Karagiannis 2010). These factors play important roles in the maintenance of chromosomal integrity and genomic stability. Defects in the above mentioned pathways can lead to genomic instability and can cause diseases like cancer (Seeber, Hauer, and Gasser 2018).

For this project, we focus on DSBs with the rationale that DSBs are a major threat to genome integrity and arguably the most deleterious lesions linked with cancer

initiation and progression, as discussed below. Moreover, several studies indicate that DSBs affect chromatin mobility (Price and D'Andrea 2013; Seeber, Hauer, and Gasser 2018).

DSB REPAIR MECHANISMS

Non-homologous end joining

Mammalian cells predominantly use NHEJ for their DSB repair throughout the cell cycle except in S/G2 phase (Weinfeld and Lees-Miller 2012). NHEJ repairs by joining the two broken ends of the DNA (Malewicz, n.d, 2010; Hefferin and Tomkinson 2005) (**Fig. 2**). This pathway is also recruited in the V(D)J recombination during the maturation of the lymphocytes and results in the diverse repertoire of immunoglobulins found in B cell and T cell receptors in T cells (Malinowski and Pastwa 2006).

Ku80 is an important protein of the NHEJ repair pathway. It forms a heterodimer with Ku70 and binds to the chromatin at the break site (Postow et al. 2008). This Ku complex helps in the stabilization of broken strands. Ku80 plays a major role in the suppression of chromosomal rearrangements (Postow et al. 2008). It is among the first proteins to be recruited to the break sites. The catalytic unit of DNA dependent protein kinase (DNA PKcs) binds Ku80 C terminus. DNA PKcs help in the tethering of the broken ends of the DNA and thus bridging the broken ends (Falck, Coates, and Jackson 2005). These two proteins (Ku80 and DNA PKcs) are very important for the successful occurrence of classical NHEJ repair. Hence, these proteins were chosen as target proteins in this study.

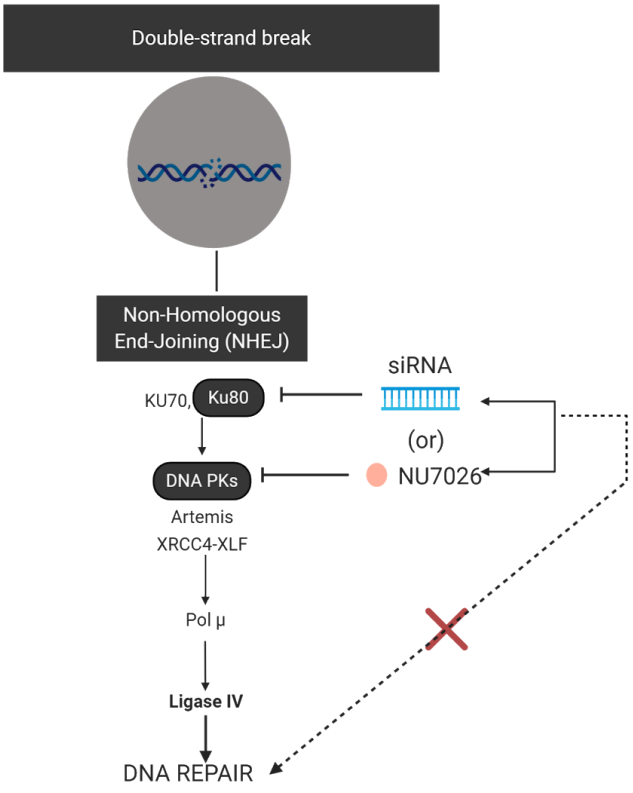


Figure 2. **Overview of the NHEJ pathway.** Ku proteins forming a heterodimer are the first to recognize the site of the damage and then recruit DNA Pks. DNA Pks autophosphylate and possess endonuclease activity (5' endonuclease and 3' endonuclease). Pol μ (Artemis) helps in the synthesis of the DNA (when DNA ends are not cohesive). Finally, the DNA ligase IV (together with structural factors XLF and XRCC4) is recruited, which reanneals the strands. In this study to examine the effect of NHEJ on chromatin mobility, the proteins Ku80 and DNA Pks were targeted. Ku80 was silenced whereas DNA Pkcs was inhibited by the drug NU7026 which binds to the catalytic subunit of DNA PK.

Homologous recombination

The HR pathway plays an important role in maintaining genomic stability. It is the pathway that is highly active in the S/G2 phases of the cell cycle. It requires nuclear exploration for the sister chromatid (homology search) to repair the damaged site. Since HR repair uses a homologous sequence as a template, it is essentially error-free, in contrast to NHEJ that can introduce mutations at the DSB sites.

HR requires the resection of the DNA, BRCA1 binds to the site and promotes the resection. MRN complex with CtIP initiates the resection of the break site, which frees the broken ends of the DNA. These free ends are stabilized by replication protein A (RPA). The next step in HR repair is homologous sequence search by RAD51 which is one of the important proteins involved in the HR repair (**Fig. 3**). RAD51 along with its family proteins like RAD51L1, RAD5L2, RAD51L3 help in the homology search and are essential for the successful occurrence of homologous recombination (Thacker 2005). RAD51 competes and displaces RPA, and stabilizes the broken strand (Thacker 2005). The other known activities of RAD51 include kick starting stalled replication forks, more specifically protecting the nascent DNA from MRE11 degradation during repair of stalled replication forks (Hashimoto et al. 2010). Hence, we chose RAD51 as the target protein for the inhibition of the HR pathway. When HR fails (e.g., due to mutations in Rad51 or other HR factors such as BRCA1), cells wrongfully use NHEJ for DSB repair, which leads to chromosomal aberrations, including chromosomal translocations. Chromosomal translocations are strongly associated with cancer, particularly chronic myeloid leukemia, burkitt's lymphoma, acute lymphoblastic leukemia, multiple myeloma, etc. (Huang and Mazin 2014; Nussenzweig and Nussenzweig 2010).

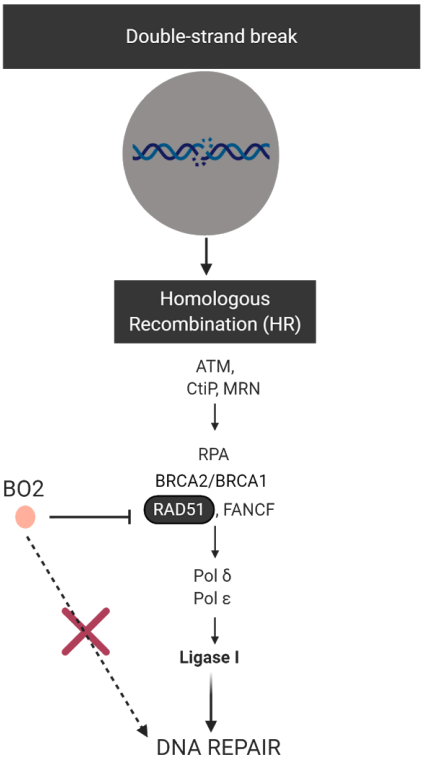


***Figure 3*. Overview of the HR pathway.** MRN (a complex of Mre11, Rad50, and Nbs1) recruits CtIP at the site of the damage and is the first to recognize the site of the damage and initiate resection of the DNA. RPA stabilizes the newly unwound single-stranded DNA that is to be repaired. RAD51 replaces RPA and helps in the search for the sister homologue for the repair. Polδ and polε are involved in the synthesis of the DNA to restore the broken sequence. Finally, the DNA ligase I seals the break. To study the effect of HR on chromatin mobility, the protein RAD51 was inhibited using the drug BO2 in this study.

CHROMATIN MOTIONS

The physical and intrinsic features of chromatin as mentioned above promote chromatin mobility. Chromatin motions have been measured in both lower eukaryotes (yeast) and mammalian cells. In both cases chromatin motions can be characterized by anomalous diffusion, meaning that chromatin diffusions obey a non-linear relationship between mean squared displacement (MSD) and time (Miné-Hattab and Chiolo 2020). MSD measures the displacement of a particle's position from its original position with respect to time (Di Pierro et al. 2018). These motions are influenced by (and are thought to influence) various mechanisms like cell cycle, DNA repair, transcription, nucleosome remodelling.

The chromatin motions can be of three types (Soutoglou and Misteli 2007):

1) Short-range motions: rapid (seconds), ATP dependent, random direction, regionally constrained motions within <1μM.
2) Long-range motions: motion observed up to several micrometers, slower (minutes), usually in the case of cell cycle, less frequently, motions are directional, ATP/motor dependent.
3) Genome reorganization: motions observed up to several micrometers, causes changes globally, takes hours/days, usually happens during cell division, differentiation, development.

In this thesis, we extensively explore the chromatin motions as a consequence of DSB repair mechanisms. We focus on a short range of motions, captured in short moves, as the DSB damage response (at least the initial steps) occurs within minutes.

CELL CYCLE AND CHROMATIN MOTIONS

Replication of DNA and cellular components occurs in distinct phases of the cell cycle, which culminates with the division of the cell into two daughter cells. The cell cycle phases consist of a S phase, where the DNA is replicated, in M phase the chromosome condensation, separation of the chromosome into two daughter cells, and cytokinesis occurs. Whereas the rest of the cellular components are replicated in gap phases called G1/G2 respectively. This complicated process involves cell cycle checkpoint proteins which help prevent cell cycle infidelity (Lyman 2003; Alberts et al. 2002).

The cell cycle status may affect chromatin motions because of the ploidy state of gene expression or replication activities. A study conducted in Hela cells on chromosome order with labelled chromosome territories showed that the chromosome territory motions during G1 were faster, compared to the later stage of the G1 phase and the motions of one chromosome territory affected the neighboring chromosome territory. Interphase to mitotic phase showed constrained chromosome territory movements (Hübner and Spector 2010; Walter et al. 2003). Studies with fluorescence tagged Lac operator reporter genes or fluorescently tagged histones

have shown that the chromatin during interphase in mammalian cells is constrained, minimal motion in mid S-phase, and increased motions in late G1/early S phase (Ellenberg 2013; Ma et al. 2019; Chuang and Belmont 2007).

DSB repair choice by HR vs. NHEJ is cell cycle-dependent (Campos and Clemente-Blanco 2020). The HR repair mechanism occurs during the S/G2 phase of the cell cycle (when a sister chromatid is available), whereas NHEJ predominantly occurs in G1. This pathway also dominates during G2 (You and Bailis 2010).

This cell cycle dependency for DSB repair highlights the need for investigations in the cell cycle's effect on chromatin mobility.

CHROMATIN MOBILITY AND GENOME MAINTENANCE

The chromatin motions observed in mammalian cells can be described by anomalous diffusion models and are highly dependent on ATP. Chromatin motions are largely an active process. As such, the depletion of ATP strongly reduces the diffusion of chromatin (Dion and Gasser 2013; Liu et al. 2015). As mentioned earlier, chromatin mobility is influenced by the cell cycle. The transcription of heterochromatin genes is accompanied by long range motions (at least in yeast), and also follows a non-linear type of diffusion (Dion and Gasser 2013). In compacted regions of chromosomes, transcriptional activity is linked with the mobility of chromatin. And finally, mobility of damaged DNA exhibits long range motions from the lesion site for repair (in yeasts and under certain circumstances in mammalian cells) (Dion and Gasser 2013). DSBs, when left unrepaired, lead to chromosomal rearrangements. DSB repair by NHEJ and HR may also depend on chromatin mobility. The HR pathway requires nuclear exploration for a sister homologue. NHEJ may exhibit spatial exploration and increased chromatin motion, in case of chromatin remodeling occurs at the DSB. The chromatin remodelling may affect the synapses of the free DSB ends or stabilization of the DSB free ends (Lottersberger et al. 2015). There is also evidence of a transient decrease in chromatin diffusion as a time dependent response (Liu et al. 2015). Thus, chromatin mobility plays a role in genome maintenance.

CHROMATIN MOBILITY IN MAMMALIAN CELLS

Chromatin mobility has been widely studied in yeast, which use the same repair mechanisms as mammalian cells (NHEJ and HR) to repair their DSBs. The nuclear exploration at the damaged site is called local mobility and the mobility at undamaged sites is known as global mobility. It has been reported that the stages of the cell cycle and the variation in the ploidy of the yeast have a significant effect on chromatin mobility (Mine-hattab et al, 2013; Soutoglou and Misteli 2007). In mammalian cells, the type of damage induced, variation of genomic organization between cell lines impact chromatin mobility have an effect over the chromatin mobility observed (Mine-hattab et al, 2013). In yeast, there is increased chromatin mobility in response to DNA damage and has been widely explored but in

mammalian cells it is largely unknown. Though yeast cells are eukaryotic in nature, they are a few key differences between yeast and mammalian cells:

1. Homologous recombination is used as a predominant type of DNA repair mechanism in *Saccharomyces cerevisiae* while NHEJ dominates in mammalian cells (Zimmer and Fabre 2019).
2. The size of the nucleus in a human cell is 80 times larger than that of *Saccharomyces cerevisiae.*
3. *Saccharomyces cerevisiae* lacks lamin and the nuclear envelope does not disrupt in mitosis (closed mitosis), in contrast to mammalian cells (Taddei et al 2013)
4. Mammalian cells have more constrained and complex chromatin organization in comparison to *Saccharomyces cerevisiae.*

These differences (and the fact that many more studies on chromatin dynamics have used yeast rather than mammalian cells) lead to a gap in knowledge on how DSB repair mechanisms influence chromatin mobility in mammalian cells. Thus, raising the need to investigate the impact of DSB DNA repair mechanisms on chromatin mobility.

MATERIALS AND METHODS

CELL CULTURE

U2OS osteosarcoma cells stably expressing photoactivatable GFP fused to histone H2A (PAGFP–H2A) and the C-terminal fragment of 53BP1 fused to mCherry (mCherry–53BP1ct) were cultured in DMEM/H14 supplemented with 10% FBS, 1 µg/ml puromycin, and 500 µg/ml geneticin at 37°C under 5% CO_2 in 35-mm glass-bottom dishes (MaTek). Puromycin and geneticin selection was to maintain expression of the two transgenes.

DNA DAMAGE INDUCTION

For induction of DSBs, cells were treated with 20 mU/ml of bleomycin (BLM; Calbiochem) for 1 hour before imaging. BLM is a radiomimetic drug that causes double-stranded breaks, even in non-dividing cells, by intercalating between the DNA strands (Dorr 1992). Alternatively, mitomycin C (MMC; 1 mM/ml) was added to the culture medium for 16 hours before imaging. MMC damage induces DNA cross links which get converted into double-stranded breaks during S phase and are predominantly repaired by HR (Lee et al. 2006).

INHIBITORS OF DSB REPAIR

For NHEJ inhibition, U2OS expressing PAGFP-H2A and mCherry-53BP1ct were treated with 10 µM of NU7026 (selleckchem) for 1 hour. NU7026 competes with the ATP domain of the DNAPks and inhibits its phosphorylation to prevent its activation (Skalitzky et al. 2003; Veuger et al. 2003).For HR inhibition, cells were treated with 10 µM of BO2 for 24 hours. BO2 disrupts the binding of RAD51 to the DNA and nuclear filament formation, thereby inhibiting HR repair (Mazin and Huang 2015).

siRNA-BASED NHEJ INHIBITION

Two days after seeding, cells were transfected with siRNA (Dharmacon; 20 **µ**M) for Ku80 or non-targeting control, using Lipofectamine 3000 (Invitrogen).

IMMUNOFLUORESCENCE FOR THE QUANTIFICATION OF 53BP1 REPAIR FOCI

Immunofluorescence was performed using antibodies against γH2AX (Millipore, 2 µg/ml) or Ku80 (Abcam,1 µg/ml). Cells were rinsed with PBS and fixed with 4% formaldehyde (Sigma) for 20 minutes, then washed with PBS supplemented with 50 mM glycine and permeabilized with TX100 (10%; Fisher bioreagents) for 10 minutes. Cells were incubated for 1 hour with the blocking buffer [10% goat serum in immunofluorescence buffer (IF:130mM Nacl, 13.2mM Na_2HPO_4, 0.1% bovine serum albumin, 0.05% NaN_3, 0.2% Triton X-100, and 0.05% Tween 20)]. After which the cells were washed with IF (3 times) for 10 minutes/wash and then were Incubated with secondary antibody anti-mouse Alexa Fluor-647 (Life technologies) for 1 hour at room temperature. The cells were washed with IF, (3 times) for 10 minutes/wash and stained with 4′,6-Diamidino-2-phenylindole dihydrochloride (DAPI; 0.5 µg/ml dilution

in PBS) for 10 minutes. Finally, the cells were rinsed with IF, and mounted in Prolong Gold Antifade (ThermoFisher). Fluorescent signals were imaged with a fluorescence microscope (Olympus IX83, using a 40x objective [NA = 0.95]). Imaging fields were randomly selected based on DAPI staining of cell nuclei. 53BP1 repair foci tagged with mCherry were quantified using a custom macro written in ImageJ (http://rsbweb.nih.gov/ij/).

WESTERN BLOT

Cells were lysed with 2% sodium dodecyl sulfate in phosphate buffered saline. The DC kit from BioRad was used to measure protein concentrations. Equal protein amounts were resolved by SDS-PAGE on precast gradient gels (BioRad), and transferred onto nitrocellulose membranes for immunoblotting. Primary antibodies used were Ku80 (Abcam, 1:1000) and H3 (Abcam, 1:1000). Horseradish peroxidase-coupled secondary antibodies were used for enhanced chemiluminescent (ECL) detection. ECL signals were captured using an Amersham 600 imager.

CHROMATIN MOBILITY

Chromatin mobility was measured by photoactivation of small chromatin microdomains using a custom laser system mounted on an inverted IX83 microscope (Bonin et al. 2018). This system is illustrated in **Fig. 4**.

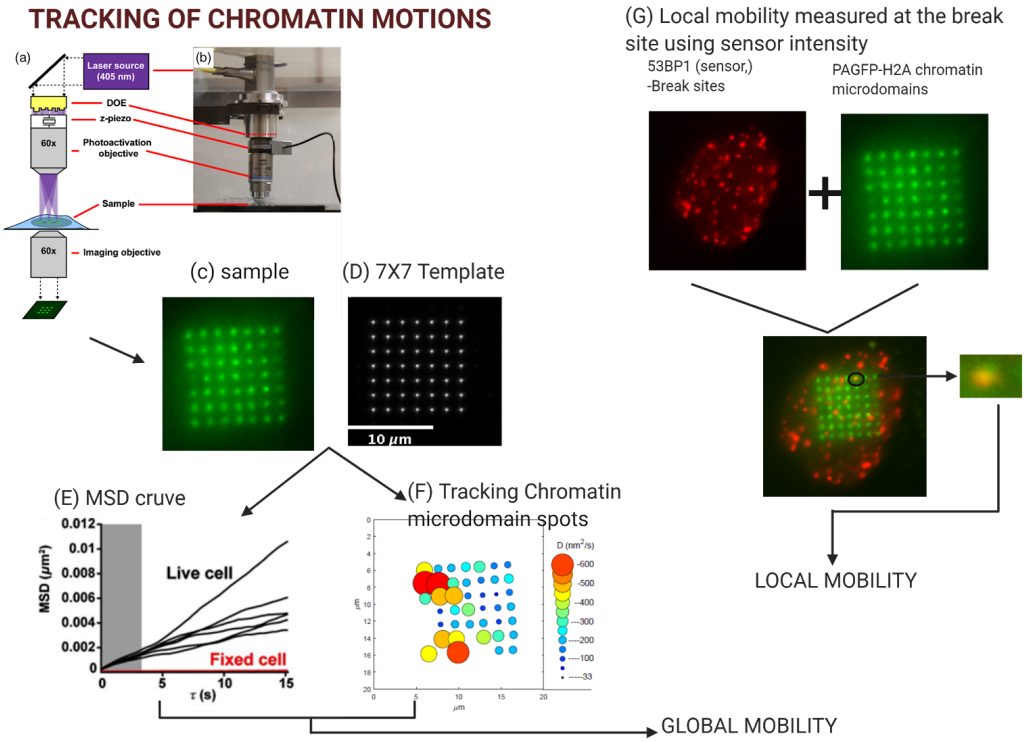


Figure 4. **Method to measure chromatin motions**. (**A**) Schematic of the instrumentation and (**B**) photograph of the custom diffractive optical element (DOE) module mounted on the condenser arm of an Olympus IX-83 inverted microscope. This structured illumination method uses a laser coupled to the DOE, which produces a 7x7 array of laser beamlets projected on the cell nucleus. (**C**) and (**D**) 49 different photo-activated GFP-H2A chromatin micro domains spots are captured at once to analyze chromatin mobility. Time-lapse moves of 200 frames for each cell in different treatment conditions are collected. (**E**) and (**F**) The chromatin diffusion (*D*) was calculated using the slope of the acquired mean squared displacement (MSD) curves. (**G**) The fluorescent images of mCherry-53BP1ct (sensor for DSB sites) are overlapped on the PAGFP-H2A chromatin microdomains images in order to guide analyses of chromatin diffusion at (or near) the break sites.

NUCLEOTIDE TRANSPORTER (NTP) FOR CELL CYCLE ANALYSIS IN LIVE CELLS

Cells were washed in tricine buffer at pH 7.4. The cells were then incubated with nucleotide transporter (SCT064; Milliporesigma,10 μM) and dUTP (CF568, #40005, biotium, 10 μM) were incubated for 10 minutes, and then replaced with DMEM/H14+10% FBS media for 50 minutes (total 60 minutes). The cells were imaged live at 37°C using a fluorescent microscope (10x and 60x oil objectives). Cells were fixed using formaldehyde and immunostained as mentioned above. Click Edu staining (fisher scientific) was done as mentioned in the Thermo Scientific protocol (Zawada et al. 2018).

NEUTRAL COMET ASSAY

Neutral comet assay (Trevigen) was performed using the protocol described in (Lu, Liu, and Yang 2017). The cells were treated with bleomycin and/or NU7026 for 1 hour before the assay. The images were captured by epifluorescence microscopy (10x objective) and analyzed using the open comet plugin in ImageJ (Gyori et al. 2014); http://rsbweb.nih.gov/ij/). Tail moment was calculated by measuring tail length multiplied by percentage of DNA present in the tail.

STATISTICAL ANALYSIS

Statistical analysis was done using GraphPad Prism version 9.0.0 for Windows. D'Agostino & Pearson test was done to determine if the data followed a non-normal distribution. When this was the case, data were analyzed using non-parametric tests like Mann-Whitney test and Kruskal Wallis test.

RESULTS

Double-stranded DNA breaks caused by the drug bleomycin remain unrepaired in the presence of DNA PK inhibitor NU7026.

We used the comet assay to validate the inhibition of NHEJ with NU7026. In this assay, the fragmented DNA from the intact DNA travels faster in the low melting agarose gel in the presence of current. In the microscopic images, the 'heads' of the comet patterns indicate the intact DNA and the tails consist of damaged DNA fragments. The DNA is stained with SYBR-GREEN for visualization and quantification. The amount of damage is measured by quantifying the intensity and length of the tail relative to the head. The graph indicates the percentage of tail moment. The images complement the plotted data and indicate the occurrence of DSBs. As expected, the cells treated with bleomycin show an increase in the amounts of DSB damage compared to the control (**Fig. 5**). Further, accumulation of DSB in the presence of BLM + NU7026 shows that NU7026 effectively inhibits DNA repair, as expected of NHEJ repair pathway inhibitor.

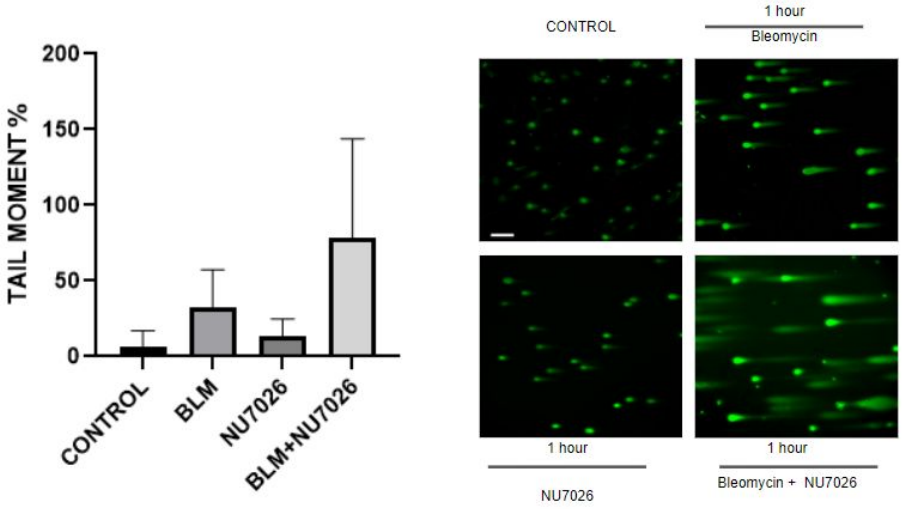
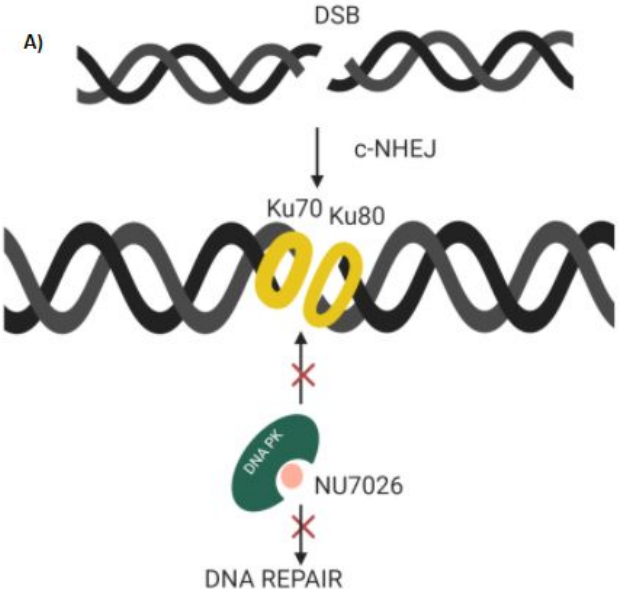
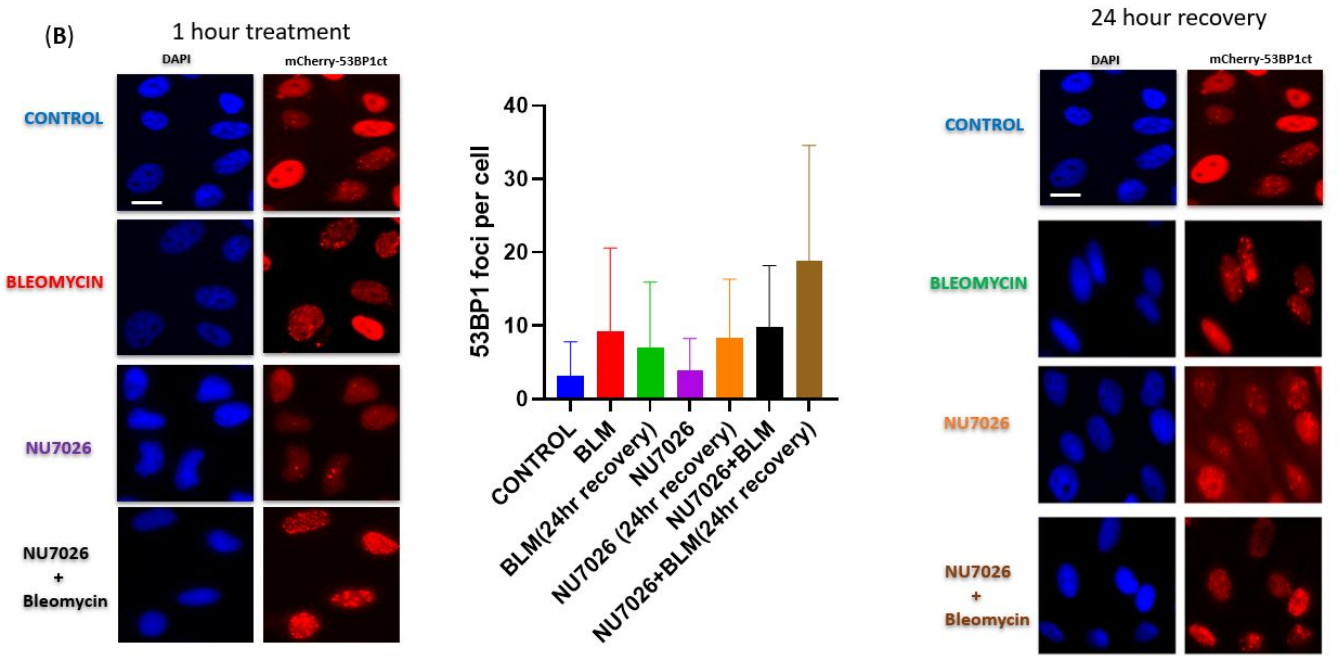


Figure 5. **Validation of NU7026 by neutral comet assay.** DSB induced by bleomycin in the absence or presence of NU7026 (DNA PK inhibitor) is confirmed by neutral comet assay. The data represent % tail moment per cell with S.D. as error bars. The induction of DSB by bleomycin is high and remains unrepaired in the presence of NU7026. Representative image panel on the right. (1 experiment, 50 nuclei per condition). Scale bar- 2µm.

NU7026 was further validated as a NHEJ pathway inhibitor by 53BP1 repair foci quantification. The average foci per cell in the cells treated for 1 hour with NU7026 were 3 foci per cell, but when left exposed to the inhibitor for 24 hours, the average foci per cell increased to 8 foci. The condition of cells treated with DNA damaging agent bleomycin for 1 hour the average foci per well were 9. After 24h recovery in the absence of the inhibitor, the number of foci decreased. In contrast, when the cells were left to recover in the media containing NU7026 without bleomycin for 24 hours, the average foci per cell further increased to 18 foci per cell (**Fig. 6B**). This clearly indicates that the NHEJ repair pathway is inhibited by NU7026, as repair foci accumulate in the presence of the inhibitor. Same observations were made when the cells were immunostained for γH2AX (**Fig. 6C**).




(B)
1 hour treatment
DAPI
mCherry-53BP1ct
CONTROL
BLEOMYCIN
NU7026
NU7026 + Bleomycin
53BP1 foci per cell
40
30
20
10
0
CONTROL
BLM
BLM(24hr recovery)
NU7026
NU7026 (24hr recovery)
NU7026+BLM
NU7026+BLM(24hr recovery)
24 hour recovery
DAPI
mCherry-53BP1ct
CONTROL
BLEOMYCIN
NU7026
NU7026 + Bleomycin

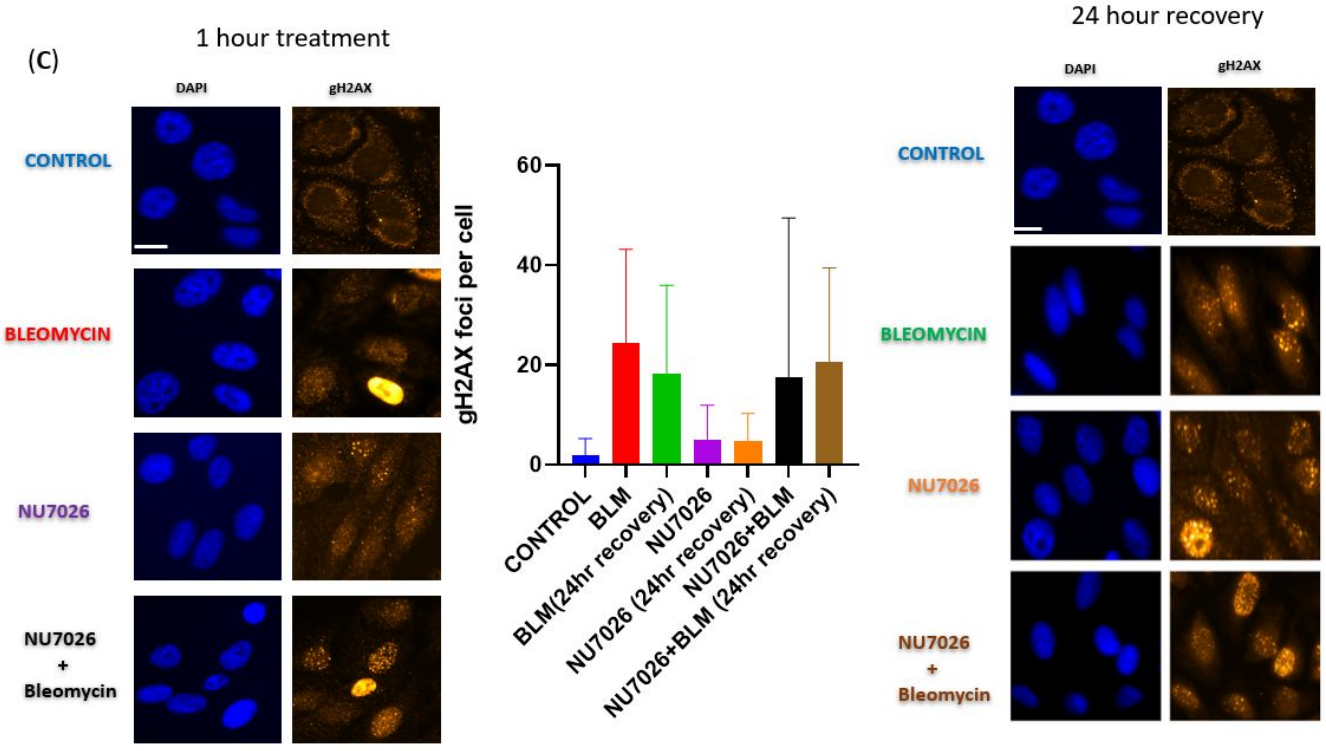

(C)
1 hour treatment
DAPI
gH2AX
CONTROL
BLEOMYCIN
NU7026
NU7026 + Bleomycin
gH2AX foci per cell
60
40
20
0
CONTROL
BLM
BLM(24hr recovery)
NU7026
NU7026 (24hr recovery)
NU7026+BLM
NU7026+BLM (24hr recovery)
24 hour recovery
DAPI
gH2AX
CONTROL
BLEOMYCIN
NU7026
NU7026 + Bleomycin

Figure 6. **Efficiency of NU7026 inhibition of the NHEJ pathway.**
(**A**) schematic of DSB repair when DNA PKcs is inhibited. (**B**) **&** (**C**) Cells were immunostained for 53BP1 and γH2AX and the repair foci were quantified. NU7026 was left in the dishes for 24 hours post treatment with 1 hour of bleomycin. The data represent foci per cell, error bars are S.D (1 experiment, 100 cells/condition). The graph indicates that in the presence of the NU7026, DSBs remain unrepaired. We see almost 50% increase in foci compared to recovery in presence of NU7026. Representative images are shown for 1 hour treatments on the right and 24 recovery with or without NU7026 for both mCherry-53BP1ct and γH2AX staining. Scale bar- 3μm.

Global chromatin mobility observed in the cells showed no significant changes by the drug treatments but chromatin motions at the DSB sites were faster than at non-break sites

DNA PKcs play an important role in the occurrence of the NHEJ pathway; this protein is essentially inhibited by NU7026 as mentioned above. To determine if NHEJ inhibition affects chromatin mobility, the global chromatin mobility was tracked using our custom DOE photoactivation system (see Methods). The selection of cells for imaging was guided with the mCherry-53BP1ct DSB sensor. The average chromatin diffusions per cell are plotted (**Fig. 7**). The differences in chromatin diffusions observed per condition were of no statistical significance. The chromatin diffusions corresponding to the sites of 53BP1 repair foci overlapping the photoactivated chromatin domains are graphed (high intensity regions) against the regions corresponding to non break sites (low intensity regions). The chromatin at the break sites was seen to move at a significantly faster speed compared to the non break sites (**Fig. 8**).

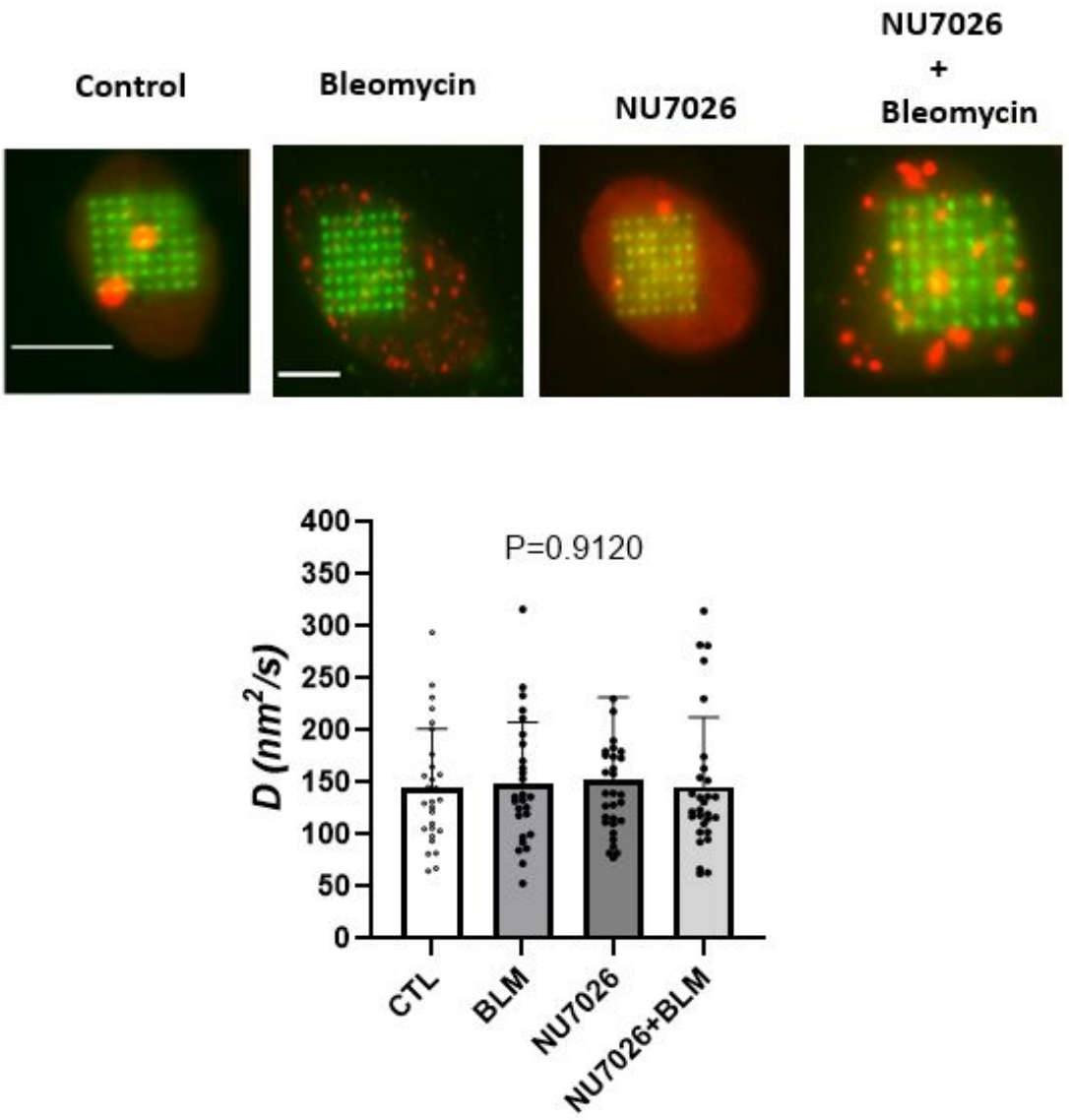


Figure 7. **Global chromatin mobility in NU7026 treated cells**: cells were chosen with the help of mCherry-53BP1ct foci to track chromatin mobility at different chromatin microdomains. The results showed no significant difference. The data represent mean and S.D as error bars (individual cell averages from 3 experiments, Kruskal wallis test, two-tailed). The image panel on top represents the cells in each condition. 10 cells/condition in each experiment, scale bar-10µm.

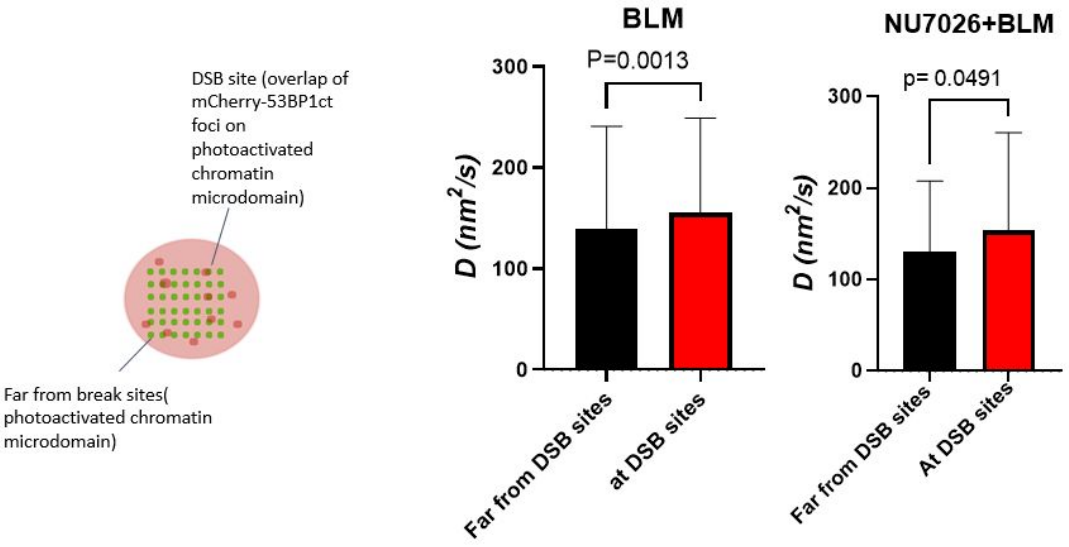


Figure 8. **Local chromatin mobility in NU7026-treated cells:** to track the chromatin mobility at the break site, we used the intensities of the mCherry-53BP1ct at each PAGFP-H2A microdomain. Microdomains at sites of high mCherry-53BP1ct intensity correspond to DSB repair foci. In the conditions with induced DSB (bleomycin, NU7026+bleomycin), chromatin mobility at DSBs shows a significant increase compared to regions far away from the break sites. The data represent the mean with S.D as error bar, (3 experiments, 10 cells/conditions in each experiment).Statistical comparisons with Mann Whitney test, two tailed.

NHEJ pathway inhibition by silencing Ku80 results in the accumulation of unrepaired 53BP1 foci.

After the successful pharmacological inhibition of NHEJ pathway, we employed siRNA-based inhibition to essentially confirm the observed effect of NHEJ on chromatin mobility. This approach avoids any off-target effect of pharmacological inhibition. Ku80 is the first protein to be recruited at the site of the repair and was therefore selected as a target for genetic inhibition of NHEJ. To validate silencing, the fluorescent intensities of Ku80 immunostaining were measured in cells transfected with siKu80 or non-targeting siRNA (siNT). The fluorescent intensities per cell are graphed in (**Fig. 9**). Silencing was further validated by western blot. The absence of a band in the lane with the sample of siKu80 cells clearly show that the Ku80 protein was successfully silenced (**Fig. 9**).

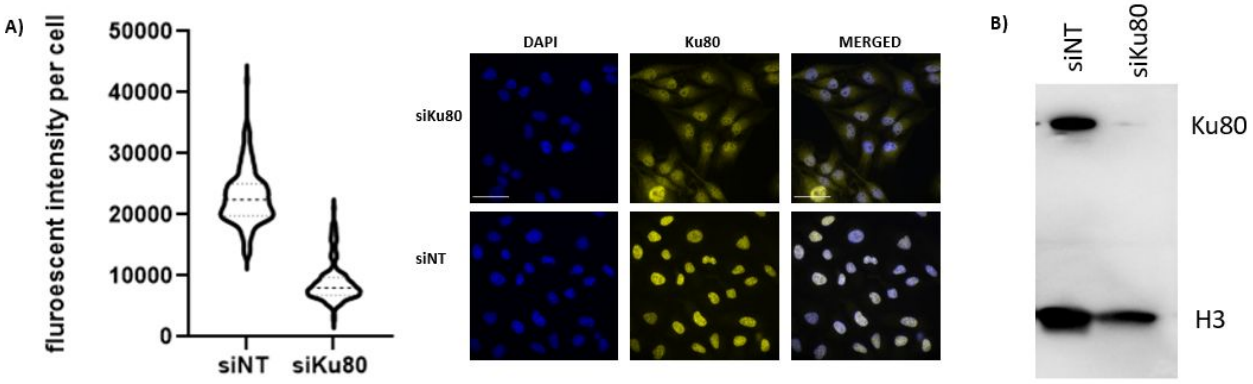
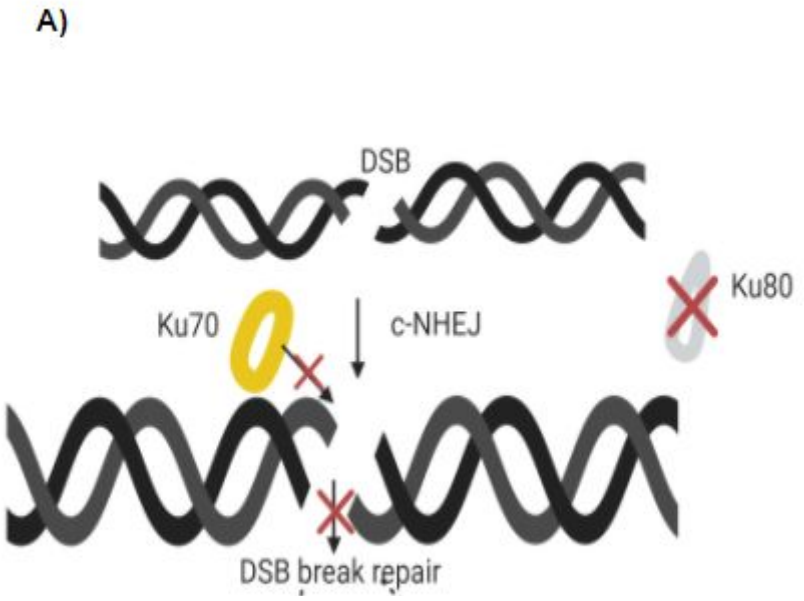


***Figure 9*. Validation of Ku80 depletion with siRNA.** (**A**) Detection of Ku80 by immunostaining in U2OS cells transfected with siKu80 or siNT. The data in the graph represent the mean fluorescent intensity and error bars are the S.D. (100 nuclei/condition, 1 experiment). Representative images are shown. Scale bar- 10**µ**m. (**B**) Immunoblot analysis of protein extracts from U2OS cells transfected as in (A) (representative result; shown immunoblot is from Dr. Locatelli). The results indicate successful silencing of the protein Ku80.

After validation of Ku80 silencing, we quantified mCherry-53BP1ct repair foci to verify inhibition of NHEJ repair pathway in the absence of Ku80. The 53BP1 foci after 1 hour of bleomycin treatment were quantified and compared in cells transfected with siKu80 and siNT. Again, BLM led to an increase in 53BP1 repair foci. This increase was more pronounced in siKu80 cells compared to controls. We also find increased repair foci in siKu80 cells in the absence of BLM, indicating that Ku80 depletion

prevents repair of endogenously-occurring DSBs (**Fig. 10**). Overall, accumulation of the 53BP1 foci (unrepaired DSB), showed that the NHEJ pathway is inhibited.



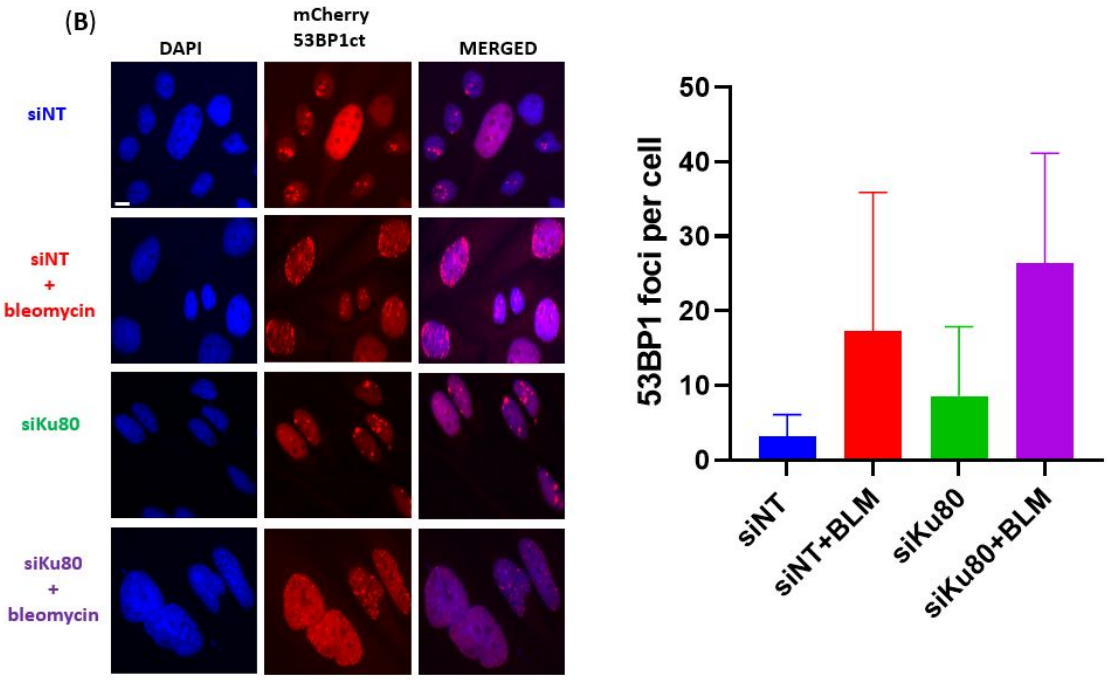


***Figure 10*. Efficiency of inhibition of the NHEJ pathway by silencing Ku80 validated by quantifying the appearance of 53BP1 repair foci.** (**A**) schematic for DSBs repair when Ku80 is silenced. (**B**) The cells were treated with bleomycin for the DSB break induction for 1 hour. The accumulation of mCherry-53BP1ct foci in the cells with silenced Ku80 and in the presence of bleomycin indicate that DSB breaks are left unrepaired. The image panel to the left illustrates quantification in the graph for each condition done. (1 experiment, 120 cells/condition). Scale bar- 3μm.

Global chromatin mobility when the NHEJ pathway inhibited by silencing Ku80 showed no significant changes but the chromatin mobility at the DNA DSB sites were significantly faster than at non-break sites

Ku80 plays an important role in the NHEJ pathway as mentioned above. The impact of NHEJ on global chromatin mobility when Ku80 is silenced showed no significant changes (see average chromatin diffusions per cell are plotted in **Fig. 11**). As described above, the cells were selected for imaging by the presence (BLM) or absence (no BLM) of mCherry-53BP1 foci. In contrast to the previous series of experiments, however, we note a tendency towards reduced global motions by BLM, as reported previously by the Vidi laboratory (Bonin et al. 2018; Liu et al. 2015). In siKu80 cells, The Chromatin diffusions corresponding to the sites of 53BP1 repair foci overlapping the photoactivated chromatin domains are graphed against the regions of non break sites or sites far away from the DSBs (**Fig. 12**). In siKu80 cells, the chromatin mobility at the break sites were faster compared to the non break sites. This effect was in line with the results obtained via pharmacological inhibition of NHEJ pathway.

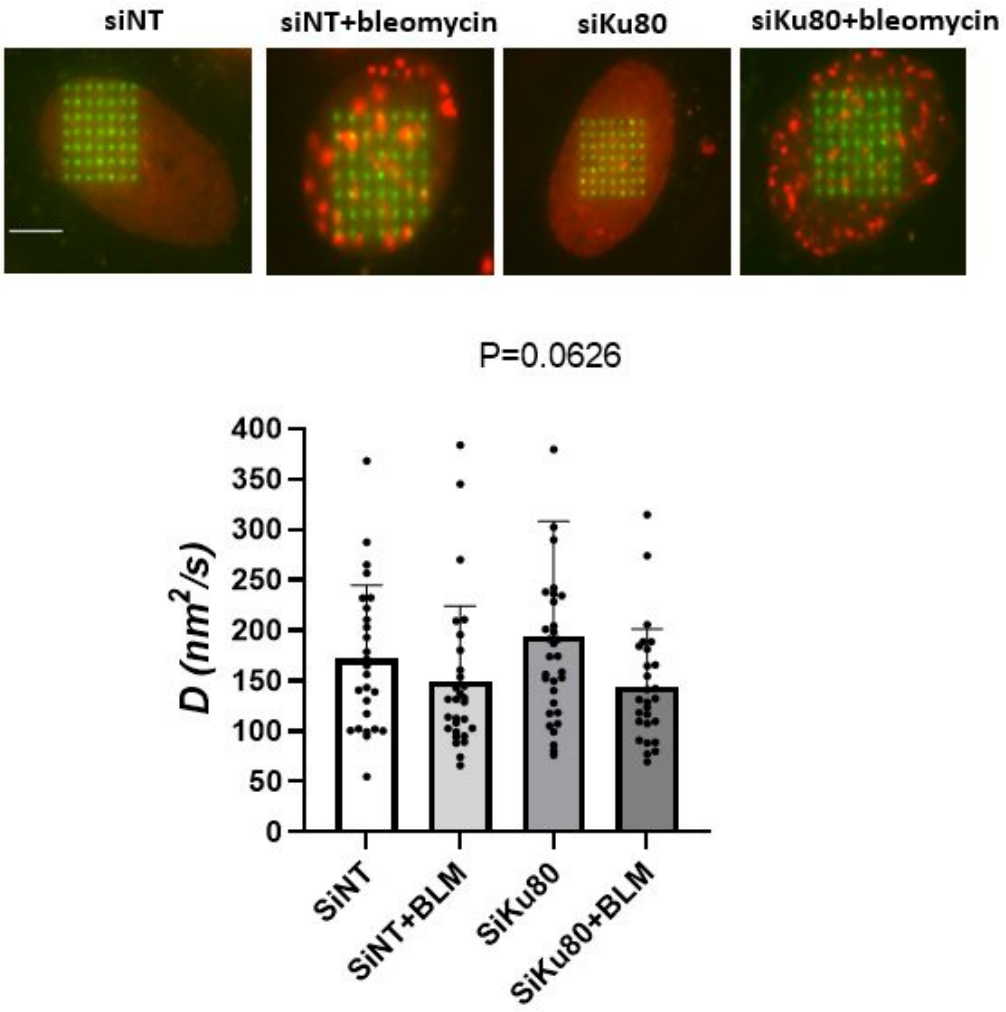


Figure 11. **Global chromatin mobility in cells with silenced Ku80:** The cells were selected by the mCherry-53BP1ct expression. Global chromatin mobility showed no significant difference across different conditions. The data represent individual cell averages from each experiment and S.D as error bars (3 experiments, 10 cells/ condition). Statistical analysis using Kruskal Wallis test, two tailed. Representative images of the condition (top). Scale bar-10 μm.

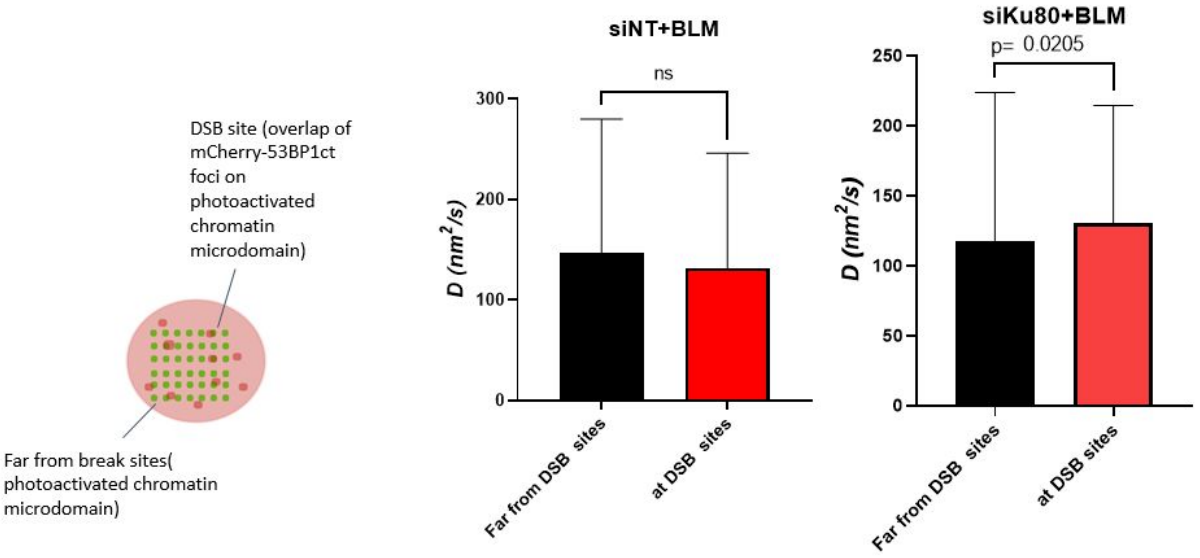


***Figure 12*. Local analysis of chromatin mobility in cells with silenced Ku80:** The break sites of cells with silenced Ku80 and treated with bleomycin show a significant increase in chromatin mobility compared to the distal sites. The break sites were chosen as mentioned above. The data represent the mean with S.D as an error bar, (3 experiments; 10 cells/condition in each experiment). Statistical comparisons with Mann Whitney test, two tailed.

Global chromatin mobility upon pharmacological inhibition of RAD51

We noticed when the NHEJ pathway is inhibited that the global chromatin mobility shows no statistically significant changes but the chromatin mobility at the break sites is generally faster compared to the non break sites. The next logical step is to address if/how the HR pathway affects chromatin mobility. We targeted RAD51 as mentioned above to investigate the effect of HR on chromatin mobility. BO2, a RAD51 inhibitor was used to inhibit HR pathway. Surprisingly, the global chromatin mobility was significantly higher in cells treated with BO2 (in the absence of detectable DSB). DSB induction with MMC restored mobility to control levels. (**Fig. 13**).

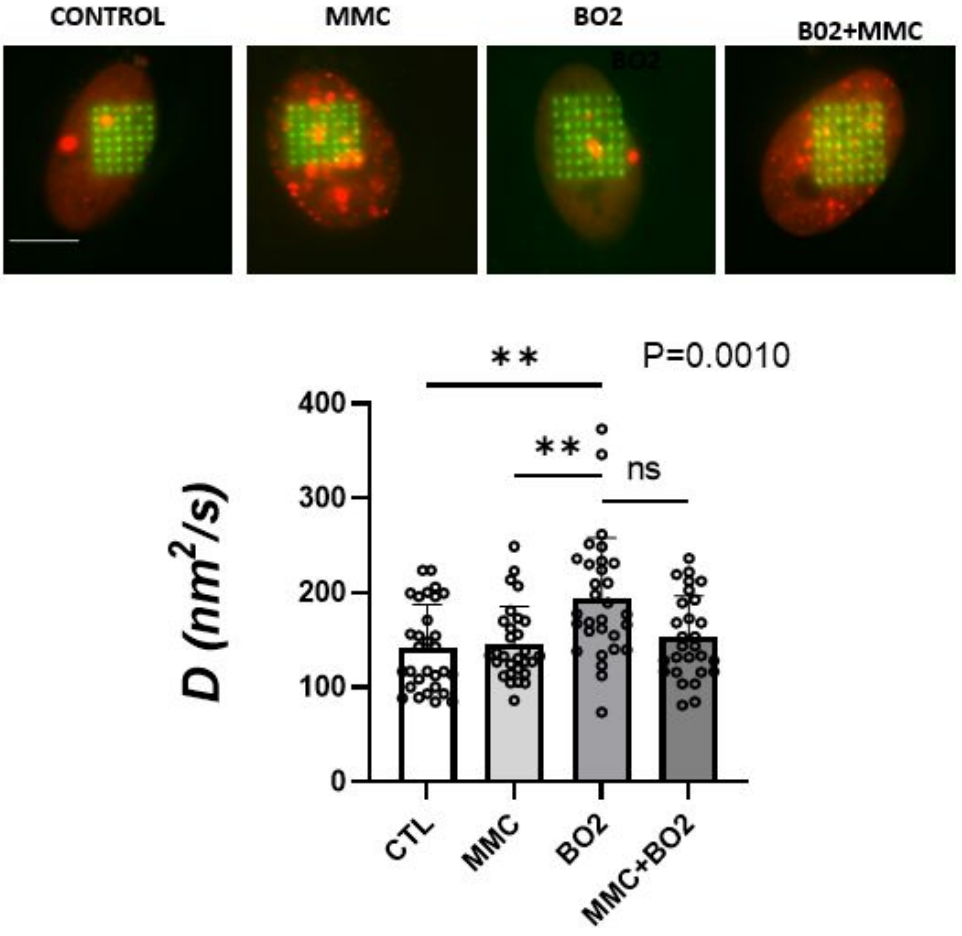


***Figure 13*. Global chromatin mobility in BO2 treated cells:** The condition of cells in the presence of BO2 and the absence of DNA damage by mitomycin C, the chromatin mobility observed was significantly faster compared to the conditions exposed to mitomycin C and control. The image panel represents (top) the conditions. The data represent chromatin diffusion averages per cell and error bars are S.D. Three experiments, 10 cells/condition in each experiment. (Kruskal Wallis test, two tailed test). Scale bar- 10 μm.

DISCUSSION

DSBs, when left unrepaired, lead to chromosomal rearrangements. DSB DNA repair mechanisms are non-homologous end joining and homologous recombination. The HR repair pathway requires nuclear exploration for homology search to repair the damaged site (Dion and Gasser 2013). Onset of a DSB causes the relocalization of the subnuclear compartment of the lesion, especially when it is strenuous to repair (Miné-Hattab and Chiolo 2020). The need for a homology search or relocalization demands chromatin to be mobile. This interdependency is poorly understood in mammalian cells. And so we studied the effect of the DNA DSB repair mechanisms on chromatin mobility.

First, we investigated the effect of NHEJ repair mechanism, as it is the predominant repair choice in mammalian cells. The presence of DNA damage by bleomycin and NHEJ pathway inhibition with an inhibitor (Britton, Coates, and Jackson 2013) and Ku80 silencing was first validated (**Figs. 5,6,9** and **10**). From this validation we observed an accumulation of 53BP1 repair foci. Several studies have shown that Ku proteins not able to activate by mutation or lack of complex formation get ubiquitinated for degradation. The inhibition of DNA PKcs has been reported to freeze the repair and not let the cell repair by either DSB repair mechanisms (Allen, Halbrook, and Nickoloff 2003; Postow et al. 2008; Weinfeld and Lees-Miller 2012; Malinowski and Pastwa 2006). These studies validate our finding accumulation of 53BP1 foci when NHEJ is inhibited.

Globally, BLM-induced DSB did not alter chromatin motions in these experiments. We note that in the second series of assays (using siRNA), there was a trend towards reduced motions by BLM (which was not seen in the first series of experiments with the NHEJ inhibitor). The Vidi laboratory has shown evidence for transient decrease in global chromatin diffusions (Liu et al. 2015). There is a possibility that each of these independent lines of experiment possess time discrepancy, which will be further investigated. In particular, we plan on using ionizing radiation (instead of BLM) to induce DSB in order to get better time resolution in the assays.

The global chromatin mobility observed in the cells (**Figs. 7** and **11**) was not altered after NHEJ inhibition, both in the presence and absence of DSBs. The Ku complex binds to the break site, stabilizing the two DSB ends (Fell and Schild-Poulter 2012). We therefore hypothesized that silencing Ku80 would increase DSB motions. Similarly, when break sites are left unrepaired, the free ends may be left unstabilized. Hence, the expected increase in the observed chromatin mobility (Lottersberger et al. 2015). However, our measure captures the motions of entire microdomains of chromatin (not only the broken ends) and suggests that NHEJ activity does not change microscale chromatin motions globally in the nucleus. However, we cannot exclude that these subtle differences were not captured in our assay. Indeed, the global chromatin mobility in a given cell is a measure of 49 photoactivated spots in the nucleus. We observe a large heterogeneity in chromatin microdomain diffusion, both between cells and within a cell nucleus (**Fig. 14**). This intracellular

heterogeneity is interesting. Each photoactivated chromatin microdomain may be located in the same chromosomal territory or in a different chromosomal territory. Although the chromatin in these chromatin microdomains of the same cell are in the same cell cycle, the nature of chromatin (either condensed or relaxed) likely to strongly affect the motions obtained at a specific site (Miné-Hattab and Chiolo 2020).

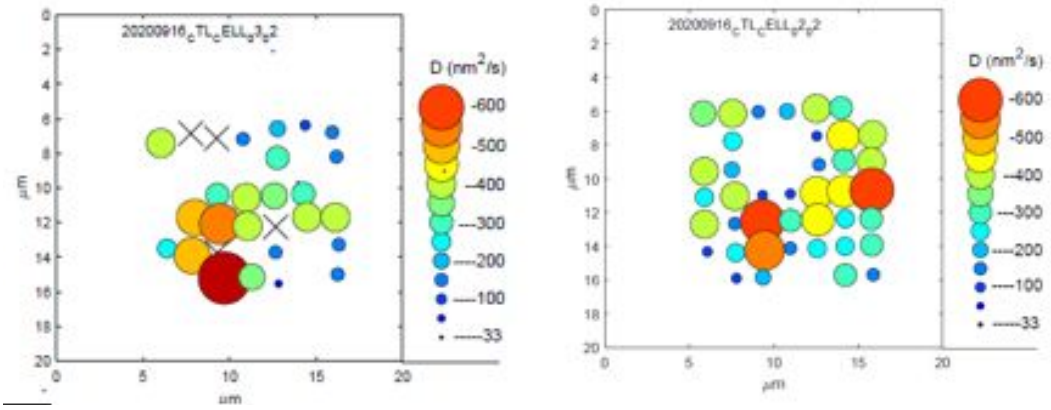


Figure 14. **Heterogeneous behavior of chromatin motions.** 'Bubble' plot of chromatin microdomain motions in two U2OS cells expressing PAGFP-H2A. The size and color of the circles reflect chromatin diffusion speed (*D*). Note heterogeneity within cells (**FIG.14**) and between cells (graphs in previous figures).

This heterogeneous behavior led to a more precise investigation on the effect of NHEJ pathway on chromatin mobility. We looked at the chromatin diffusions at the DNA break sites, the site where there is an overlapping of mCherry-53BP1ct repair foci on the photoactivated chromatin microdomain (PAGFP-H2A). This guided analysis led us to discover that there is an increase in the chromatin mobility at the DSB sites when compared to the nuclear regions devoid of damage (**Figs. 8** and **12**).

Why is the chromatin mobility at the break sites faster? In the presence of DSB, the break sites are bound to 53BP1. 53BP1 prevents the resection required for occurrence of HR. 53BP1 has been known to promote chromatin mobility (Lottersberger et al. 2015). As a consequence of 53BP1 accumulation to the break site, there may be an increase in the local mobility observed. To test this hypothesis, we will repeat these measurements in the absence of 53BP1 (using another DSB sensor instead of mCherry-53BP1ct).

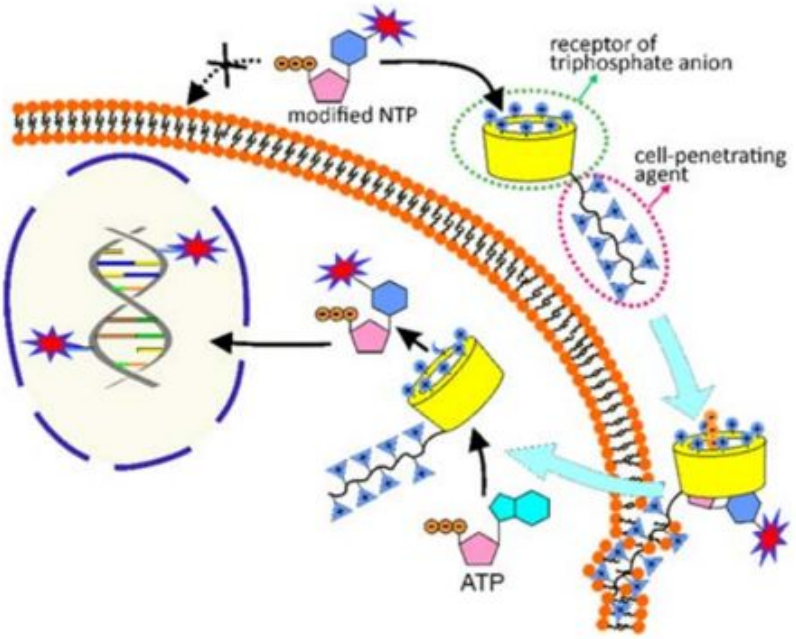
Unexpectedly, we observed a significant increase in the global chromatin mobility when the HR pathway is inhibited, in the absence of induced DNA damage. RAD51 is an important protein, involved in the HR pathway. It helps in the search of sister chromatids for the repair and displaces RPA (Thacker 2005). Other activities of RAD51 include inhibition of SSA (single-strand annealing mechanism), protection of the nascent DNA formed during the replication fork, and re-starting of stalled

replication forks (Hashimoto et al. 2010; Mason et al. 2019). In yeast, in the absence of RAD51, the broken end of the chromosome can search and invade its homologous template (Malkova, Ivanov, and Haber 1996). *Hypothesis*: when there is no drug-induced damage, the effect we see may reflect cells in S phase that may experience a deficit in the recruitment of RAD51 at stalled replication forks. Usually, RAD51 stabilizes the single free strand and (like in yeasts) its absence may compromise the stabilization of the forks. Also, the effect of BO2 on chromatin mobility may reflect a yet unrecognized function of RAD51 as the favourable concentration of BO2 used in our research may only inhibit 40% of RAD51 foci formation. This needs further exploration, in particular using silencing of RAD51, to determine potential off-target effects of BO2.

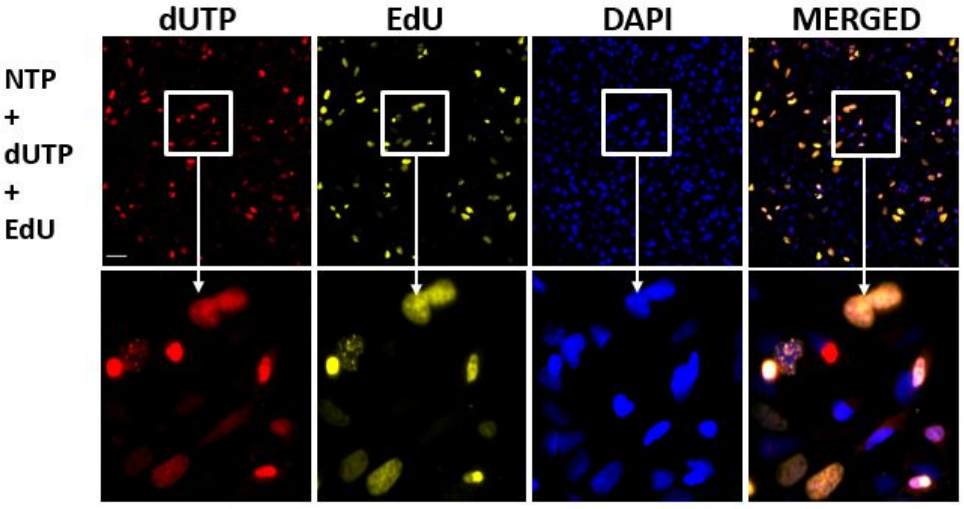
As DSB DNA repair mechanisms are cell cycle dependent, it will be important to consider cells in different cell cycle phases in follow up experiments to precisely explore the mechanisms regulating chromatin mobility. We were able to implement a novel approach to guide analyses in live cell imaging experiments based on cell cycle.

This assay relies on a nucleotide transporter system to highlight cells in S-phase (Zawada et al. 2018). The synthetically derived molecule acts as a transporter molecule, binds to the supplied fluorescent molecule tagged dUTP and is transported inside the cell. When the cell incorporates the fluorescent dUTP, we are able to observe it visually by live cell imaging within 10 minutes of the exposure to dUTP+ nucleotide transporter molecule (NTP) (Zawada et al. 2018). As our research requires live cell imaging, we believe that this system would be of great help. The NTP system (with fluorescent nucleotides) was compared against the gold standard - staining with EdU. The NTP + nucleotides staining showed that the transporter aided the entry of fluorescent dUTPs in the cells. Cells in S phase could be clearly identified (**Fig.15**).

(A)


receptor of
triphosphate anion
modified NTP
cell-penetrating
agent
ATP

(B)


dUTP
EdU
DAPI
MERGED
NTP
+
dUTP
+
EdU

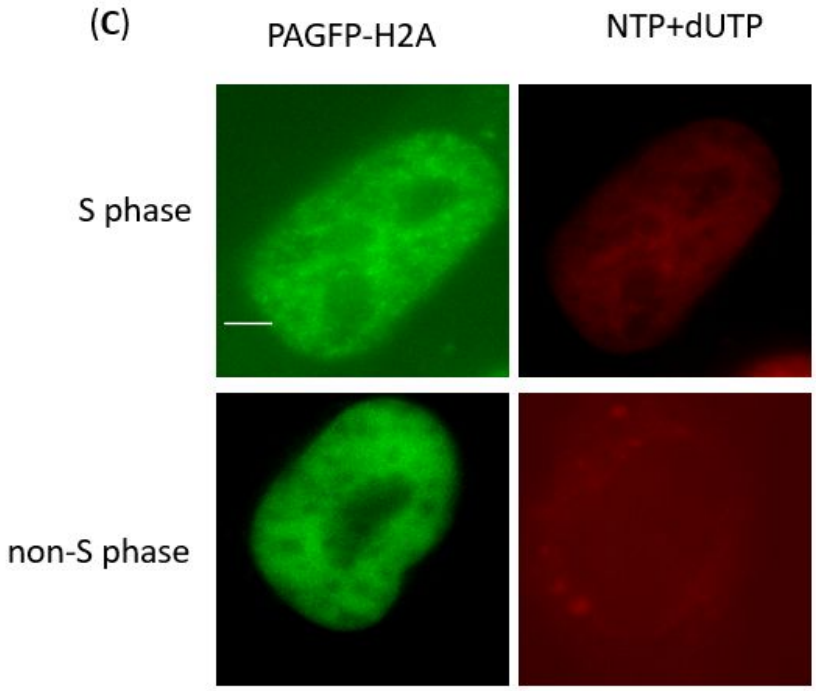


Figure 15. **Validation of nucleotide transport system against EdU staining** (**A**) A schematic of the nucleotide transporter (NTP) mechanism in the presence of dUTP (Zawada et al. 2018). (**B**) Representative images with their zoomed-in images for the key experimental conditions. Scale bar- 2µm. (**C**) Live cell imaging of NTP+dUTP with one cell with and one cell without dUTP fluorescence. Presence of the nucleus is indicated with the GFP-tagged H2A. Scale- 10 µm.

This NTP system can be easily incorporated and used to reduce the heterogeneity of chromatin motions due to cells being in various cell cycle phases. We can easily identify the cell in S-phase. This would be our next phase of investigation.

CONCLUSION

This thesis explored the interdependent relationship between chromatin mobility and DNA DSB repair mechanisms. We find that chromatin mobility is not altered globally, when the NHEJ pathway is inhibited. Surprisingly, global chromatin mobility measured in the presence of an HR inhibitor (but no induced damage) was significantly faster compared to untreated cells. Most interestingly, our spatial analysis of chromatin dynamics reveals that chromatin diffusion at the break sites is significantly faster compared to the non break sites. Further investigations on the relationships between NHEJ or HR and chromatin motions will require cell cycle-based guided analysis. The cell cycle-based selection of cells will not only give more insight on the DSB DNA repair mechanisms in specific contexts but also shed light on the different effect each cell cycle phase has on chromatin mobility. We anticipate that this study and its continuation will further our understanding of chromosomal translocations, a type of mutations that lead to multiple cancers, since the biogenesis of translocations is thought to necessitate chromatin motions.

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EDUCATIONAL QUALIFICATION

Master of Science in Biomedical Sciences August 2019 – December 2020
Current GPA: 3.32/4
Wake Forest University, Winston Salem NC

Bachelor of Technology in Cancer Biotechnology August 2015 – May 2019
GPA: 3.71/4
B.S. Abdur Rahman Crescent Institute of Science and Technology, Chennai India

CERTIFICATIONS

Medical Biomaterials, IIT Madras – Chennai NPTEL – 2019

THESIS

- "Effect of DSB DNA repair Mechanism on chromatin mobility", November 2019- December 2020 Masters (Advisor : Dr. Pierre-Alexandre Vidi, Assistant Professor, Cancer biology)

AWARDS & HONOURS

- Awarded **Gold Medal** for securing the Highest GPA in the department of cancer biotechnology (2015-2019).
- Secured **2nd place** in the Undergraduate research symposium (poster presentation, topic: Comparison of lipid content between different Indian brands of sunflower oil), in School of life sciences, B.S. Abdur Rahman crescent institute of science and technology, 2016.
- **Won** "Innovation and Research Symposium For students" (Paper presentation topic: "Screening of Herbal Matrix Metalloproteinases inhibitors to control Metastasis."), in School of life sciences, B.S. Abdur Rahman crescent institute of science and technology, 2019.

PROJECTS

- Screening of herbal Matrix metalloproteinase inhibitors to control Metastasis, 2018- 2019 Undergraduate, (Advisor: Dr. S. Hemalatha, Professor and Dean, School of Life Sciences).
- Epidemiological study: Impact on digestive system due to altered circadian rhythm, 2018.
- Ginger -Extraction of Alkaloid, Phytochemical assay by TLC and its antimicrobial effect, 2017
- In silico analysis of P53, 2016
- Comparison of lipid content between different Indian brands of sunflower oil, 2016.

TEACHING EXPERIENCE/ WORK EXPERIENCE

- **Research Lab technician 1, Vidi laboratory, Wake Forest Baptist health medical center, Winston-Salem, NC** July 2020- present
  - Weekly organization of the lab
  - Part-time
- **BIO3342- INTRODUCTION TO MOLECULAR BIOLOGY** 11th - 13th February 2020
  - SUPERVISOR: Dr. David Kump, ph.D.,
  - Gave a lecture on the topic "DNA damage and repair and Transfection – technique"
  - Prepared assignment for the familiarization of the jargon and definitions of the words.
  - Clicker questions to comprehend students' level of understanding.
  - Addressed Junior and Senior students of Biology major (WSSU)

- **Intern at National Institute of Mental Health and Neuroscience (NIMHANS), Bangalore, India.**
  May2018 – June 2018

  - Worked in electrophysiology lab, autonomic function test lab, cell culture lab, sleep lab and behavior labs.
  - Observed and learnt laboratorial techniques.
  - Attended Classes on neurophysiology and Neuroanatomy.

- **Content developer, Wise Corner Laboratories Pvt Ltd, Chennai, India**
  January 2018 – May 2018
  - Collection of Data on recent advances made in CRISPR CAS-9 system
  - Workshop organizer and content developer
  - Part-time

POSTER AND PAPER PRESENTATIONS:

- Poster presentation on the topic "Effect of DSB DNA repair mechanisms on chromatin mobility", American Society of cell Biology, USA, December 2020.
- Poster presentation on "Screening of herbal matrix metalloproteinases", National Conference on Biochemistry and therapeutic of Diabetes and cancer treatment and challenges, Loyola college, Chennai, 2019.
- Poster presentation on "Screening of herbal matrix metalloproteinases" Recent Trends in bioinorganic chemistry, Loyola College, India, 2019.
- Paper presentation on "Screening of Herbal Matrix Metalloproteinases inhibitors to control Metastasis", Innovation and Research Symposium For students, School of life sciences, B.S. Abdur Rahman crescent institute of science and technology, 2019.
- Poster presentation on "Comparison of lipid content between different Indian brands of sunflower oil", School of life sciences, B.S. Abdur Rahman crescent institute of science and technology, 2016.

ORGANIZATIONS:

- Organized & was the coordinator for the national level symposium, Exon in the year 2016 & 2018 for school of life sciences, B.S. Abdur Rahman crescent institute of science and technology.
- Held the position of Vice President of Biotech association, School of life sciences, B.S Abdur Rahman crescent institute of science and technology,2016.
- Member of Crescent Innovation and Incubation Center (CIIC),India.
- Student member of the Society of Pharmaceutical Education & Research (SPER), India.

REFERENCES

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   Wake Forest University,
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