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DNA Double Strand Breaks: To Repair, or Not to Repair

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Dedication

I dedicate my thesis work to my mom, who's also a mentor in my life. As a dedicated doctor, she guides me to explore the intricacies of medicine and fuels my interest in basic medical science. She has been my source of inspiration and strength whenever I thought of giving up. I will be forever grateful for her unconditional love and continued spiritual, emotional, and financial support.

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List of Abbreviations

DNA	Deoxyribonucleic Acid
DSB	Double-Strand Break
ROS	Reactive Oxygen Species
SSB	Single-Strand Break
IR	Ionizing Radiation
NHEJ	Nonhomologous End-Joining
HR	Homologous Recombination
SSA	Single-Strand Annealing
ICL	Interstrand Crosslink
ssDNA	Single-Stranded DNA
RPA	Replication Protein A
D-loop	Displacement Loop
dHJ	Double Holliday Junctions
ATM	Ataxia Telangiectasia Mutated
ATR	ATM Rad3-Related
DDR	DNA-Damage Response
CHK1	Checkpoint Kinase 1
hRad51	Human Rad51
dsDNA	Double-Stranded DNA
PLC	Phospholipase C
DAG	Diacylglycerol
IP_3	Inositol Trisphosphate
PKC	Protein Kinase C
dNTP	Deoxyribonucleoside 5'-Triphosphates
ORC	Origin Recognition Complex
Cdc	Cell Division Cycle
pre-RC	Pre-Replication Complex

Cdt1	Chromatin Licensing And Dna Replication Factor 1
MCM2-7	Minichromosome Maintenance Proteins
CDK	Cyclin-Dependent Kinase
DDK	Dbf4-Dependent Kinase
Cdc45	Cell Division Cycle 45
PCNA	Proliferating Cell Nuclear Antigen
RFC	Replication Factor C
Polα	DNA polymerase α
MMR	Mismatch Repair
Hop2	Homologous Pairing 2
Mnd1	Meiotic Nuclear Divisions 1
Polδ	Polymerase δ
BER	Base Excision Repair
ID	Insertion/Deletion
UV	Ultraviolet
TDG	Thymine DNA Glycosylase
NER	Nucleotide Excision Repair
SSBR	Single-Strand Break Repair
PARP1	Poly(ADP-Ribose) Polymerase 1
pADPr	Poly(ADP-Ribosylation)
DNA-PKcs	DNA-Dependent Protein Kinase Catalytic Subunit
XRCC4	X-ray cross - complementing Group 4
XLF	XRCC4-like factor
BRCA2	Breast Cancer Associated Gene 2
MRN	Mre11-Rad50-Nbs1
PI3	Phosphatidylinositol 3
PIKK	PI3K-like Protein Kinase
FAT	FRAP-ATM-TRRAP
FATC	FAT Carboxy-Terminal

HEAT	Huntingtin, Elongation Factor 3, Protein Phosphatase 2a, TOR1
Mdc1	Mediator Of DNA Damage Checkpoint Protein 1
GFP	Green Fluorescent Protein
DR	Direct Repeat
FBS	Fetal Bovine Serum
MTT	1-(4,5-Dimethylthiazol-2-yl)-3,5-diphenylformazan
SDS-PAGE	Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis
S6K1	S6 kinase beta-1
GSK3β	Glycogen Synthase Kinase 2
HGF	Hepatocyte Growth Factor
EGF	Epidermal Growth Factor
MDM2	Mouse Double Minute 2 Homolog
PRD	PIKK-Regulatory Domain

Abstract

DNA damages that cause double-strand breaks (DSBs) to the chromosome are most harmful. Subsequent choices have critical consequences for cell fate. Without repair, cells will face certain death. Low-fidelity repair will introduce mutations that could transform the cells, leading to carcinogenesis.

How cells make the decision is not well-understood. A single DSB can lead to apoptosis for some cells, whereas others can repair up to 25 DSBs and survive. It has been postulated that decision to repair DSBs is a stochastic process.

In the nucleus, DSBs elicit a cascade of signaling events that require the recognition, protection, processing, and subsequent repair of these breaks. Repair through homologous recombination (HR) process preserves genomic stability. The proteins involved in HR, such as MRN, ATM, RAD51, BRCA1/BRCA2 and others have been extensively characterized. Loss of function mutations of these proteins renders cells unable to repair DSBs through HR and increases propensity for genomic mutagenesis.

We hypothesize that nuclear signaling events are insufficient for cellular decision to repair DSBs through HR. In addition, the decision also relies upon the presence of pro-survival factors and subsequent activation of signaling pathways. Previous studies have indicated that phosphorylation of proteins such as BRCA1, BRCA2, or RAD51 might play a role in the regulation of HR activity. An example involves the phosphorylation of RAD51 by kinases downstream of phosphatidylinositol 3-kinase-related kinase (PI3K) pathway. However, the functions of many signaling molecules in DNA damage response are still uncertain. Using specific inhibitors of protein kinases, we aim to delineate the signaling molecules involved during HR repair.

Using a cellular HR-reporter assay, we demonstrate that the activation of mitogenic MAP kinase pathway has no impact on HR activity, whereas PI3K pathway is critical for cellular decision of HR repair. Among the downstream PI3K pathways, inhibition of Akt and PDK significantly suppresses HR repair.

Our results indicate that extracellular stimuli can regulate DNA repair process, especially HR. These findings could have broad implications in cancer therapy and gene therapy. Kinase inhibitors could enhance the cancer killing ability of DSB inducers such as platinum derivatives and topoisomerase inhibitors. On the other hand, growth factors could enhance the gene-editing efficacy through enhanced HR in techniques relying on HR such as CRISPR.

I. Introduction

Damage of the genome of a cell is inevitable and continuous since some normal cellular processes cause DNA damage (Negritto, 2010). These damages produce several different consequences: base lesions, intra- and inter-strand cross-link, DNA-protein cross-link, and both single- and double-strand breaks (DSBs) (Mehta and Haber 2014). The source of DNA damage can be subdivided into two main types – endogenous damage and exogenous damage. Normal metabolic by-products of cells such as reactive oxygen species (ROS) can cause endogenous oxidative DNA damage: ROS predominantly introduces DNA base leading to single-strand break (SSB) formation, and DSBs arise following replication past ROS-induced lesions (Woodbine et al, 2011). Exogenous damages on DNA are generally caused by external agents such as ultraviolet radiation from the sun, ionizing radiation (IR) and anticancer chemotherapeutic drugs. Topoisomerase inhibitors such as camptothecin and etoposide induce the formation of SSBs and DSBs by trapping covalently linked topoisomerase-DNA cleavage complexes. Ionizing radiations creates DNA SSBs by producing radiolysis radicals that attack the sugar-phosphate backbone, and at high dose of irradiation, two such nicks are generated in complementary DNA strands within one helical turn leading to DSBs, in which the phosphate backbones of the two complementary DNA strands are broken simultaneously (Mehta and Haber, 2014).

Though DSBs arise at a lower frequency than SSBs, they are biologically significant if unrepaired or misrepaired because inaccurate repaired DNA can lead to mutations or larger-scale genomic instability through the generation of dicentric or acentric chromosomal fragments. These genome changes may have tumorigenic potential or even can be sufficient to induce apoptosis (Jackson, 2014). Accordingly, to counteract

deleterious threats on the genome posed by DSBs, mammalian cells have evolved multiple complex repair pathways. These includes nonhomologous end-joining (NHEJ), a process that allows the break ends to be directly ligated without the need for a homologous template; homologous recombination (HR), a process in which a homologous sequence acts as a repair template; and single-strand annealing (SSA), a process initiated when a double strand break is made between two repeated sequences oriented in the same direction (Weinstock, 2006).

Homologous recombination (HR) is found in all forms of life, and it provides high-fidelity, template-dependent repair or tolerance of complex DNA damages including DNA gaps, DNA DSBs, and DNA interstrand crosslink (ICLs). HR also plays a prominent role in faithfully duplicating the genome by providing critical support for DNA replication and telomere maintenance (Li and Heyer, 2008). HR repairs DNA before the cell enters mitosis (M phases). It occurs in the S and G_2 phases of the cell cycle, during and shortly after DNA replication, when sister chromatids are more readily available. A DSB in the DNA generates region of single-stranded DNA (ssDNA) ends, which invade a homologous chromosome to copy genetic information into the donor chromosome and serve to attract the recombinase, either Rad51 or Dmc1, and its associated ancillary factors. ssDNA tail is firstly bound by the **R**eplication **P**rotein **A** (RPA) protein and then displaced by RAD52 for yeast or BRCA2 for mammals, which promotes the formation and stability of Rad51 – DNA presynaptic filaments. The ssDNA- RAD51 complex finds the intact homologous double-stranded DNA and facilitates the exchange of identical strands and the hybridization of complementary strands. DNA polymerase fills in the gap and moves the displacement Loop (D-loop), forming the double Holliday junctions (dHJ). The resolution

of a dHJ can produce crossover or non-crossover products (Guirouilh-Barbat, 2010) (Sung, 2006) (Krejci et al, 2012) (Figure.1).

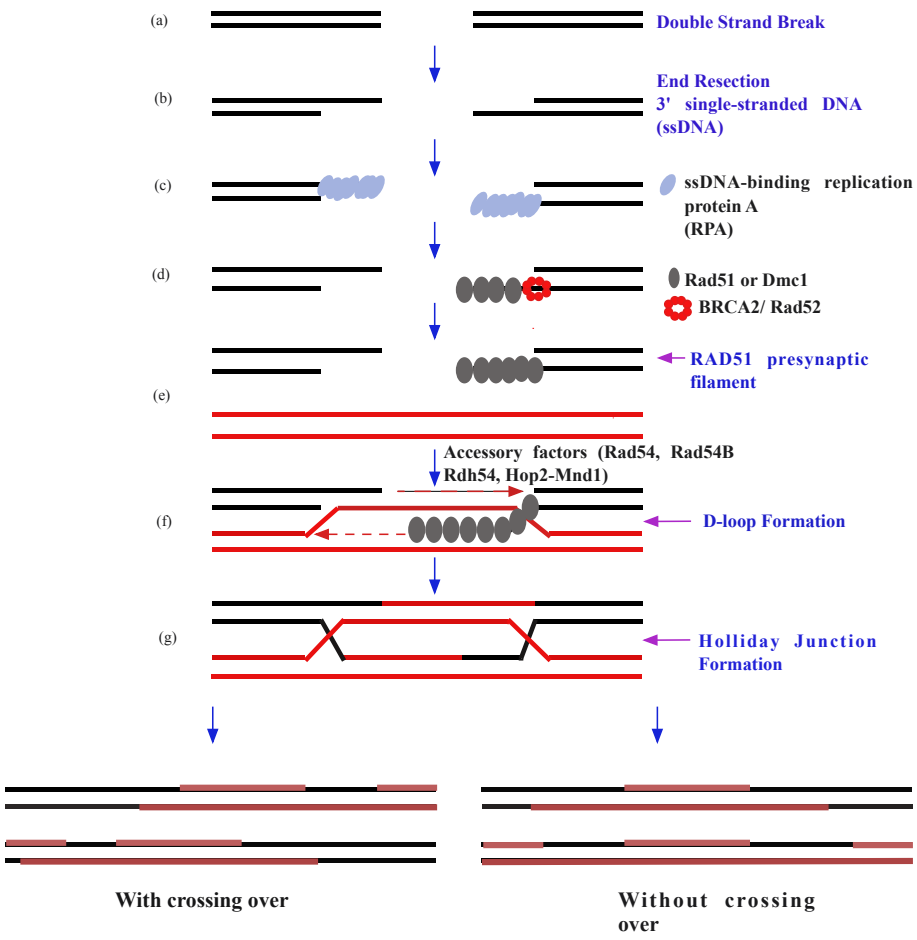


Figure 1.Model for double-strand break repair by homologous recombination
In mammalian cells, DSBs in the DNA generates regions of ssDNA under the control of the RAD50/MRE11/NBS1 complex associated with CtIP. The ssDNA first interacts with the RPA protein forming a "landing tract" for RAD52 or BRCA2, which facilitate the loading of key protein for HR, RAD51, resulting in the formation of Rad51 presynaptic filament. The presynaptic filament then binds duplex DNA to form the synaptic complex which searches for DNA homology in the duplex DNA molecule. With the aid of accessory factors such as Rad54, Rad54B, Rdh54 and Hop2-Mnd1, the ssDNA invades the homologous region in the duplex to form D-loop. The Holliday junctions are then formed. The directional resolution of the Holliday junctions occurs by crossing over independent or crossing over dependent mechanisms. Adapted from Guirouilh-Barbat, Wilhelm & Lope, 2010.

Upon the formation of DNA-damage, sensors detect DNA lesions and subsequently activate apical **a**taxia **t**elangiectasia **m**utated (ATM) and ATM Rad3-related (ATR) kinases and their recruitment to the DNA-damage sites, the process called DNA-damage response (DDR) signaling. Checkpoint kinase 1 (CHK1) is one of the key downstream molecules. In response to DNA damage, it is phosphorylated by ATR kinase at Ser345 primarily to arrest the cell cycle in S ant at G_2/M phases to promote DNA repair before cell division. Meanwhile, CHK1 can also be phosphorylated by other kinases such as PKB/AKT and MAPKAPK at different sites. When activated, CHK1 phosphorylates a variety of intracellular substrate proteins, including the central molecule in HR pathway, the recombinase Rad51 (Narayanaswamy. *et al*, 2016).

As shown in Figure 1, HR is catalyzed by a class of enzyme known as recombinases, either Rad51 or Dmc1, the activity of which is tightly regulated by other factors (Sung and Klein, 2006). For human HR, human Rad51 (hRad51) is a key protein, which forms a helical nucleoprotein filament on RPA bound ssDNA and such presynaptic filament performs several of the most basic steps of HR: a search for homologous double-stranded DNA (dsDNA) and DNA strand exchange (Bugreev and Mazin, 2004). For eukaryotic orthologs, in the presence of ATP Rad51 forms a helical filament on DNA because the activities for DNA-binding and DNA strand exchange require the bound of ATP. Though ATP hydrolysis plays crucial functions in promoting the disassembly of the filament for bacterial RecA protein, the hydrolysis process is not required for eukaryotic orthologs. (Li, 2007) (Bugreev and Mazin, 2004). Though the role of the ATPase activity of Rad51 protein remains obscure, it is evident that in response to DNA damage, Rad51 protein catalyzes the annealing of resected DNA ends to homologous DNA in the presence of ATP. The

phosphorylation of Rad51 on site Ser192 is mediated by the DNA-damage- responsive protein kinase Mec1. Thus, Rad51 phosphorylation on Ser192 is crucial for Rad51 function, which provides a potential mechanism for how Mec1-mediated phosphorylation of this site could influence HR (Flott *et al.*, 2011).

Moreover, the mechanism of Rad51 protein activation by Ca^{2+} significantly stimulates DNA strand exchange activity of Rad51 protein. The Rad51-ATP-ssDNA filament becomes quickly converted to an inactive Rad51-ADP-ssDNA form because of the relatively rapid ATP hydrolysis and slow dissociation of ADP. To restore the active Rad51-ATP-ssDNA filament, Ca^{2+} ion exerts its stimulatory effect to modulate the ATPase activity of Rad51 protein by slowing down the ATP hydrolysis rate (Bugreev and Mazin, 2004). As a ubiquitous second messenger, calcium functions in wide-ranging physiological and biochemical roles including cell proliferation and components of cytoskeleton. In addition, calcium ion plays important roles in the cellular signaling pathway, especially for phospholipase C (PLC). Diacylglycerol (DAG) and inositol trisphosphate (IP_3) produced by PLC catalyzed reaction are two second messengers that function as substrates for synthesis of other important signaling molecules. While DAG remains bound to the membrane, IP_3 is released as a soluble structure that diffuses through the cytosol to bind IP_3 receptors, which causes the cytosolic concentration of calcium to increase and cascade of intracellular changes and activity. For example, calcium and DAG activate protein kinase C (PKC) to phosphorylate other molecules to lead to altered cellular activity. These findings provide a potential mechanism for the way in which PLC-mediated calcium signaling pathway could influence HR either.

Mechanisms of regulation of HR are not completely understood yet. Based on the current studies, it has been suggested that both Mec1-mediated phosphorylation of Rad51 on Ser192 and PLC-mediated calcium signaling pathway are possible to regulators of Rad51, which subsequently might influence HR process. There are two potential phosphorylation sites, Ser192 and Thr197 on Rad51. Ser192 is reported to be phosphorylated by Mec1 kinase. However, it is still unknown whether other kinases can phosphorylate Ser192 and/or Thr197 on Rad51 to regulate its activity. In addition to Rad51 as a key protein, the HR machinery is known to involve other critical proteins, such as the highly conserved MRN complex, whose core contains the proteins Mre11, Rad51 and Nbs1, which are required for ATM activation and consequently for timely activation of ATM-mediated pathways (Uziel et al, 2003*)*. Thus, the regulation of ATM protein kinase might also influence HR. Furthermore, it remains to be determined whether other protein kinases, distinct from Mec1, ATM and PKC, also play significant role in modulating the HR machinery, in relationship to cell survival and apoptosis and cell cycle progression.

II. Background

A. DNA Replication Machinery

Each parental strand serves as a template for the synthesis of a new complementary daughter strand in DNA replication by a semiconservative process. As a central enzyme, DNA polymerase catalyzes the joining of deoxyribonucleoside 5'-triphosphates (dNTPs) to form the growing DNA chain. Proofreading mechanisms are involved in maintaining that the accuracy of replication is compatible with the low frequency of errors that is required for cell reproduction. DNA replication machinery has three major steps:

A.1. Initiation

A trans-acting 'initiator' factor starts the process of replication by directing assembly and loading of the replicative machinery onto particular genomic loci called origin of replication in both space and time. In short, replication initiation includes four steps. 1)During the G1 phase of the cell cycle, the start sites are demarcated by the Origin Recognition Complex (ORC) and the cell division cycle 6 (Cdc6) helicase-loader. 2) To form the pre-replication complex (pre-RC) at the origins of replication, with chromatin licensing and DNA replication factor 1 (Cdt1), an origin bound ORC•Cdc6 complex facilitates the reiterative recruitment of an inactive form of the replicative helicase, minichromosome maintenance proteins (MCM2-7). Cyclin-dependent kinase 2 (CDK) and Dbf4-dependent kinase (DDK) are used to activate the pre-RC complex, which then helps transition the pre-RC to the initiation complex prior to the initiation of DNA replication and leads to replisome assembly and origin formation. 3) In S-phase, other initiation factors such as cell division cycle 45 (Cdc45) and GINS are recruited. The helicase is activated to drive fork progression as the formation of the pre-initiation complex (pre-IC), and single-

stranded DNA-bound Cdc45•MCM2-7•GINS(CMG) complex. The loading of Cdc45 onto chromatin allows the assembly of other various replication proteins such as DNA polymerase α, replication protein A (RPA) and proliferating cell nuclear antigen (PCNA) onto chromatin. GINS complex is essential for the interaction of MCM and Cdc45 at the origins of replication. Cdt1 is degraded in a PCNA-dependent manner. Its specialized PIP motifs, which has the greater affinity for PCNA and is responsible for making the protein a substrate for E3 ubiquitin ligase $CRL4^{Cdt2}$, is bound to a PCNA ring. $CRL4^{Cdt2}$ facilitates the poly-ubiquitylation of Cdt1 leading to its degradation. Therefore, Cdt1 is not available to re-license origins (Boehm, 2016) in S-phase. 4) A bidirectional replication fork is generated, and DNA is unwound to expose template DNA (Parker, 2017).

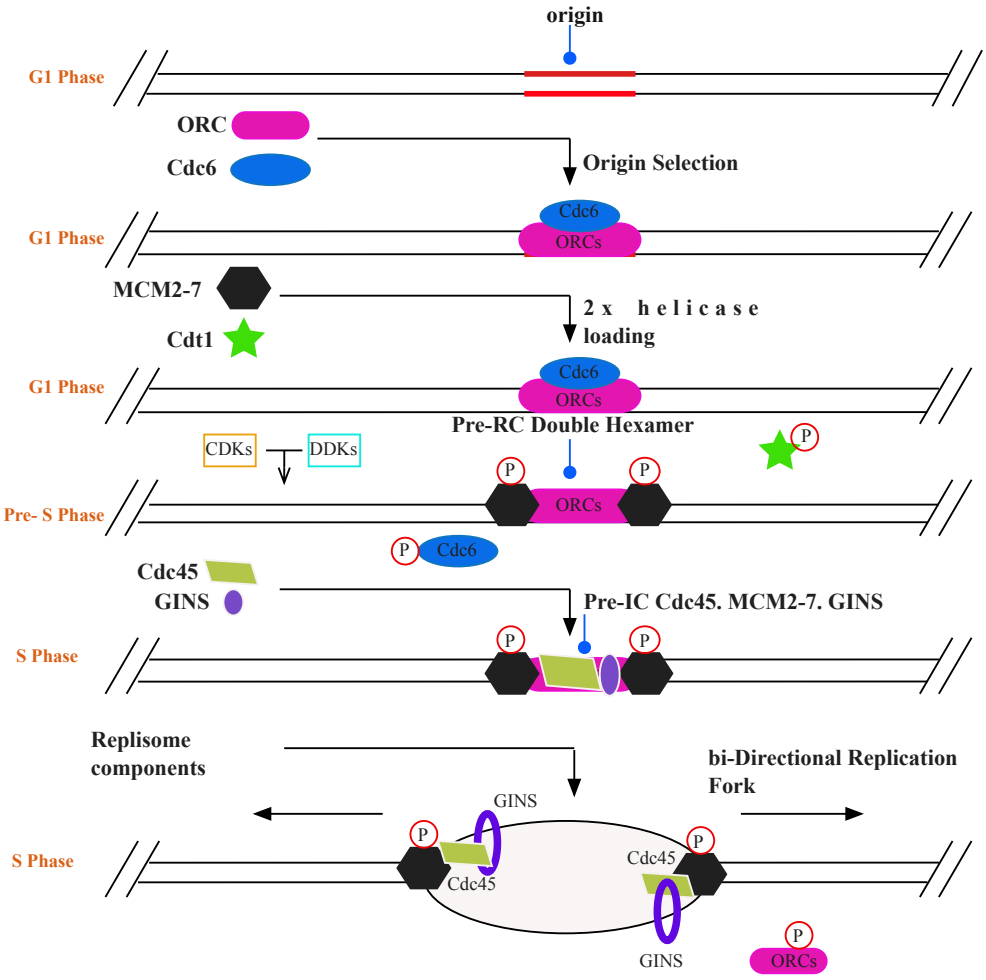


Figure 2. Mechanistic outline of DNA replication initiation in eukaryotes.

1) The starts sites are demarcated by the ORC and Cdc6
2) An inactive form of the replicative helicase MCM2-7 is loaded reiteratively to replication origins by ORC•Cdc6 and the Cdt1 chaperone. The pre-RC complex is formed
3) CDK/DDK-dependent phosphorylation of pre-replicative proteins. Cdc6 and Cdt1 are removed from the nucleus. GINS and Cdc45 are loaded onto replication origins and the helicase is then activated by the formation of pre-IC complex.
4) DNA is unwound. Generation of a bidirectional replication fork. Replication is initiated

"P" represents phosphorylation.

Adapted from Parker, 2017.

A.2. Elongation

The cells enter the S phase of the cell cycle upon the formation of the initiation complex. The initiation complex interacts with a constellation of other proteins including DNA polymerases and becomes a replisome. The parental duplex DNA is unwound by replisome into a two single-stranded DNA template or the replication fork, which is responsible for the replication of the entire genomic DNA. The base pairing and chain formation that move along single-stranded DNA are catalyzed by DNA polymerases. The nascent DNA strand is extended by reading the template strand and the incorporation of the proper nucleotides. These activated free deoxyribonucleotide triphosphates (dNTPs) are added to the exposed 3'-hydroxyl group on the last incorporated nucleotide. Though DNA replication occurs in the opposite directions in the two new strands, leading and lagging strands, at the replication fork, all DNA polymerases synthesize DNA in the 5' to 3' direction. The PCNA, DNA sliding clamps for eukaryotes, is loaded onto primer-template junctions site via replication factor c (RFC) complex and associates with the polymerases to promote their processivity.

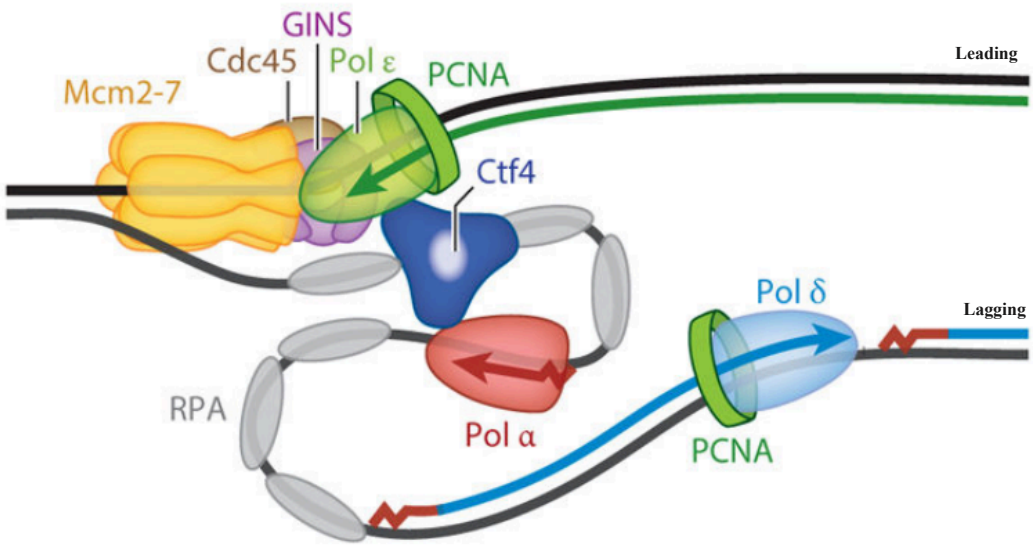


Figure 3.DNA replication at Replication Fork. Adapted from Burgers, 2017.

On the leading strand synthesis, before extending further with DNA, DNA polymerase α (Polα) first synthesizes an RNA primer. Polymerase ε then takes over and adds nucleotides to the template strand continuously (Figure 3).

Synthesis on the lagging strand is initiated with the formation of the primer by Polα, and is followed by loading of PCNA RFC, which subsequently recruits polymerase δ (Polδ) extending the primer to complete the short sequences called Okazaki fragment. The RNA primer is removed by strand displacement; namely, Polδ displaces the 5'end for the previous Okazaki fragment, which contains the RNA primer and a small segment of DNA. Fen1 then cleaves the RNA-DNA single strand flap generated by Polδ and leaves the nick between two Okazaki fragments, which is sealed by DNA ligase I (Figure 3).

A.3. Termination

When two replication forks meet on the same stretch of DNA, the termination of DNA replication occurs. During the process, the forks converge until all intervening DNA is unwound and any remaining gaps are filled and ligated. Since the eukaryotic cells contain a circular chromosome, the two DNA molecules remain linked together after the replication of the entire molecule. Type II topoisomerase is used to remove any remaining catenanes. The replication proteins are unloaded (Dewar, 2017).

B. Maintenance High Fidelity for DNA Replication

The balance between incorrect and correct DNA synthesis has significant biological consequence. DNA replication machinery faithfully replicates the genomes despite facing constant challenges from cellular metabolism and the external environment. The incorrect nucleotides, if not corrected, could have dire consequence. The high fidelity of DNA replication is maintained through three fundamental mechanisms. The high-fidelity of replicative DNA polymerases, proof-reading abilities of exonuclease domains of polymerases, and mismatch repair. The first two properties reside within DNA polymerases δ and ε, contributing to the error rate of the replication machinery in the range of 10^{-7} to 10^{-8}. The MMR improves the fidelity about two orders of magnitude. The three significant determinants for replication fidelity (Kunkel, 2004) are further summarized below.

B.(a). Proofreading

Upon misincorporation of an incorrect nucleotide, two mechanisms can lead to the excision of the wrong nucleotide. The replicative polymerase that inserts a wrong nucleotide

dissociates, which allows another protein with intrinsic 3' → 5' proofreading exonuclease activities to excise 90-99.9% of incorrectly incorporated mononucleotides and then replace with the correct nucleotide to continue synthesis by the replicative DNA polymerase. Meanwhile, genome stability requires DNA synthesis to fill gaps created when lesions are excised. Gap filling process during mismatch repair, nucleotide excision repair, and long patch base excision repair (BER) is performed the intrinsic proofreading activities of T4 Pol, Pol δ, Pol ε and T7 Pol polymerases (Kunkel, 2004).

B.(b). High Fidelity of DNA Replication Polymerases

Nucleotide selection step is another major determinant for overall fidelity for DNA polymerases. The ability to insert the correct nucleotide and requires several conformational changes to achieve stabilization of the transition state (Kunkel, 2004). The DNA polymerase first forms a complex with DNA, a nucleotide and two metal ions at the active site of the enzyme. The binding process and the stabilization of the incoming nucleotide are accomplished in the nucleotide binding pocket, which includes the interactions between the nucleotide and the template bases, the polymerase active-site residues, the fingers domain of polymerase, the metal ions, and the 3'-terminal hydroxyl group of the primer strand. A dNTP-induced conformational change within the polymerase fulfills these interactions, which is referred to as an induced fit mechanism. When an incorrect nucleotide is bound within the nucleotide binding pocket, the phosphodiester bond will not be formed due to the lack of proper geometry within the active site. Therefore, the incorrect nucleotide will leave the pocket. The nucleotide selection fidelities of Pol α, Pol δ, and Pol ε are around one error per $10^4 – 10^5$ incorporated nucleotides (Ganai, 2016).

B.(c). Mismatch Repair

Base-base mismatch and insertion/deletion (ID) misparis generally arise during DNA replication. DNA mismatch repair (MMR) is then used to correct DNA mismatches and reduces the number of replication – associated errors, thereby prevents the occurrence of mutations in dividing cells. In prokaryotes, the hemi-methylated dGATC sites are used to discriminate daughter and template strands since the daughter strand is transiently unmethylated and hMutSα preferentially recognizes base-base mismatches and ID mispairs of 1 or 2 nucleotides while hMutSβ recognizes longer ID mispairs. Such strand-specific nick generated via hMutSα or hMutSβ and hemi-methylated dGATC creates a starting point for excision of the mispaired base. In human MMR, since the new DNA strand has a primer terminus that PCNA and RFC are bound to, the interaction between hMutSα/ hMutLα with PCNA/RFC also provides the means for identifying the newly – made strand. The hMutLα endonuclease is activated to cleaves the new strand after binding to RFC and PCNA. Exo 1, a 5' to 3' exonuclease is recruited and cleaves the DNA strand from the 5'end to the nick made by hMutLα to remove the mismatch. The gap is filled and sealed with Pol δ and DNA ligase (Li, 2007).

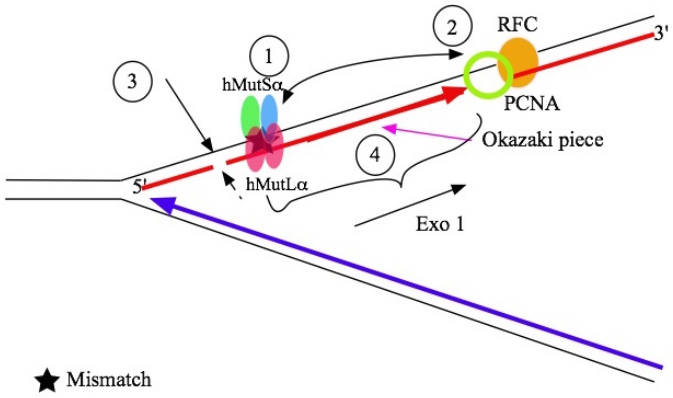


Figure 4. Mechanisms for MMR

1) hMutSα/ hMutLα binds to the Mismatches
2) Communication between hMutSα/ hMutLα and RFC and PCNA at the primer terminus DNA chain
3) The hMutLα endonuclease is then activated
4) Exo 1 is recruited and cleaves the DNA strand from the 5'end to the nick made by hMutLα
5) (Not Shown) Pol δ and DNA ligase fill in and seal the gap.

Adapted from Mismatch Repair, ACSU. Buffalo.edu Accessed March 24, 2019

C. Source of DNA Damage

DNA insults result from either cellular metabolic process (endogenous sources), which includes hydrolysis, oxidation, alkylation, and mismatch of DNA bases, or environmental factors (exogenous sources), which contains ionizing radiation (IR), ultraviolet (UV) radiation, and various chemicals agents (Hakem, 2008). CpG to TpG transition mutation is generated by the spontaneous deamination of 5'- methylcytosine, which converts C to T and is recognized by thymine DNA glycosylase (TDG). Guanine is oxidized by ROS to form 8-oxoG, which can pair with adenine and then generate G to T mutations. DNA polymerase errors induce MMR, and failure to complete MMR will cause incorrect base substitutions. Cyclobutane pyrimidine dimers are generated by exposure to UV light. The dimers are recognized by nucleotide excision repair (NER) factors and excised by XPG

and XPF flap endonucleases. The G to T transition mutations at CC dinucleotides are caused by the failure to complete NER (Tubbs, 2017).

D. DNA Damage Repair Mechanisms

D.(a). Single-Strand Break

SSBs, which are discontinuities in on strand of DNA double helix due to the loss of a single nucleotide and damaged 5'- and / or 3'- termini at the site of the break, are the most common types of DNA damage. These arise in cells at a frequency of tens of thousands per cell per day. These damages are directly caused by intracellular metabolites and spontaneous DNA decay and will cause a severe threat to genetic stability and cell survival if not repaired appropriately. Chromosomal single-strand break repair (SSBR) is a rapid and efficient process. SSBs are detected by the binding and activation of poly(ADP-ribose) polymerase 1 (PARP1), which promotes rapid accumulation of downstream repair factors. PARP1 accelerates SSBR in multiple possible ways. Firstly, it promotes the focal accumulation or stability of SSBR protein complexes at chromosomal SBs through interaction with dedicated pADPr-binding motifs. Furthermore, PARP1 regulates chromatin structure since histone proteins are targets for poly(ADP-ribosylation). Therefore, pADPr synthesis can disrupt nucleosomes and then regulate higher-order chromatin compaction. Moreover, PARP is suggested to promote DNA gap filling during long-patch repair and to generate ATP for the DNA ligation step. Finally, since PARP1 is a transcriptional regulator, so it might accelerate SSBR rates by affecting the level of SSBR proteins (Caldecott, 2008).

D.(b). Double-Strand Break

DSBs are lesions formed when both strands of the DNA duplex are broken, which are highly toxic. DSB is a major driver in carcinogenesis due to its repair or mis-repair that could cause genome rearrangements. DSBs are also related to developmental, immunological, and neurological disorders as well as cell death. Though exogenous agents such as IR and chemicals can cause DSB, DNA replication is a major source of DSB. When encounter unrepaired DNA lesions, the forks will stall and then collapse, leading to DSBs. A one-ended DSB also forms when replication fork encounters a nick in the template DNA. A single unrepaired DSB could lead to cell death. Organisms have evolved a range of DSB repair mechanisms to prevent these severe consequences and to support a critical role in maintaining genetic stability. HR and NHEJ are two major DSB repair pathways (Chapman, 2012).

D.(b).(1) Nonhomologous end-joining

Though it is dominant in G_0/G_1 phase, NHEJ occurs throughout the cell cycle, while HR only functions in late S/G_2 phase since the full set of sister chromatid is generated during the S phase of interphase. NHEJ is initiated by Ku70/Ku80 protein complex (Ku heterodimer), which recognizes and binds DSBs ending, and DNA-bound Ku protects ends from nuclease digestion but does not stop ataxia telangiectasia mutated (ATM) activation or signaling. DNA-dependent protein kinase catalytic subunit (DNA-PKcs) is then recruited by DNA-bound Ku, which binds to broken ends and phosphorylates itself and other targets including Artemis, a nuclease that trims the DNA ends, to generate the DNA-PK complex and activate the activity of DNA-PK. This kinase activity facilitates

recruitment of the DNA ligase complex including DNA ligase IV, X-ray cross - complementing Group 4 (XRCC4) and XRCC4-like factor (XLF)/cernunnos, which contribute to end processing and seal the DNA break (Kakarougkas, 2014).

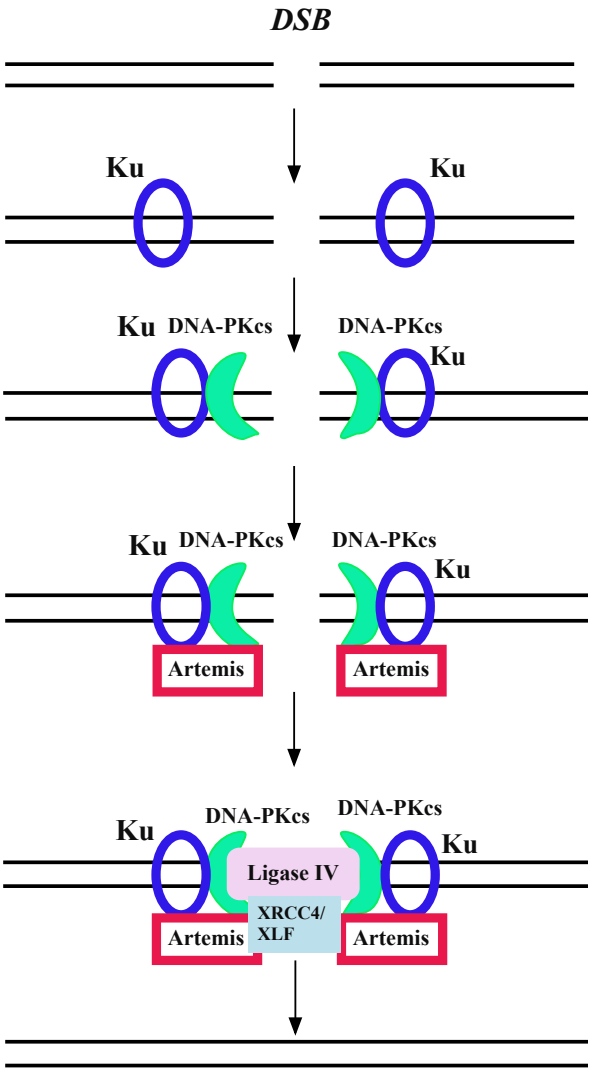


Figure 5. Mechanism for NHEJ Repair

1) DNA End-binding and Bridging: Initiated by the recognition and binding of the Ku70/Ku80 heterodimer to the broken DNA ends, which then recruits the catalytic subunit of DNA-PKcs
2) Terminal Ending Process: DNA-PKcs phosphorylates itself and bound Artemis protein, and the Artemis/DNA-PKcs complex have nuclease activity to cleave 5' and 3' DNA overhangs
3) Ligation: DNA ligase IV, XRCC4 and XLF carries the ligation step

Adapted from Davis, 2013

D.(b).(2) Homologous Recombination

As depicted in Figure 1, HR uses an undamaged template to re-generate any sequence of information lost at the DSB site. 5' to 3' end resection generates a 3'ended single-strand region, which initiates the mechanism of HR. RPA binds to the ssDNA tail rapidly to prevent the formation of secondary structures, and it is displaced by RAD51 subsequently via a Breast Cancer Associated Gene 2 (BRCA2) – dependent process. RAD51 promotes invasion onto the undamaged template and strand displacement to induce D-loop formation, which is used to generate a Holliday junction and a heteroduplex molecule. Finally, the resolution of Holliday junctions produces either cross-over or non-cross-over products depending on the direction of resolution and completes the HR process (Kakarougkas, 2014).

E. Coupling of Homologous Recombination and Cell-cycle Checkpoints

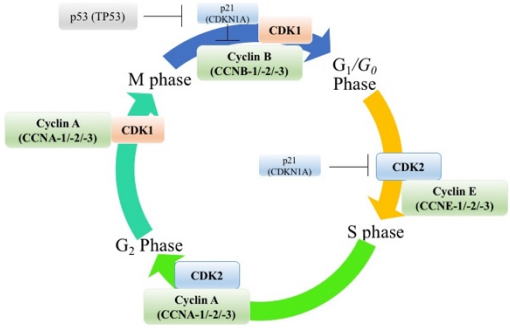


Figure 6. Cell Cycle Control. Adapted from Cancer Genetics, CuboCube.

Cell-cycle checkpoints are critical control mechanisms in eukaryotic cells to establish order and timing of cell cycle progression, to ensure complete genome duplication even in the faces of damage stress, and proper division of cells. The cell cycle encompasses two major phases including interphase, which contains G_1 (gap phase 1), S (DNA synthesis) and G_2 (Gap phase 2), and M (mitosis) phase (Figure 6). Cyclin-dependent kinases (CDKs)

function as the central regulators in cell cycle progression. These serine/threonine kinases that phosphorylate key substrates to promote DNA synthesis and mitotic progression, thereby drive cell cycle progression (Barnum, 2014). There exist two essential checkpoints in the cell cycle to ensure the integrity of daughter cells, G_1/S checkpoint, and the G_2/M checkpoint.

E.(i). G_1/S checkpoint

The G_1/S checkpoint is used to control the commitment of eukaryotic cells to transition through the G_1 phase to enter the S phase, during which DNA replication occurs, therefore, the proliferation of irreparably damaged cells is prevented. In higher organisms, the G_1/S checkpoint is regulated by p53, which is a tumor suppressor and transcription factions, and its down-regulation plays a significant role in tumorigenesis. p53 and its negative regulatory protein, Mouse double minute 2 homolog (MDM2) or E3 ubiquitin-protein ligase Mdm2, are phosphorylated directly or indirectly on N – terminal serine-15 by damage response kinase ATR, ATM, and DNA-PKcs to enhance p53 stability and DNA binding activity. ATM kinase targets to DSB by interacting protein **M**re11-**R**ad50-**N**bs1 (MRN) and the interacting protein Ku70-Ku80 complex are used for DNA-PKcs. The activated p53 is stabilized through protein from MDM2, and it regulates transcription of CDK inhibitor $p21^{Waf1}$/Cip1. Therefore, G_1 CDKs are inhibited, and G_1/S checkpoint is arrested, to enable repair of DNA damage before DNA replication (Barnum, 2014) (Deckbar, 2010).

DNA damage checkpoints are also controlled by checkpoint kinase Chk1. Two master checkpoint kinases, the ATM (ataxia-telangiectasia mutated) and ATR (ataxia telangiectasia mutated and rad3-related), are activated by DSBs in mammalian cells. ATM

and ATR kinases phosphorylate and activate the transducer kinases Chk2 and Chk1, which are activated by all known forms of DNA damage, restricted to post-replicative lesions, and it is more efficient in S- and G_2-phase.

After phosphorylated by ATR, BRCT domain (for BRCA1 carboxyl terminus) mediator proteins are recruited and serve for the recruitment of Chk1, which is released to maintain the mitotic CDK Cdc2 in its Y15 phosphorylated and inactive state. The phosphatase Cdc25A, which regulates inhibitory phosphorylation of Cdk1/Cdk2, is phosphorylated by Chk1. The activity of Cdc25 is then decreased. Therefore, the activated pathway for G_1 - S is arrested due to the inhibition of Cdc25A – mediated activation of cyclin E – Cdk2 (Barnum, 2014) (Deckbar, 2010).

E.(ii). G_2/M checkpoint

Upon the detection of DNA damage, the G_2/M checkpoint prevents cells from entering mitosis and provides an opportunity for repair. Cdk1 is the master regulatory kinase that controls cell entry into mitosis. In G_2 phase, cyclin B is kept in the cytoplasm by binding to nuclear export CRM1. When cells approach the G_2/M boundary, cyclin B is phosphorylated to prevent its binding to and consequently stops being exported. The accumulation of cyclin B in the nucleus induces entry into mitosis. Similar to G_1/S checkpoint, p53-independent pathways involving ATM, ATR-dependent activation of Chk1 and Chk2 can rapidly arrest G_2 – M phases by phosphorylating Cdc25 and stimulating its binding to 14-3-3 proteins to anchor it in the cytoplasm (Stark, 2007). Moreover, p53 is also required for prolonged G_2 arrest since human Cdc25 gene is a target for p53-dependent transcriptional repression (Clair, 2004).

To survive the inevitable adverse challenges by extrinsic and intrinsic stresses and pass on intact genetic information to subsequent generations, cells have developed a highly organized and coordinated response, DNA damage response (DDR), to ameliorate genotoxic stress. DDR demands cell's ability in sensing and signaling problem in its DNA, arresting cell-cycle progression and activating appropriate DNA repair mechanisms to eliminate cells with unrepairable genomes. Like other classical cell signaling pathways such as receptor tyrosine kinase cascade, DDR signaling pathway regulates and coordinates the cellular response to DNA damage by using signal sensors, transducers, and effectors. While ligands binding to receptors activate classical signaling pathway, the DDR signaling pathway is activated by aberrant DNA structure. These structures could originate from nucleotide base damage, DNA strand breaks, and replication stress. The sensors involved in the DDR signaling pathway are proteins that can recognize DSBs or stalled/stressed DNA replication forks. The transducers include ATM, ATR, and their downstream kinases that can phosphorylate effectors (Marechal, 2013).

In mammalian cells, the ATM, ATR and DNA-PKcs kinases are the three most upstream DDR kinases. The catalytic kinase domains are located at the C-terminus, exhibit homology to the phosphatidylinositol 3 (PI3) family of lipid kinases (PI3Ks), and thereby belongs to PI3K-like protein kinases (PIKKs) family (Figure 7). As mentioned before, DNA-PKcs play a role primarily in the NHEJ pathway, while ATM and ATR activate the second wave of phosphorylation through the activation of Chk1, Chk2 and MK2 protein kinases in DSBs. These three kinases are all serine/threonine-directed and have a preference of Ser/Thr – Glu motifs, which phosphorylate hundreds of proteins in response to DNA damage (Paull, 2015).

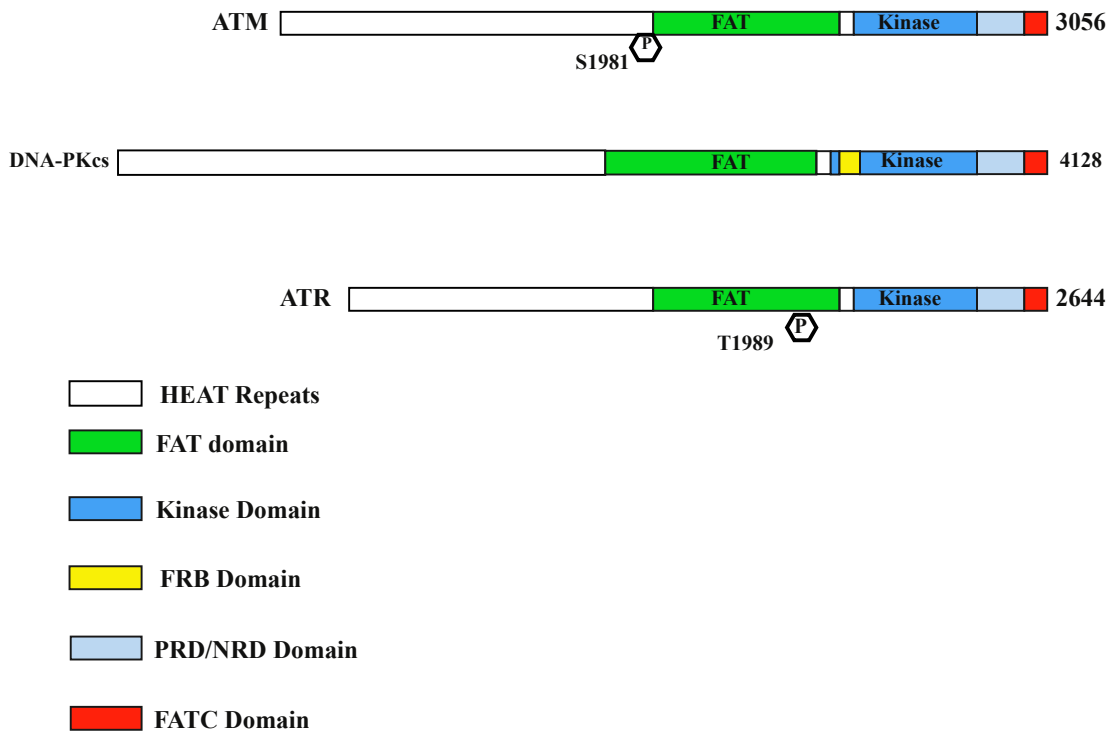


Figure 7. Structural Basis of PIKKs (ATM, DNA-PKcs, and ATR) Functional Diversity

PIKKs share a similar domain organization. The kinase domains are located near carboxyl-termini and contain the catalytic loops of Class-I PI3K and therefore they are categorized separately from the classic protein kinases. The kinase domains are flanked by conserved **F**RAP-**A**TM-**T**RRAP (FAT) and FAT carboxy-terminal (FATC) domains; these two domains are essential for ATR and ATM activation and mutations will hamper their kinase activity. In addition, PIKK contains PIKK-regulatory domain (PRD), and their N – terminus bears numerous α-helical Huntingtin, elongation factor 3, protein phosphatase 2A, TOR1 (HEAT) repeat motifs, which act as a scaffold to mediate the interactions of ATM and ATR with proteins that regulate their catalytic activity. Under non-stress conditions, ATM exists as dimers or oligomers and it is released in monomeric form in response to DNA damage. ATR forms a heterodimer with its obligatory partner ATR-interacting protein (ATRIP). In response to DNA damage, ATR and ATM are autophosphorylated near their FAT domains (Marechal, 2013) (Awasthi, 2015).
Adapted from Marechal, 2013, Figure 2.

E.(iii). Ataxia Telangiectasia mutated and rad3-related (ATR) Kinase

As a master regulator of DNA damage signaling, upon activation in S-phase, ATR can suppress firing of replication origins and restrains DNA synthesis; while activated in G_2-

phase, it can promote the G2/M cell-cycle arrest (Buisson, 2017). Not only required for the full checkpoint response to DSBs, but ATR also responds to a broad spectrum of other DNA damages, especially those interfering with DNA replication. ATR is recruited to DSBs via RPA-coated single-stranded DNA (RPA-ssDNA), which is generated by the nuclease-mediated resection of DSBs and this ssDNA is also necessary for the HR pathway (Shiotani, 2009). ATR regulates DSB repair in homologous recombination. Most recent studies from Buisson suggest that ATR regulates HR through controlling the S59-S64 "phosphorylation switch. HR occurs in two consecutive phases: the resection and ATR activates driven by high CDK activity in the first, and ATR then suppresses CDKs and phosphorylates HR substrates in the second. Since this CDK – to – ATR switch is necessary for optimal BRCA1 – PALB2 interaction and other possible HR events, therefore, ATR can promote HR by phosphorylating HR substrates and inhibiting CDKs, directly coupling the checkpoint to HR (Buisson, 2017).

ATR in Homologous Recombination – Mediated Repair

BRCA1 associates and co-localizes with RAD51 in nuclear foci in mitotic cells, and it acts as a recombination mediator or co-mediator to promote ssDNA resection via interaction with CtIP, those observations indicate that BRCA1 functions in HR repair (Zhang, 2013). ATR promotes HR-mediated repair via phosphorylating PALB2 and enhancing its localization to DNA lesions via interaction with BRCA1 after DNA damage. Additionally, BRCA1 phosphorylation and its interaction with TOPBP1 are mediated by ATR to promote HR since BRCA1 is stabilized at lesions during S-phase (Kim, 2018).

E.(iv). *Ataxia – telangiectasia mutated (ATM)*

ATM kinase is critical for the initial response and the subsequent ATR activation, and it is primarily activated by DSBs and coordinates checkpoint activation and DNA repair in response to DSBs. The inactive dimeric ATM is monomerized into active monomers by MRN complex, which is highly conserved and plays critical roles in DSB in all organisms. MRN complex also acts as a cofactor for ATM catalytic activity that promotes the activity of ATM in physiological conditions in which magnesium is the primary cation. Thus, the ATM kinase is activated via the MRN complex (Paull, 2015). After the initial activation by DSBs, ATM executes specific functions around the breaks via chromatin-mediated mechanisms involving H2AX, Mdc1, and other proteins (Shiotani, 2009) (Figure 7).

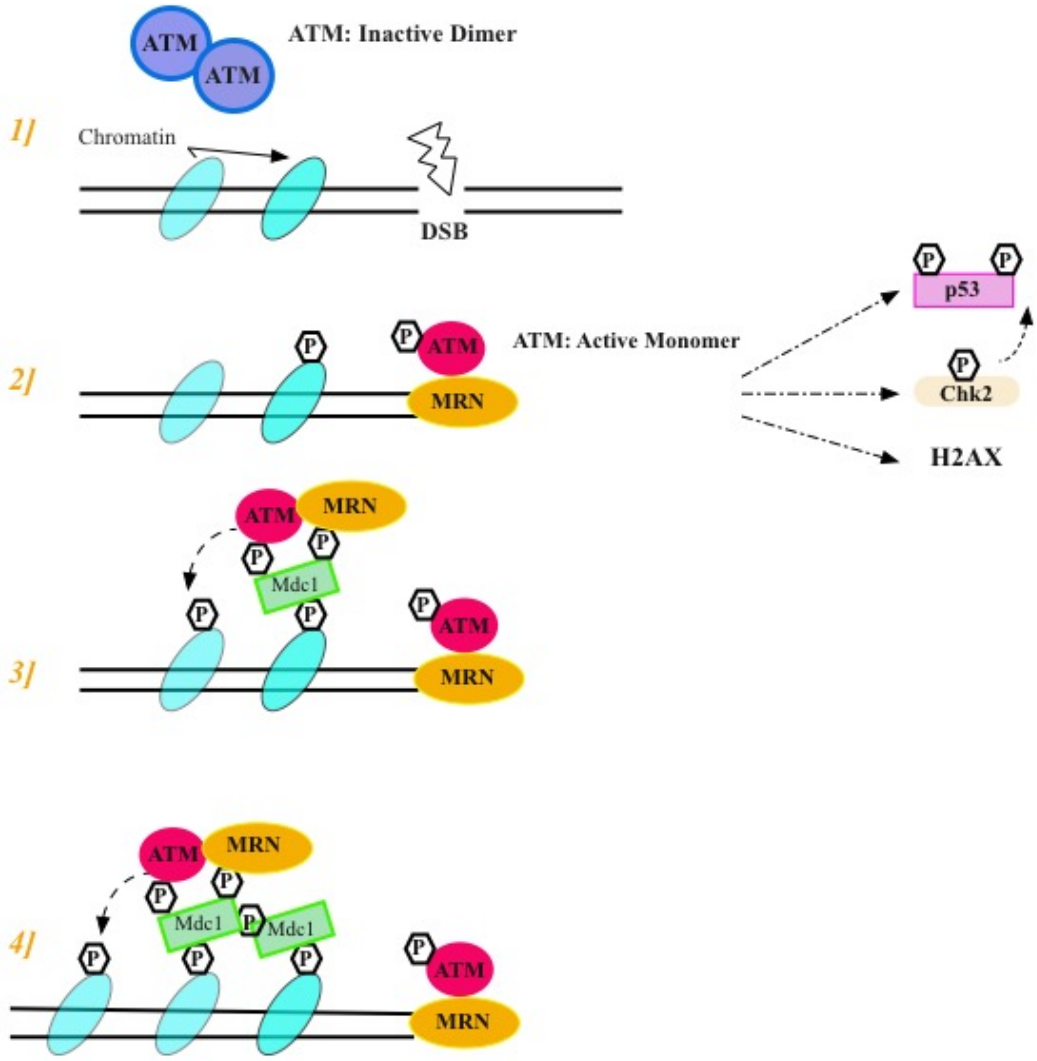


Figure 8. Activation of ATM by DSBs. 1] The DNA ends and chromatin are recognized by ATM. The MRN complex functions as a sensor of DNA ends and direct activator of the ATM kinase that converts inactive dimeric ATM to active monomer. Meanwhile, autophosphorylation of ATM occurs at residue Ser1981 coincides with the activation of ATM monomerization. 2] The activated ATM phosphorylates substrates including Chk2 and p53. H2AX in flanking nucleosomes is also phosphorylated. 3] Mediator of DNA damage checkpoint protein 1 (Mdc1) recognizes phosphorylated H2AX (γH2AX) to trigger a feed-forward loop that spreads activated ATM and γH2AX over large chromatin domains 4] (Marechal, 2013).

Activation of DSB-Signaling Cascade and p53 by ATM

Figure 8 illustrates that the activated ATM can phosphorylate substrates including Chk2 and p53, which then activates the DSB-signaling cascade and the activity of p53 protein. Figure 9 provides more details in ATM serine/threonine kinase and the functions of ATM-dependent signaling events in response to DNA damage.

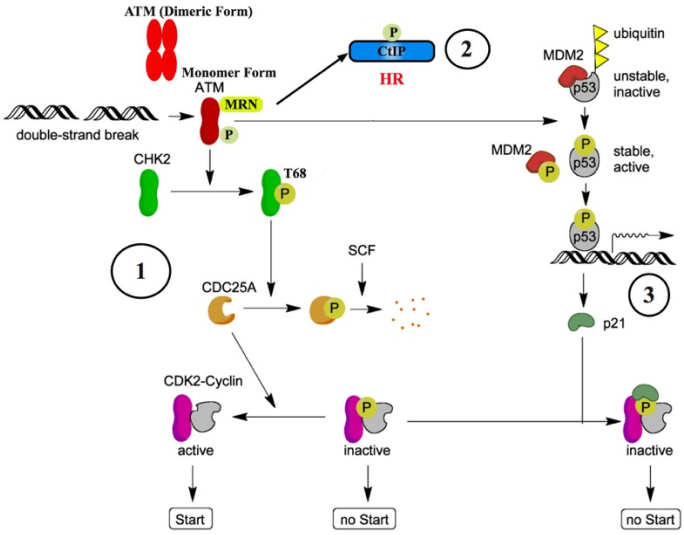


Figure 9. ATM-Mediated three types of Response to DNA Double Strand Breaks.
Adapted from ATM serine/threonine kinase, Wikipedia.

ATM phosphorylates and activates hundreds of substrates for DSB-signaling cascade, and the most typical and well-characterized example is the Chk2 kinase. The Chk2 protein contains a Ser/Thr – Glu cluster domains including five Ser-Glu and two Thr-Glu motifs that satisfy the primary substrate motif for ATM kinase. Following DNA damage, the inactive monomers of Chk2 are phosphorylated by ATM directly at residue Thr68, a consensus site in the SCD domain and undergo dimerization, and autophosphorylation of Ser516 within the kinase domain, which is required for full kinase activation. Cdc25A and Cdc25C, two members from the Cdc25 phosphatase family, promote cell cycle progression by activating Cdk2 and Cdk1 via dephosphorylation of inhibitory phosphorylation at Thr14 and Tyr15. Chk2 counters these actions by phosphorylating and inactivating Cdc25A, and

thus prevents G_1/S transition. Besides, Chk2 phosphorylation creates a binding site for 14-3-3 proteins at Ser216 in Cdc25C, which causes the persistent cytoplasmic Cdc25C localization and thus prevents the G_2/M transition since the Cdk1/Cyclin B complex cannot be activated (Ahn, 2004).

Secondly, a portion of (~20%) IR-induced DSBs are repaired by a pathway requiring ATM as MRN-dependent ATM activation in order to trigger phosphorylation of DNA-PKcs to promote Artemis recruitment and activation. ATM promotes HR either through stimulating DNA-end resection, which is a crucial determinant of choice for DSB repair pathway. DSB-end processing to produce tracts of ssDNA is essential to invade another DNA molecule after binding by the recombinase RAD51(Blackford, 2017)[37]. Mechanistically, ATM phosphorylates the MRN-interacting protein CtIP (C-terminal binding protein 1 interacting protein), which promotes the resection of DSBs and are essential for meiotic recombination (Makharashvili, 2015).

Finally, in response to DNA damage, the tumor suppressor p53 is stabilized for the activation of a transcriptional program that can cause cell-cycle arrest in G_1 phase, senescence, or apoptosis. ATM phosphorylates p53 on multiple sites that include S15. The phosphorylation of S15 inhibits the interaction of p53 with the ubiquitin ligase MDM2, resulting in the stabilization and activation of p53, which promotes the transcription and translation of Cdk inhibitor p21 in response to DSBs. The increased p21 level helps to keep Cdk activity low to maintain longer cell cycle arrest in G_1 phase (Blackford, 2017).

F. Overall Goal of the Study

Experiments in this thesis are to explore and test the thesis that extracellular environment affects the cellular decision for DNA repair, especially homologous recombination repairs of DSBs. The link between the extracellular signal to HR is examined by using specific inhibitors for cytoplasmic kinases. DSBR reporter assay is developed to detect HR induced by DSB with DR-GFP reporter. DR-GFP substrate is used to monitor HR. It contains two differentially mutated **g**reen **f**luorescent **p**rotein (*GFP*) genes, which are oriented as **d**irect **r**epeats (DR): the recognition site for the rare-cutting I-*Sce*I endonuclease on the upstream repeat and 5' and 3' truncated *GFP* fragment on the downstream repeat. Transient expression of I-*Sce*I causes DSB in the upstream GFP gene and HR to repair DSB finally results in GFP^+ cells, which can be quantified by flow cytometry (Nakanishi *et al.*, 2011)[15]. In this thesis, I hypothesize that nuclear signaling events are insufficient for the cellular decision to repair DSBs through HR. Besides, the decision also relies upon the presence of pro-survival factors and subsequent activation of signaling pathways. Several previous studies have indicated that the phosphorylation of proteins including BRCA1, BRCA2, or RAD51 might play a role in the regulation of HR activity. However, the functions of many signaling molecules in DNA damage response are still uncertain. Using specific inhibitors of protein kinases, I aim to delineate the signaling molecules involved in HR repair pathway.

III. Material and Methodology

1. Cell Culture

Human kidney cell line 293T/HEK 293T (ATCC® CRL-11269™) and 293T – DR, which are 293T cells stably transfected with pDRGFP plasmid via Lipofectamine™ 3000 Transfection Reagent Invitrogen (ThermoFisher Scientific), were cultured in Dulbecco's Modified Eagle's Medium (DMEM) and supplemented with 10% heat-inactivated fetal bovine serum(FBS) (Lonza, Waltham, MD), 2mM L-glutamine and 1X Penicillin – Streptomycin in humidified incubator in 37℃, 5% CO_2 and 95% humidity. These reagents were purchased from Invitrogen (Waltham, MA).

2. MTT Assay

80% - 90% confluent cells were enzymatically detached by Trypsin-EDTA solution (Sigma-Aldrich) and counted by hemocytometer. In general, 1000 cells/100µl/well were seeded in 96 – wells plates for 24 hours before any treatment. After the addition of several kinase inhibitors to the desired concentrations, the cells were incubated in the CO_2 incubator. After 72hrs exposure, 20 µl of 5mg/ml 1-(4,5-Dimethylthiazol-2-yl)-3,5-diphenylformazan (MTT) stock solution was added and MTT reactions proceeded in the dark for 4hr. 100µl of 10% SDS in 0.01M HCl (Septisetyani, 2014) was subsequently added to dissolve formazan crystals. To accelerate the dissolution of crystals, the plates were then shaken on a nutating platform and incubated in the dark overnight. The absorbance was read at 450nm and 595nm set as reference wavelength on a Multi-Mode Microplate Reader (FilterMax F5). All doses were set up in triplicate and experiments were repeated in three times to yield consistent and reproductive results.

2.2. IC_{50} Determination

Seven concentrations of each kinase inhibitors were used in this experiment. Similar to above MTT assay, 293T cells were seeded in 96-well plates at a density of 1000 cells/100µl/well 24hrs before treatment with different inhibitors at a specific concentration range. After 72hrs exposure, the cells were analyzed via MTT cell proliferation assay. The dose - response curves for the determination of IC_{50} values were generated in GraphPad Prism 7 (GraphPad Software Inc. La Jolla, CA, USA).

Table 1. Concentration Range for Each Kinase Inhibitor

PI3K: Buparlisib (BKM120)	0.1µM – 2.0µM (Netland, 2016)[42]
Mek: GSK1120212	0.5nM – 5.0nM (Gilmartin, 2011)[43]
Akt: Afuresertib. HCl	0.5µM – 20µM (Spencer, 2014)[44]
Met: AMG337	0.1µM – 1.5µM (Du, 2016)[45]
EGFR: Gefitinib	0.25µM – 20µM (Noro, 2006)[46]
PKC: Bisindolylmaleimide I	0.5µM – 8.0µM (Sigma)
PDK1: GSK2334470	0.5µM – 20µM (Yang, 2017)[47]

2.3. Cell Proliferation Assay

The specific concentrations for each kinase inhibitor used in the following experiments were determined by MTT cell proliferation assay. After determination of IC50 values (specific for 293T cell line) for each kinase inhibitor, 293T WT cells were seeded in other 96-well plates at same density (1000 cells/100µl/well) 24hrs before treatment with one low concentration dose and one high concentration dose. The concentrations of each kinase inhibitor were chosen based on the IC50 values and previous studies. The cells were analyzed after 72hrs exposure. The experiments conducted in triplicate sets and assessed by ANOVA with Tukey's Multiple Comparisons test with the $p - value < 0.05$ considered significant.

Table 2. Dosing Concentrations

Inhibitor	Dose 1 (Low Concentration)	Dose 2 (High Concentration)
PI3K: Buparlisib (BKM120) (Anderson, 2015)[48]	0.1μM	0.25μM
Mek: GSK1120212 (Abe, 2011)[49]	0.5nM	2.0nM
Akt: Afuresertib. HCl (Yamaji, 2017)[50]	1.0μM	3.0μM
Met: AMG337 (Du, 2016)[45]	0.1μM	0.3μM
EGFR: Gefitinib (Khalil, 2017)[51]	0.25μM	0.5μM
PKC: Bisindolylmaleimide I (Ingeborg, 1999)[52]	0.5μM	2.0 μM
PDK1: GSK2334470	1.0μM	3.0μM

2.4. Cisplatin and Kinase Inhibitor Combination Assay

293T cells were seeded in 96-well plates at a density of 1000 cells/100μl/well 24hrs before treatment with different inhibitors at specific cisplatin concentrations range. The concentrations of each kinase inhibitors were maintained whereas, and the concentration range for cisplatin was from 1.0μM to 100μM. The cells were analyzed after 72hrs exposure, and the dose-response curves were generated in GraphPad Prism 7 (GraphPad Software Inc. La Jolla, CA, USA).

3. Western Blot

80-90% confluent 293T cells were starved in media without serum overnight. Next morning, several kinase inhibitors were added for one hour prior to stimulation by the addition of FBS to a final concentration of 10%. Cells were harvested 30 minutes later and lysed in RIPA buffer with protease and phosphatase inhibitor (Halt from ThermoFisher). The Bradford Protein Assay (BioRad, Hercules, California) was used to determine the protein content of each sample. Treated lysates were separated by **s**odium **d**odecyl **s**ulfate-**p**oly**a**crylamide **g**el **e**lectrophoresis (SDS-PAGE), and then transferred to nitrocellulose membranes (BioRad) for 2 hours at 100 V with the electrophoretic tank transfer apparatus

(Bio-Rad). The membranes were then blocked with 5% nonfat milk in TBST buffer (20mM Tris-HCl pH7.4, 150mM NaCl and 0.05% (v/v) Tween-20) and probed with primary antibodies overnight at 4°C. The primary antibody used in this experiment was anti - hPhospho-p70 S6 Kinase (Cell Signaling Technology #9205) in 1: 1000 dilution with TBST buffer. The membrane was then detected by the secondary label antibody or secondary antibody with an enzyme such as horseradish peroxidase that is detected by the signal it produces corresponding to the position of the target protein. Finally, the signals were captured on a film that developed in a dark room (Mahmood, 2012)[62]. Multiple film exposures were made by using chemiluminescence substrates (ThermoFisher Scientific, NJ), which enables optimization of signal to noise. The quantification of the Western Blot was conducted by ImageJ and GraphPad Prism 7 (GraphPad Software Inc. La Jolla, CA, USA).

4. <u>DR-GFP Homologous Recombination Assay</u>

DSB recombination reporter pDR-GFP and I-*Sce*I endonuclease expression vector pCBASceI were generated following standard protocols (QIAGEN Plasmid Maxi Kit)[53]. The 293T stable cell lines were established by transfecting with DR-GFP plasmid. After plating these transfected cells overnight, a plasmid harboring I – Sce (pCBASceI) was transfected by lipofectamine 3000, and selective inhibitors were added 2 to 4 hours post-transfection. The number of GFP positive cells were counted and analyzed by flow cytometry and quantified as a percentage of total cells. All doses were set up in triplicate and experiments were repeated in three times to yield consistent and reproductive results. Fisher's exact test and ANOVA with Tukey's Multiple Comparisons test assessed the results with p-value < 0.05 considered significant

IV. Results

Effect of Kinase Inhibitors on Cells Growth In Vitro

All the kinase inhibitors chosen in this experiment have been studied extensively, and FDA approval has been obtained for Trametinib and Gefitinib, for cancer treatment. The rest of the inhibitors have also been clinically tested. MTT assays verify the ability of each inhibitor in suppressing cell proliferation with 293T cells. The IC_{50} values of each kinase inhibitor for 293T cell line, as calculated by MTT assay and GraphPad Prism 7 (Sebaugh, 2011), are summarized in Figure 10. 293T cells are highly-sensitive to buparlisib [$IC_{50} < 1$ μmol/L], less sensitive to GSK1120212, and Bisindolylmaleimide I [1μmol/L < IC_{50} < 10μmol/L], and least sensitive to Afuresertib , AMG337, Gefitinib and GSK2334470 [$IC_{50} >$ 10 μmol/L]

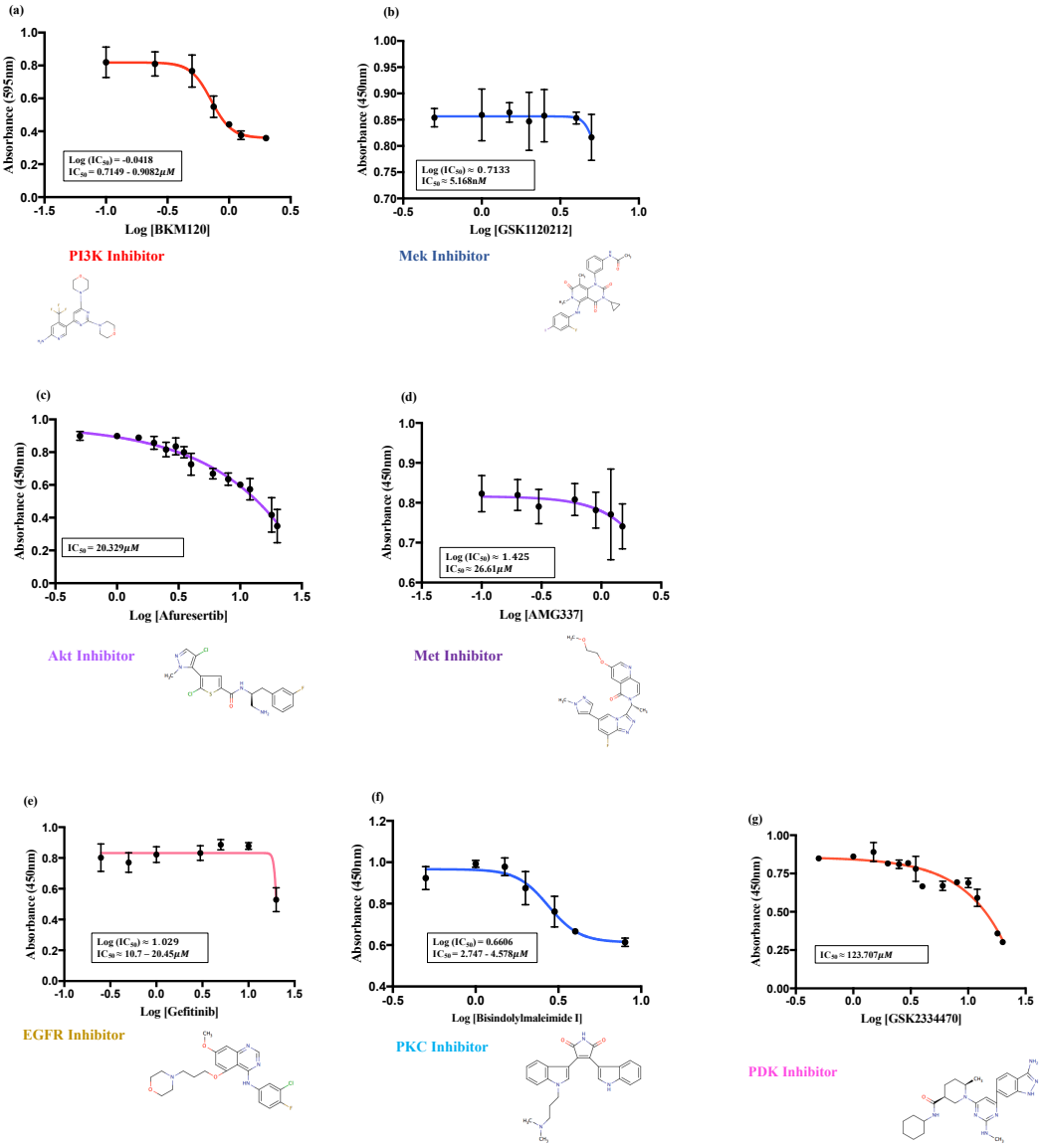


Figure 10. IC50 values for 293T cell lines using the MTT assay.

For 293T cell lines, the IC_{50} value for BKM120 (PI3K inhibitor) is 0.91μM, for GSK1120212 (Mek Inhibitor) is 5.17nM, for Afuresertib (Akt inhibitor) is around 20.33μM, for AMG337 (Met inhibitor) is 26.61μM, for Gefitinib （EGFR inhibitor) is 10.7 to 20.45μM, for Bisindolylmaleimide I (PKC inhibitor) is 2.75 to 4.58μM, and for GSK2334470 (PDK1 inhibitor) is above 100μM

Highly-sensitive: buparlisib [IC_{50} < 1 μmol/L]

Intermediate-sensitive: GSK1120212 [1nmol/L < IC_{50} < 10nmol/L], and Bisindolylmaleimide I [1μmol/L < IC_{50} < 10μmol/L]

Resistant : Gefitinib, Afuresertib, AMG337, and GSK2334470[IC_{50} > 10 μmol/L]

For this experiment, the ultimate goal is to investigate the effect of each inhibitor on HR of DSBs. The concentrations of each kinase inhibitor chosen in this thesis should exhibit robust inhibition of its cognate target without inhibition of cell proliferation. Two concentrations for each inhibitor were chosen to show the minimal impact on cell growth in our IC_{50} characterization process. These concentrations have also been investigated for the ability to inhibit each respective kinase. The impact of these chosen inhibitor concentrations was further analyzed by MTT assay. These data demonstrated that for PI3K, Mek, Akt, Met, and EGFR kinase inhibitors, there exist no significant difference between controls with low and high concentration dosing groups, while for PKC and PDK1 kinase inhibitor, the high concentration treatments slightly inhibit cell growth (Figure 11). However, based on the results from ANOVA with Tukey's multiple comparisons, for PKC and PDK1 kinase inhibitor, since the high concentration treatments have no significant difference with low concentration treatments, the adjusted P values for these two tests are only slightly smaller than 0.05 (0.04 for PDK1 and 0.01 for PKC), and there are only three test groups for each treatment, therefore, some statistic deviations might exist. More important, the high concentration dose is chosen below IC_{50} values for Bisindolylmaleimide I and GSK2334470. In the following experiments, all 293T cell lines are treated with high concentration dose.

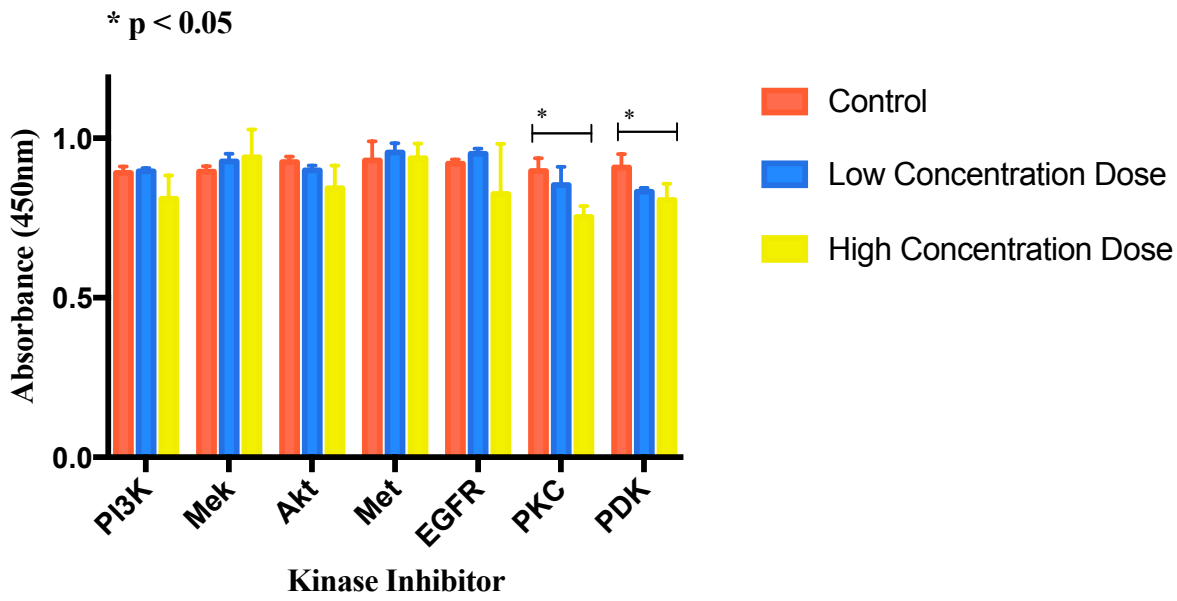


Figure 11. Impact of Chosen Inhibitor Concentrations on 293T cell proliferation.
Both IC_{50} values and cell viability for each treatment group are analyzed by the MTT Assay. All collected data are then processed by ANOVA with Tukey's Multiple Comparisons test with p value < 0.05 considered significant.

The inhibitors suppress activity of kinases while the cell growth conditions are not affected.

The inhibitors chosen in this thesis mainly affected two pathways, the MAP kinase, and PI3 kinase cell signaling pathway, and there are significant overlaps as well as cross-communications between them. However, inhibitors for downstream kinase substrates still possess unique responses. The activation of ribosomal protein S6 kinase beta-1 (S6K1) was used as the first readout. Though the phosphorylation of Thr229 on p70S6K (mainly by PDK-1) is activated by signals that can also activate the PI3K pathway, there exist other phosphorylation sites on p70S6K aside from Thr229.

293T cells were firstly serum-starved for 24 hours, and the cells were incubated with respective inhibitors for one hour. Then FBS was added to a final content of 10% for 30 minutes before harvest and lysis. The cell lysates were analyzed for p70S6K

phosphorylation by Western blot analysis. Western Blot bands were quantified via ImageJ, and the results were plotted by GraphPad Prism 7 (Figure 12).

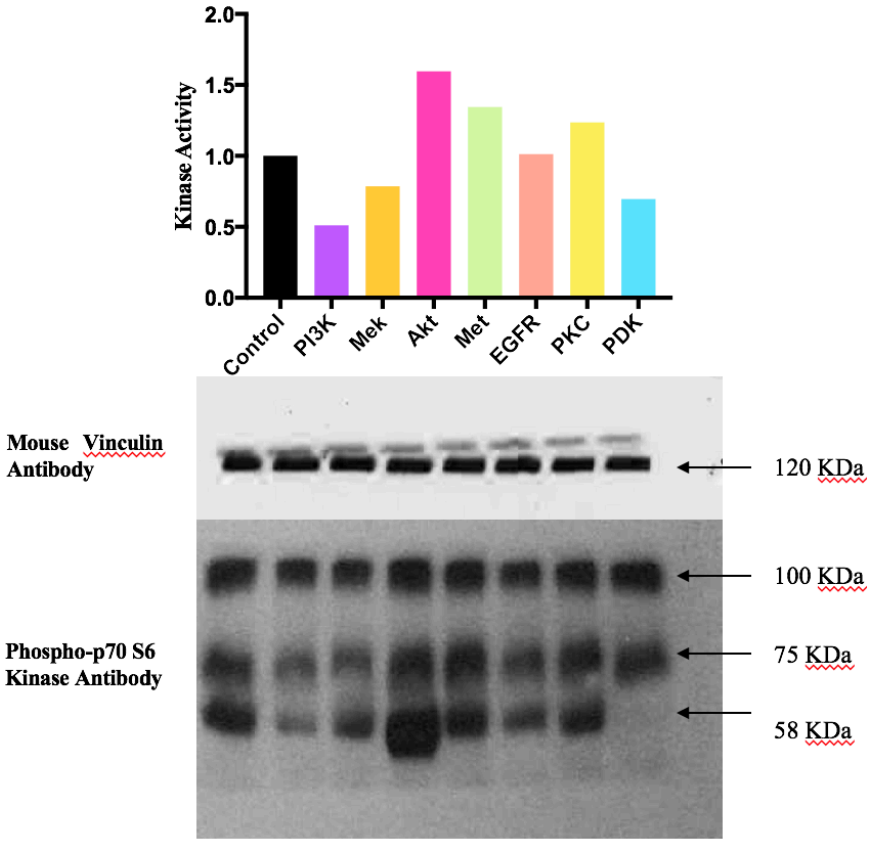


Figure 12. Western Blot Analyses: Effect of Kinase Inhibitors on p70S6K phosphorylation in 293T cells

The lanes from left to right, the sequence of bands is: Control, BKM120 (for PI3K), GSK1120212 (for Mek), Afuresertib (for Akt), AMG337 (for Met), Gefitinib (for EGFR), Bisindolylmaleimide I (for PKC), and GSK2334470 (for PDK). The bottom two bands correspond to two isoforms of p70S6K. Mouse vinculin antibody was used for loading control.

Three of the inhibitors display significant inhibition of p70S6K phosphorylation in 293T cells: 0.25μM BKM120, 2.0nM GSK1120212, and 3.0μM GSK2334470. Interestingly, the phosphorylation of p70S6K on Thr229 seemed to be elevated by the AKT inhibitor, which requires further investigation. On the other hand, though PI3K pathway is downstream of the majority of receptor kinases, neither Met inhibitor nor EHFR inhibitor showed significant inhibition of p70S6K phosphorylation, since the growth factor rich media with 10% FBS will inevitably activate PI3 kinase pathway (Figure 12).

PI3K/Akt/PDK/mTOR pathway suppress the Homologous Recombination Repair in 293T cells

To detect DSB-induced HR, DR-GFP reporter was transfected to 293T wild type cells using LipofectAMINE 3000 (Invitrogen), and stably transfected 293T – DR cell line is produced by the selection with puromycin – resistance (1.5μg/ml). Two differentially mutated GFP genes oriented as direct repeats composes DR-GFP reporter: the recognition site for the rare-cutting I-*Sce*I endonuclease is in upstream repeat, while a 5' and 3' truncated GFP fragment is located in the downstream repeat. DSB in the upstream GFP gene is caused by transient expression of I-*Sce*I and HR repair results in GFP^+ cells (Figure 13.a), which can be visualized and imaged by fluorescence microscopy (Figure 13.b) and quantified by flow cytometry. In this thesis, the number of GFP positive cells in each sample represents the efficiency of HR.

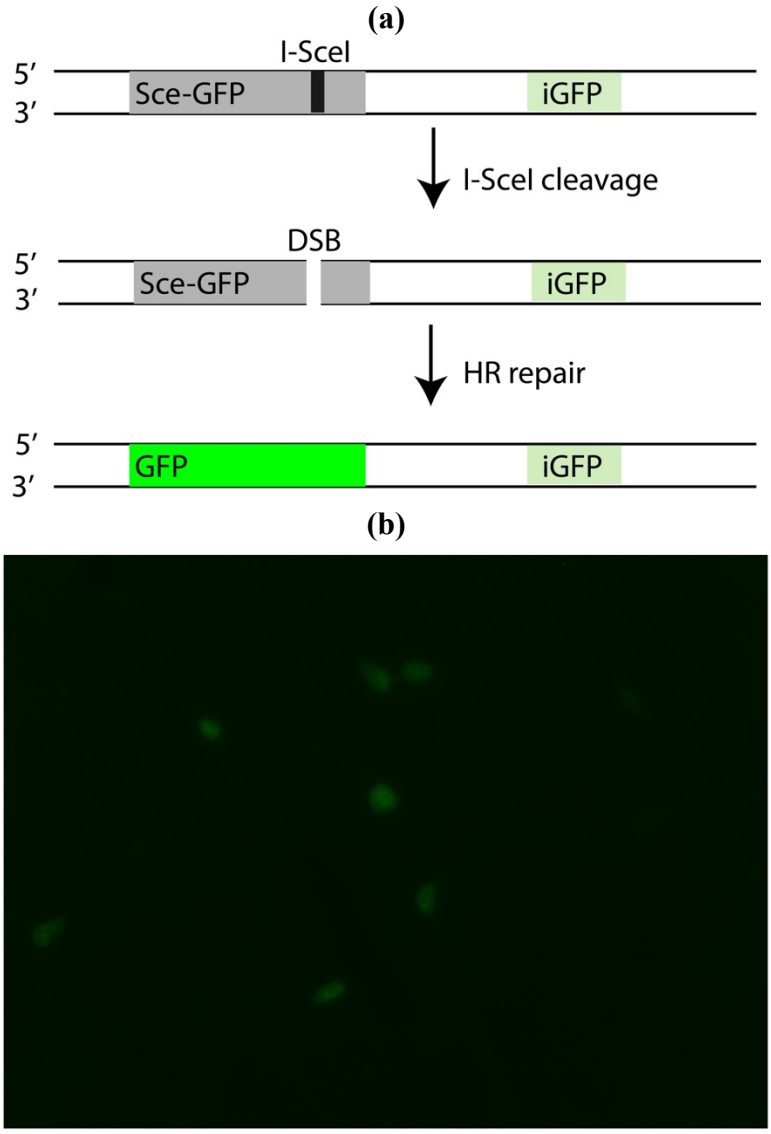


Figure 13. Design of HR Assay.
(a) To analyze the impact of different signaling pathways, a 293T cell lines stably transfected with DR-GFP plasmid was established. After plating these cells overnight, a plasmid harboring I-Sec (pCBASceI) is transfected by lipofectamine 3000. Selective inhibitors were added 2 to 4 hours post-transfection. Percentage of fluorescent cells was analyzed by flow cytometry and quantified as a percentage of total cells. (b) GFP+ cells can be visualized and imaged by fluorescent microscope. The example in this figure is from PI3K treatment group.

GFP+ cells quantifications are processed by flow cytometry, and the raw data is then analyzed via Fisher's exact test and Unpaired T-test with the p-value < 0.05 considered significant (Figure 14, 15). For Chi-Square test or Fisher's exact test, p values are calculated based on the total number of cells, while in unpaired T-test, p values are generated by the comparisons the percentage of GFP positive cells in control group with other treatment groups (Figure 16). Chi-Square reveals whether there is a relationship between two treatments and T-test is used to test whether the difference between two means is zero.

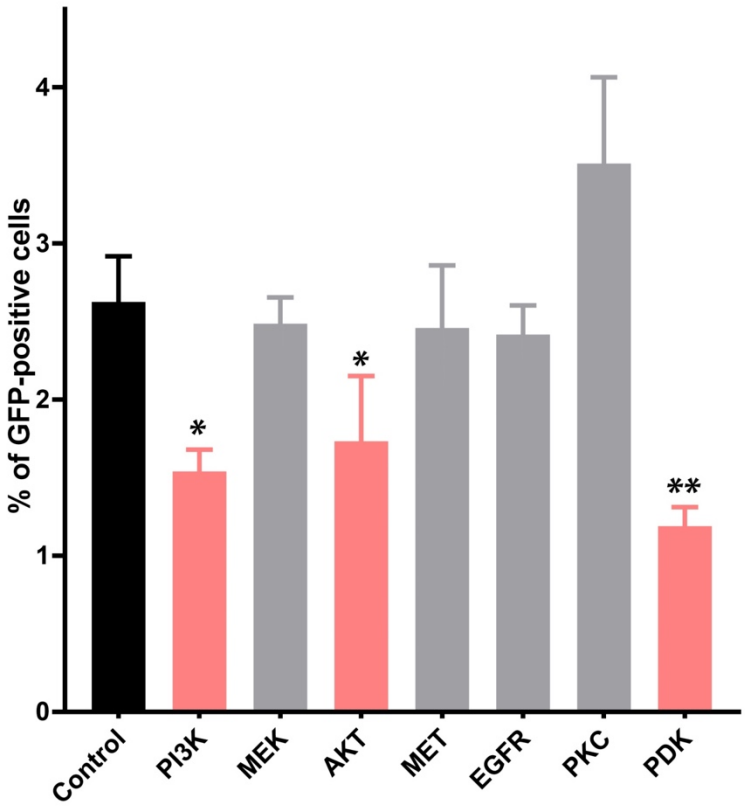


Figure 14. Impact of Inhibitors on HR Repair of DSBs (T Test).
Using sub-lethal concentrations of each inhibitor, their respective impacts on homologous recombination repair of DSBs were analyzed by the DR – GFP assay following the protocol in Figure 13(a). Numbers of GFP positive cells in each treatment group are compared with numbers in control groups and p values are calculated via ***ANOVA with Tukey's Multiple Comparisons test.*** Any p values less than 0.05 is considered statistically significant. Data summarized in ** represents very significant with p values between 0.001 to 0.01, in * represents significant with p values between 0.01 to 0.05.

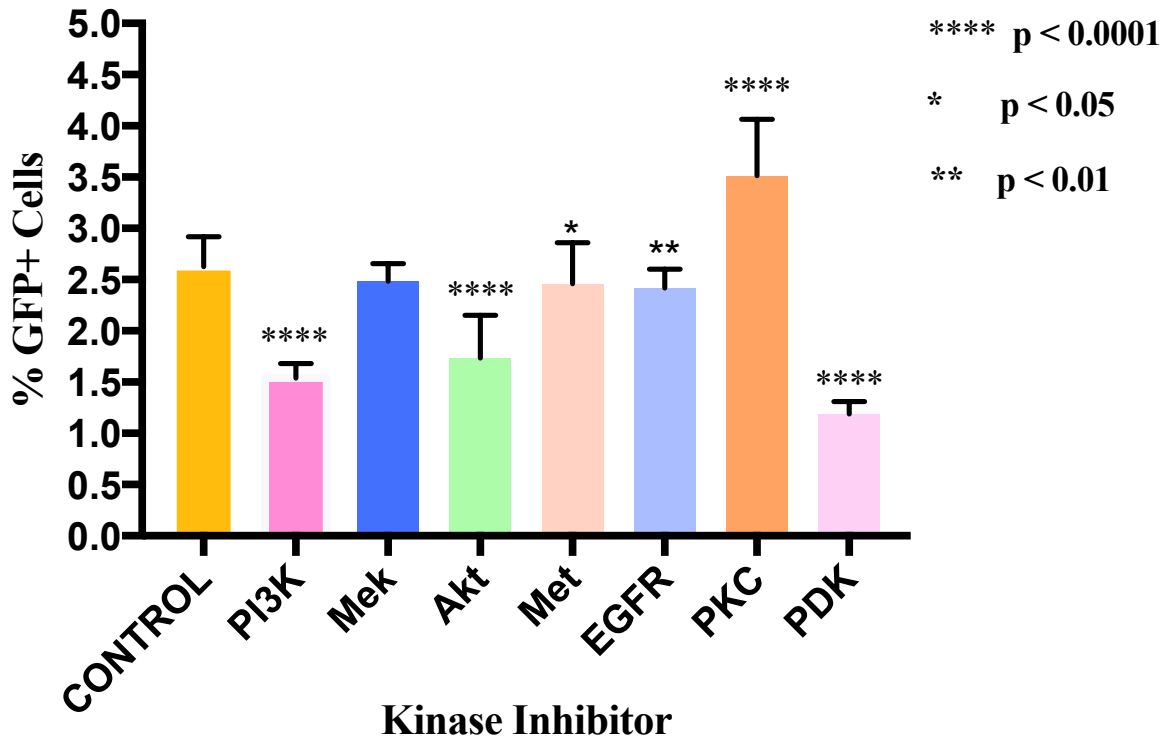


Figure 15. HR Repair is regulated by several cell signaling pathway (Chi-Square). Numbers of GFP positive cells in each treatment group are compared with numbers in control groups and p values are calculated via ***Fisher's exact test***. Any p values less than 0.05 is considered statistically significant. Data summarized in **** indicates extremely significant difference with p values less than 0.0001: ** represents very significant with p values between 0.001 to 0.01. More detailed results are shown in Figure 16. Figure 14, 15 and 16 present data from 1.22 trial.

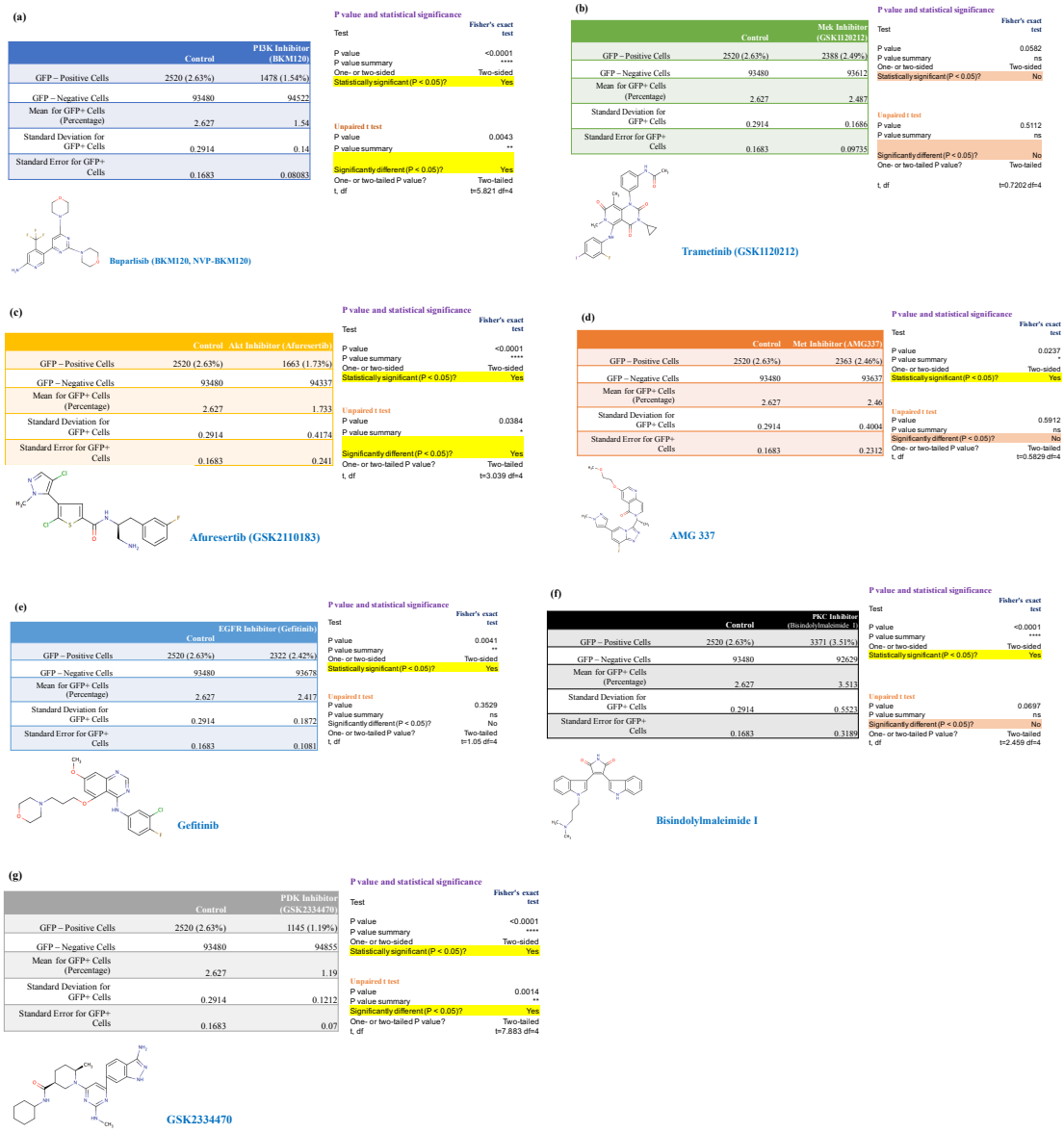


Figure 16. Detailed Statistic Analysis for kinase inhibitor suppressed HR.
P values are calculated from Fisher's Exact test (Chi – Square) and Unpaired T - Test

Collectively, these results indicate that inhibition of PI3K directly or its downstream pathway (Akt and PDK) compromise homologous recombination repair of DSBs. These effects are reproducible with a different batch of cells, and consistent with the earlier report that mTOR could regulate HR (Chen, 2011). Therefore, it could be inferred that PI3K/Akt/PDK/mTOR cascade regulates the mechanisms of homologous recombination for DSBs.

Effects of MAPK/ERK or Mek pathway to homologous recombination

Trametinib (GSK1120212) was used to investigate the impact of MAP kinase pathway on HR repair. It was suggested that the inhibition of the Mek (also known as MAPK/ERK) or **G**lycogen **s**ynthase **k**inase 2 (GSK3β), which is regulated by Mek pathway and respond to glucose, might enhance homologous recombination efficiencies in cells (Lin, 2014). However, current results from this experiment demonstrate the inhibition of this pathway has minimal, if any, impact on HR repair of DSBs. The experiments were repeated four times independently, each time with triplicates. No significant numerically or biologically differences exist between control and experimental groups even if numerically substantial (Figure 17).

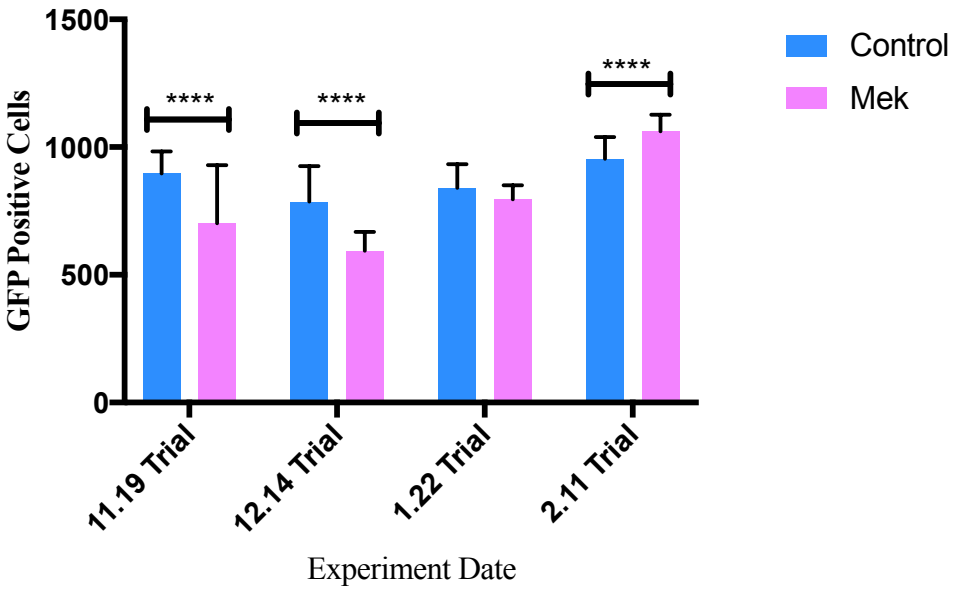


Figure 17. Mek Inhibitor GSK1120212 affects HR Efficiency significantly
Any p values less than 0.05 is considered statistically significant. Data summarized in **** indicates extremely significant difference with p values less than 0.0001.

Regulation of DSB repair in HR via Growth Factors (Epidermal growth factor EGF, Hepatocyte growth factor HGF

PI3K pathway is activated by myriad of pro-growth and/pro survival factors, especially these that activate receptor tyrosine kinases, which include epidermal growth factor (EGF), vascular endothelial growth factor (VEGF), fibroblast growth factors (FGF), insulin, platelet-derived growth factors (PDGF), hepatocyte growth factor (HGF), and many others. In 293T cells, the expression of EGF receptor is minimal and 293T cells display minimal response to EGF stimulation. On the other hand, 293T cells express significant HGF receptor, c-Met, and is generally response to HGF stimulation for subsequent PI3K activation is the absence of serum (Viticchie, 2015). Met is a receptor tyrosine kinase that can activate a wide range of different cellular signaling pathways after binding with its ligand, HGF, which acts as a pleiotropic factor and cytokine to promote cell proliferation,

survival, motility, scattering, differentiation, and morphogenesis (Organ, 2011). EGFR is a transmembrane protein that functions as the receptor for members of the EGF family of extracellular protein ligands. We first tested EGF, in general as a negative control, for any potential effect of growth factors on HR. In this experiment, we added 100 ng/ml EGF post-transfection in the presence of 10% FBS, which could further complicate the situation as FBS contains variety of growth factors. Our assay indicates EGF had minimal impact on HR with numerically less than 10% reduction while statistically significant (Figure 17). The effects of other growth factors including HGF are planned.

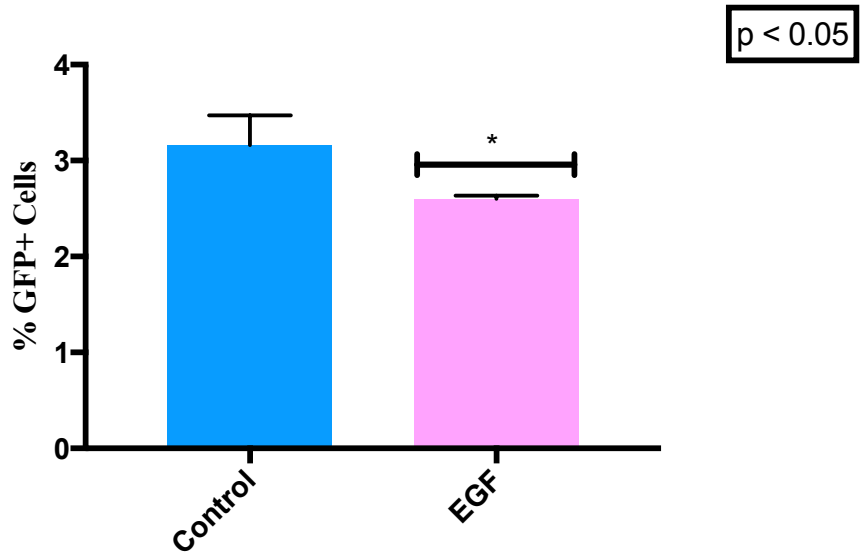


Figure 18. Addition of EGF did not increase the efficiency of HR in 293T cells.
Any p values less than 0.05 is considered statistically significant and summarized in *.

The Effect of PKC Inhibitor

Though the statistical analyses show that the inhibition of PKC kinase even increases the efficiency of HR significantly, the results might be confounded by the overlap between orange and green color because the PKC inhibitor possesses bright orange color, which could complicate the fluorescence flow cytometric analysis. While the observation that

inhibition of PKC kinase by Bisindolylmaleimide I resulted in the increasing of GFP positive cells may suggest novel mechanisms of PKC – regulated HR, control experiment indicated that Bisindolylmaleimide I itself was a fluorescent molecule and could have interfered with the measurement of GFP.

We incubated untransfected 293T cells with Bisindolylmaleimide I and analyzed the fluorescence signal. The cytometry analysis indicated the presence of GFP-signal (1.05%, Figure 19). Therefore, the effect of PKC inhibitor Bisindolylmaleimide I should be interpreted with caution. Alternative inhibitors will be used for future analysis.

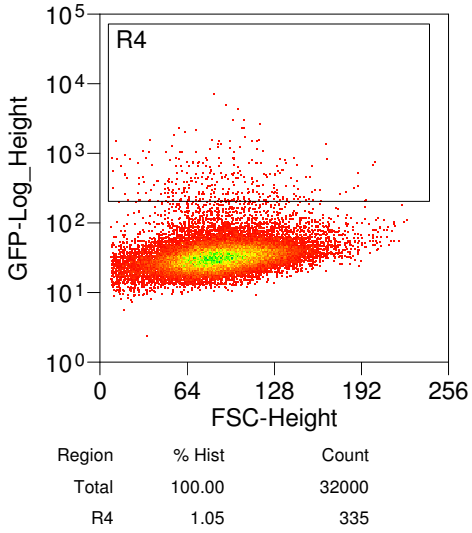


Figure 19. Ratio of "GFP positive cells" in 293T wild type cells.
293T wild type cells were treated with 2.0 μM of Bisindolylmaleimide I overnight, and the number of green cells is counted by flow cytometry. Since no DR and I-*Sce*I plasmids were transfected, theoretically, non GFP positive cells should exist in wild type sample. The conflict result might indicate the problem exists in the choice of kinase inhibitor.

Cellular Decisions to Cisplatin – Induced DNA Damage

Platinum derivatives such as *cis*-Diaminedichloroplatinum (II) (Cisplatin), are a class of potent chemotherapeutic agents that treats a variety of cancers for decades (Sears, 2012). As the primary biological target of cisplatin, N^7 position of purine bases in DNA molecule forms covalent bonds with the platinum atom of cisplatin. 1,2- or 1,3- intra-strand adducts

and a lower percentage of inter-strand adducts are then formed, distorting of DNA double helix, which interferes with DNA replication and transcription. The cellular proteins that can recognize this alteration in the DNA structure are used to repair cisplatin-induced DNA damage (Basu, 2010). However, resistance development is the most significant challenge for cisplatin application. It has been reported that up-regulation of homologous recombination or translesion synthesis play critical roles in cisplatin resistance (Aloyz, 2002). If HR plays a significant role in DNA damage repair caused by cisplatin, then inhibition of HR by kinase inhibitors could enhance its toxicity. We conducted the combination studies on 293T cells.

WT – 293T cells were treated in combination of cisplatin and kinase inhibitors. Though the results are preliminary, some interesting conclusions can be drawn. The dose-response curves suggested that protein kinases regulated the cellular responses to cisplatin-induced DNA damage. With the addition of kinase inhibitors, 293T cells are more sensitive to cisplatin (Figure 20, 21), and three of kinases have been identified that they can regulate cellular decisions to repair DSBs in homologous recombination pathway (Figure 14, 15). More specifically, first, these inhibitors that showed no impact on HR, including against EGFR, MET, and MEK, have no effect on cisplatin toxicity. Second, inhibitors that affect HR, including against PI3K, AKT, and PDK, all display moderate enhancement of cisplatin toxicity. Third, PKC inhibitor, though displayed no impact on HR, showed the strongest enhancement of cisplatin toxicity. One possible explanation is the fact that PKC is critical for regulating Pol h activity (Peddu, 2018). Now we are in the process to investigate whether the downstream cellular effects of cisplatin treatment have an impact on HR catalyzed repair of a non-cisplatin-damaged DSBs.

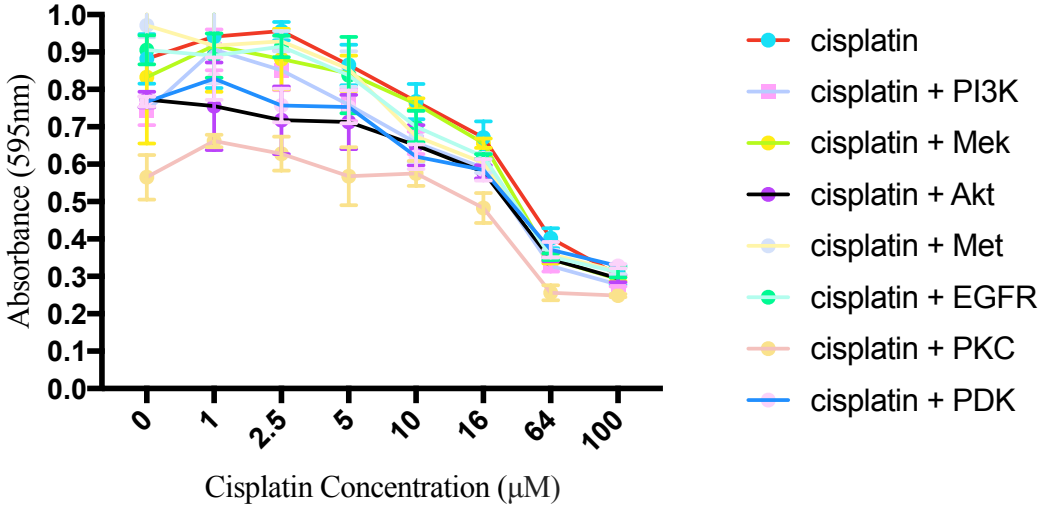
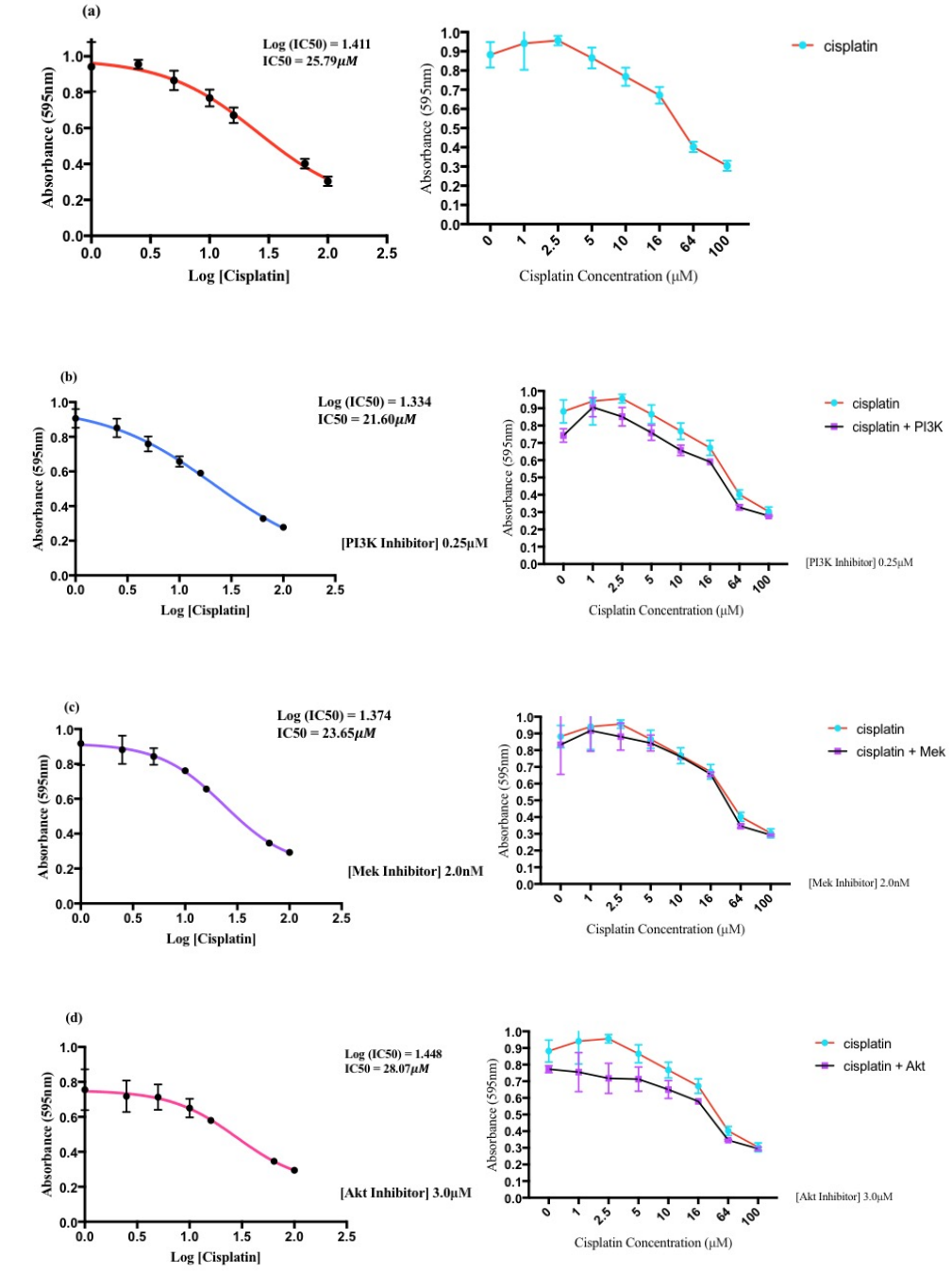


Figure 20. Cell Survival Assay for the Combination of Cisplatin and Kinase Inhibitor
WT-293T cells were incubated with cisplatin and kinase inhibitors for 72 hours. The concentrations of each kinase inhibitors were maintained (High Concentration Listed in Table 2) whereas, and the concentration range for cisplatin was from 1.0μM to 100μM. More details are shown in Figure 21.


(a)
Log (IC50) = 1.411
IC50 = 25.79μM
Absorbance (595nm)
Log [Cisplatin]
cisplatin
Cisplatin Concentration (μM)
(b)
Log (IC50) = 1.334
IC50 = 21.60μM
[PI3K Inhibitor] 0.25μM
cisplatin
cisplatin + PI3K
(c)
Log (IC50) = 1.374
IC50 = 23.65μM
[Mek Inhibitor] 2.0nM
cisplatin
cisplatin + Mek
(d)
Log (IC50) = 1.448
IC50 = 28.07μM
[Akt Inhibitor] 3.0μM
cisplatin
cisplatin + Akt

(Continue)

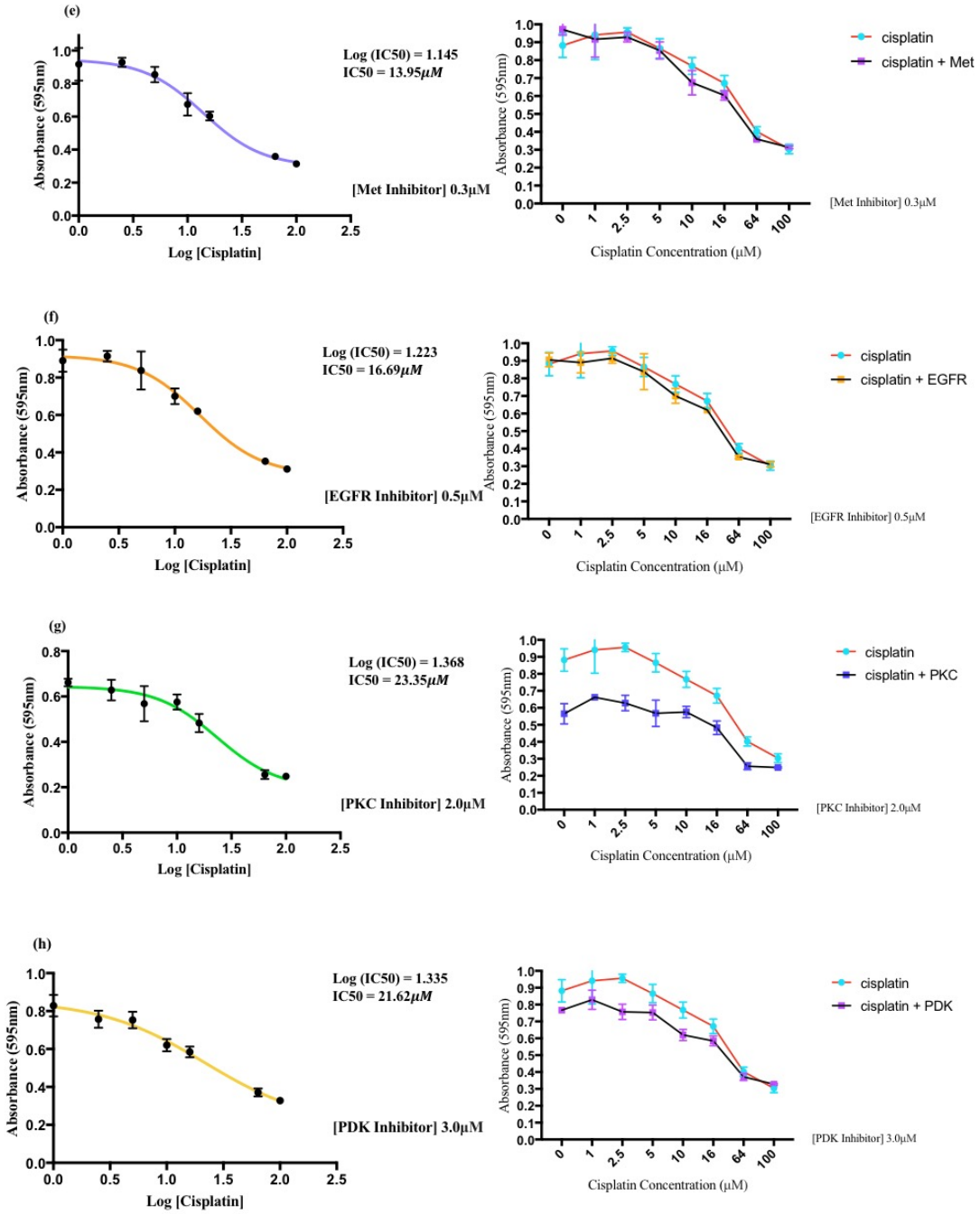


Figure 21. More Detailed IC50 curves and Dose-Response Curves for the Cell Survival Assay for the Combination of Cisplatin and Kinase Inhibitors.

V. Discussion

i. Cellular Decision to Repair DSBs by Homologous Recombination is regulated by cytoplasm signaling event.

The concentrations of each kinase inhibitor used in this experiment should suppress the phosphorylation status for a specific kinase substrate but not inhibit cell growth. The sub-lethal concentrations of each inhibitor are determined by IC_{50} curves (Figure 10) and cell proliferation assay (Figure 11) so that each will have a minimal impact on cell proliferation. To avoid possible bias, the 293T cells in the presence of two different concentrations of each inhibitor for a similar time-frame as DR-GFP assay. Western Blot analysis was conducted to ensure that the inhibitor concentrations employed have a significant impact on respective signaling pathways. First, phosphorylation of S6K (p70S6K) was selected as the initial readout of activation of PI3K pathways. S6 kinase beta-1 (S6K1), a serine/threonine kinase, acts downstream of PI3 kinase pathway (Figure 22), and are targets of Akt, PDK1 (Figure 22), and Mek (Figure 25). Since Akt and PDK1 are activated and phosphorylated by the activation of PI3K (Figure 22), therefore, the antibody of S6K used in this experiment can indicate the effect on signal transduction for the inhibition of PI3, Akt and PDK1 kinases (Figure 12). The selected antibody MAB8964 (Cell Signaling) is targeted against phosphorylation of T229, a direct target of PDK. The results indicate that 0.25 μM BKM120, 2.0 nM GSK1120212, and 3.0 μM GSK2334470 inhibited the phosphorylation on T229 of S6K. It is interesting to note that inhibition of AKT increased the phosphorylation of T229, the direct target of PDK. We plan to use other antibodies for the detection of other potential phosphorylation sites, especially direct targets of AKT and mTor. Nevertheless, the inter-relationship between Akt and PDK1 is under-explored. One

possibility is that Akt is phosphorylated and activated by PDK1, the blocking of Akt pathway might not influence PDK1 pathway (Figure 22). Thus, with the addition of Akt inhibitors, S6K can still be phosphorylated by PDK1, which might cause the increase signal detected by Western Blot. Met and EGFR act as the high-affinity tyrosine kinase receptor for **h**epatocyte **g**rowth **f**actor (HGF) and **e**pidermal **g**rowth **f**actor (EGF). Though both Met and EGFR are up-stream of the PI3K - Akt pathway, the activation of PI3 kinase is not only depended on the Met or EGFR. Therefore, to detect the effect on signal transduction by the addition of Met or EGFR inhibitor, the specific antibody for kinase receptor itself should be used. Since S6K is not involved in the downstream pathway for PKC kinase, another specific antibody for phosphorylated PKC kinase will be used in the future to improve this experiment.

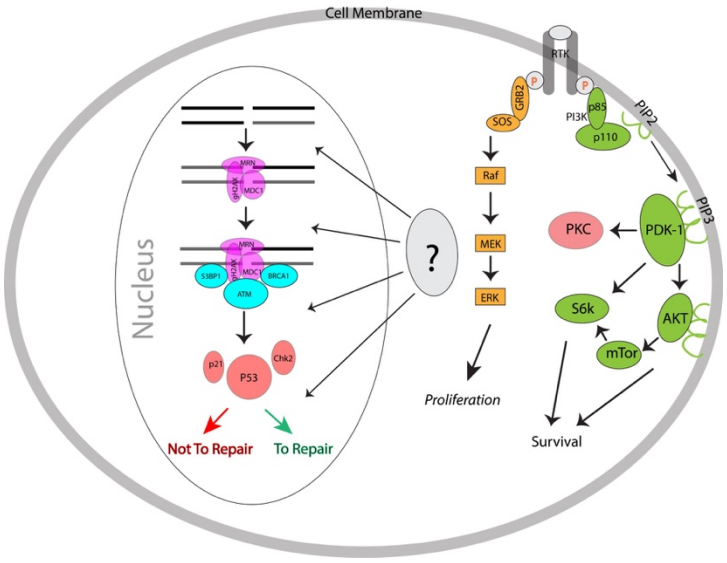


Figure 22. Extracellular Cell Signaling Pathway have Impact on Cellular Decision for DNA Repair.
These factors transduce signals through cytoplasmic kinase cascades to affect the decision of whether to repair a DSB.

DSB – induced HR was measured by DR-GFP reporter in this research. Nakanishi 's HR-Reporter was modified to analyze the impact of different signaling pathways (Nakanishi, 2011). DR-GFP plasmid is transfected in wild type 293T cells. This vector consists two defective GFP genes: the arrow indicates the I – *Sce*I endonuclease restriction site located in the first GFP genes and the cells are GFP negative. After generating a stable 293T DR cell line, I – *Sce*I endonuclease expression vector is then transfected. Cellular expression of I – *Sce*I causes a DSB and the downstream wild-type GFP sequence now is used as a template for HR repair, which results in GFP positive cells. Selective inhibitors or growth factors (EGF) were added 2 to 4 hours post-transfection and percentage of fluorescent cells was analyzed by flow cytometry and quantified as a percentage. The flow – chart of DR-GFP assay is shown in Figure 23.

Figure 23. Flow – chart of DR-GFP assay with the treatments of Kinase Inhibitor

The cells with transfected DR-GFP plasmid was selected by 1.5μg/ml of puromycin, To estimate the transfection efficacy of pCBASceI (I-Sec), an internal control vector that constitutively expressed a reporter protein (GFP in this thesis) was used to estimate the transfection efficiency for DR – GFP plasmid. This GFP – expressing plasmid had similar insert size with DR – plasmid and was transfected into a separate well of WT 293T cells. The fluorescence microscopy image in Figure 24 the appearance of GFP fluorescence from the living, viable cells (Plasmids 101), and routine transfections all results in 6—80% efficacy under conditions we used.

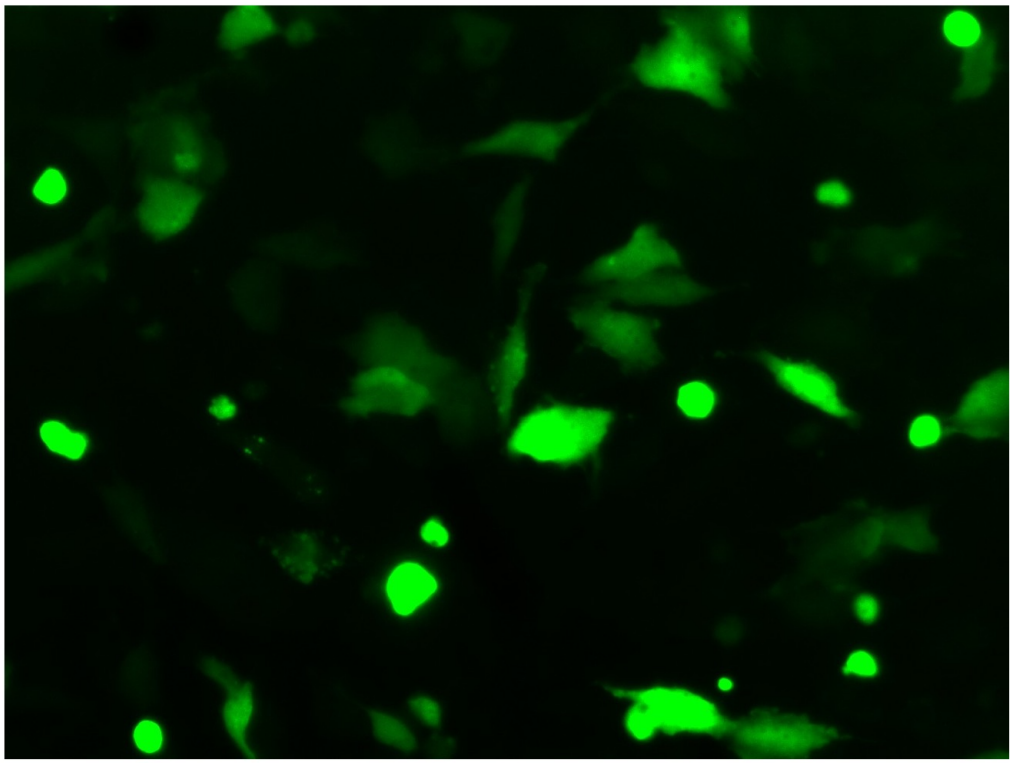


Figure 24. Measure Transfection Efficiency by Internal Control Vector.
The GFP – expressing plasmid used has similar insert size with our test plasmids, DR – GFP and I - SceI. As a positive control, this internal control vector is used when transfected with DR – GFP plasmid and I – SceI plasmid. The transfection of internal control vector and test plasmid occurred at the same time and under same conditions, but in separated wells.

One critical question remains in the assessment of HR defect. There are two factors for the significant inhibition of HR activity. Granted, all differences have to be statistically significant. However, a statistically significant difference only indicates that the difference is unlikely to have occurred by chance, but does not mean the such difference is biologically large, important, or significant (Dosits.org, 2019). The concept of biological relevance is explored that before studies are initiated the nature and size of relevant biological changes or differences is defined, and those pre-defined relevant biological effect will be used to design studies (EFSA Scientific Committee, 2011). In clinical settings,

it is observed 16% cutoff in loss of heterozygosity can define the benefit of PARP inhibition in ovarian cancer treatment (Morgan, 2018).

In our results, the p-values calculated by Fisher's exact test (Figure 15, 17) demonstrated that the addition of kinase inhibitors affected the efficiency of HR significantly, these differences might not be all biologically significant. The activity of PI3K family members is activated after growth factors and cytokines binding to the transmembrane receptors, and phosphatidyl-inositol di-phosphate (PIP2) is then phosphorylated to generate PIP3 at the plasma membrane. PIP3 recruits Akt and PDK1 that containing a pleckstrin homology (PH) domain to the membrane. When Akt isoform is activated and phosphorylated by PDK1, the activity of mTOR is then activated, which can regulate HR repair following DSBs (Liu, 2014). Meanwhile, PI3K activity plays a critical role for Rad51 recruitment since the inhibition of PI3K decreases Rad51 focus formation (Juvekar, 2012), and Akt1 can stimulate HR repair of DSBs via a Rad51 – dependent manner (Mueck, 2017). Based on current studies, we can hypothesize that the efficiency of HR would decrease with the addition of PI3, Akt and PDK kinase inhibitors. The statistics results (Unpaired T-test and Fisher's exact test) corroborate the initial hypothesize. For PI3K/Akt/PDK1 pathway, a statistically significant difference means biologically important.

The activation of mitogenic MAP/ERK kinase or Ras – Raf – MEK – ERK pathway showed minimal impact on HR activity in our assays. **E**pidermal **g**rowth **f**actor **r**eceptor (EGFR), a receptor – linked tyrosine kinase, is activated by extracellular ligands. The EGFR becomes phosphorylated on tyrosine residues, which provides the binding site for the SH2 domain of the GRB2 docking proteins. Guanine nucleotide exchange factor SOS binds the SH3 domain of GRB2. SOS is activated when the GRB2 – SOS complex docs to

the phosphorylated EGFR, and activated SOS promotes the removal of GFP from Ras. Ras binds to GTP and become active, which then activate the protein kinase activity of Raf kinase. Mek is phosphorylated and activated by RAF kinase, and mitogen-activated protein kinase (MAPK) is activated and phosphorylated by Mek (Figure 25).

Attempts to investigate the impact of MAPK on HR activity by other research groups produced mixed results. The existence of multiple MAPK pathways further complicates potential results interpretation. Human ERK1/2 is phosphorylated at Tyr204/187 then Thr202/185 by Mek1/2, and it catalyzes the phosphorylation of cytoplasmic and nuclear substrates including transcription factors (Roskoski, 2012). Similar to other MAP kinases, the p38 MAP kinase pathway is activated by MAP kinase kinases (MKKs) and exerts a negative influence on ERK1/2 signaling and therefore, HR repair (Golding, 2007). Though in MAPK/ERK pathway, ERK is a positive regulator for HR repair whereas JNK and p38 have both collaborative and antagonistic effects on cellular response to DSB – induced HR, the interrelations between them are currently being investigated in more detail.

Our results with Mek inhibitor GSK1120212 were repeated four times independently, each time with triplicates. GSK1120212 is an allosteric inhibitor of Mek1/2 activity. To inhibit MEK-Dependent ERK phosphorylation, Mek1/2 activation is inhibited by GSK1120212, which prevents Raf phosphorylation of Mek on S217 (Gilmartin, 2011). Based on its properties, we initially hypothesized that the inhibition of Mek kinase would decrease the efficiency of HR. However, our results showed GSK1120212 did not have consistent impact on HR (Figure 17). More experiments will be conducted in the future to investigate the more detailed mechanism that MAPK signaling pathway regulates HR repair.

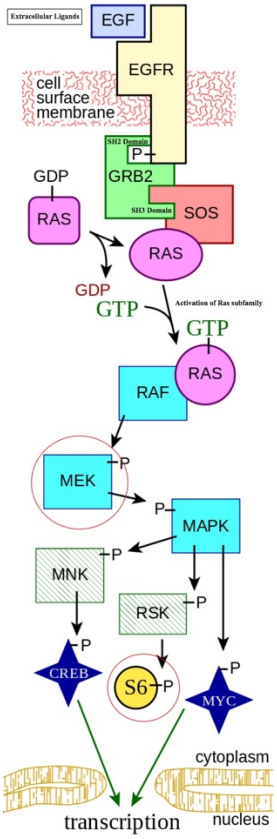


Figure 25. Key steps in MAPK/ERK pathway.
Though Mek is not in the downstream pathway of PI3K, since S6K activation is controlled by insulin – dependent mTOR – mediated phoshphorylation and insulin – independent MAP kinase (Zhang, 2013), the antibody against phosphorylated S6K can be used to detect the effect on signal transduction via Mek pathway. Adapted from MAPK/ERK pathway, Wikipedia.

EGFR is involved in several signaling pathways including Ras/Erk and PI3K/Akt, which might affect the HR repair process. Theoretically, EGFR and its downstream signaling can affect the intracellular distribution of DNA repair proteins including DNA – PKcs, and ATM, which are the most upstream kinase for HR repair (Meyn, 2009). As a competitive inhibitor, Gefitinib prevents ATP binding to the ATP – binding pocket in the kinase domain of EGFR. Therefore, both PI3K/Akt and Ras-Raf-Mek-Erk pathways are shut down (Uchida, 2007). We conjectured gefitinib treatment would suppress HR repair significantly. The experimental results did not support our assumption, and such statistically significant differences (Figure 15) did not indicate the real biological relevance. Similary with EGFR,

c – Met is also a receptor tyrosine kinase that activates a wide range of different cellular signaling pathways after binding with its ligand, **h**epatocyte **g**rowth **f**actor (HGF) (Organ, 2011). HGF induces c – Met phosphorylation, and activates PI3K/Akt signaling pathway (Usatyuk, 2014). The activation of Ras/Erk or PI3K/Akt pathway is not only dependent on the activated EGFR or c - Met and the fetal bovine serum used in cell culture media contains lots of other embryonic growth promoting factors that can also stimulate PI3K/Akt and MAPK signaling pathway. The results from Western Blot (Figure 12) also indicates that the S6K pathway is still at least partially activated. Therefore, the function of Met and EGFR in HR repair cannot be determined yet.

Since the EGF activates the PI3K – Akt cascade signaling pathway, which regulates HR repair, the efficiency of HR is supposed to increase after incubating with EGF. The transfected 293T cells were incubated with 100ng/ml of EGF, but the ratio of GFP positive cells did not increase (Figure 18). One of the possible reasons is 293T cell line does not highly express the wild type EGFR gene since the number of binding sites of EGFR is only 0.01×10^6 (Derer, 2012). To improve specificity and sensitivity for screening drugs targeted to EGFR, the A431 cell line, which highly expresses EGFR and largely depends on the EGFR/MAPK pathway, should replace with 293T cell line (Zhang, 2010).

ii. Conclusion and Future Direction

1. Improvements in the Future

Experiments employing specific antibodies against EGFR and Met should be conducted in the future to gain additional information and insights concerning the roles of the activation of Met and EGFR play in the modulation of HR in293T cells. Similarly, it is noteworthy that the phospho-p70S6 kinase antibody only detects endogenous levels of S6K when

phosphorylated at Thr421 and Ser424. We are working with a p70S6K antibody that can detect endogenous levels of total S6K protein. Based on the results from both phosphorylated S6K and S6K antibodies, we can test whether the phosphorylation of kinase proteins regulates HR repair. Since S6K is not involved in the downstream pathway for PKC, the antibodies against phosphorylated PKC and PKC itself should be used in the future. Furthermore, S6K activation is also controlled by PDK1, which does not require the activation of Akt. Therefore, instead of using an antibody against S6K, the antibody against Akt itself might be a better choice in the future.

To detect the effects of growth factor in HR pathway, the serum – and phenol red - free cell culture media should be used instead of the media with 10% of FBS because growth factor rich media with 10% FBS will inevitably activate PI3 kinase or MAP kinase pathway, which will interfere with the final results. For the choice of PKC inhibitor, if we continuously work with bisindolylmaleimide I that contains bright orange color, the technique named "fluorescence compensation" should be used in flow cytometry to ensure that the fluorescence detected derives from the fluorochrome that is being measured.

Finally, experiments using a variety of cancer cells should be considered to compare and contrast HR control, in response to environmental chemicals and synthetic chemicals with clinical potentials.

2. Conclusion and Future Direction

Even though additional research is required, our results suggest that there is a differentiation between PI3K and MAPK pathway in terms of impact on HR. The activation of mitogenic MAP kinase pathway has minimal impact on HR activity, whereas

PI3K pathway plays a critical role for cellular decision to repair a DSB by HR. Among the downstream PI3K pathways, inhibition of Akt and PDK significantly suppresses HR repair. Though still in early stage, our results (Figure 20, 21) imply that the sub-lethal concentrations of certain kinase inhibitors could be combined with chemotherapeutic agents, cisplatin, that cause DSBs for management of cancers. Certain protein kinases involved in this thesis can regulate the cellular responses to cisplatin-induced DNA damage that the cells become more sensitive (with lower IC_{50} values) to cisplatin after adding kinase inhibitors.

VI. Appendices

A fluorochrome is a fluorescent chemical compound that raises from the ground state to an excited state when absorbing light, and return to the ground state by the emission of a quantum of light. In flow cytometry, laser light, which produces light in the UV and visible range, is used to excite the fluorochromes. Within a flow cytometer, it is possible to separate combinations of fluorochromes based on the excitation and emission properties of fluorescent compounds (Sinobiological.com, 2019)[58]. GFP, a member of the fluorochrome family, can be attached to other specific proteins to form a fusion protein and then synthesized in cells after transfection of a plasmid carrier. In this research, mutated GFP gene is transfected to 293T wild type cells by pDR-GFP reporter, and HR repair corrects this mutation and results in GFP positive cells, which can be detected and separated by flow cytometry (Figure 13b). However, energy transfer occurs whereby excitation of one compound causes the other to fluoresce if two fluorochromes are closely associated and the acceptor molecule has an absorption spectrum that overlaps with the emission spectrum of the donor molecule. In that case, the effective emission maximum will be shifted, and fluorescence from more than one fluorochrome may be detected.

Spectral overlap might cause the great increase in "GFP positive cells" in bisindolylmaleimide I treatment groups. Since bisindolylmaleimide I is a cell – permeable inhibitor, its bright orange color might stain 293T cells. The structure of bisindolylmaleimide I (Figure 10f, 16f) contains two combined aromatic groups with a delocalized conjugated π system, which can be found in a typical fluorochrome molecule. When treated 293T wild type cells with bisindolylmaleimide I, after enzymatically detaching by Trypsin – EDTA solution and washing by **p**hosphate **b**uffered **s**aline (PBS),

the collected cells still contain orange color. These cells are sorted by flow cytometry using a green/yellow- green laser, and there exist 1.05% of green cells (Figure 19). Since none plasmid were transfected to wild type sample, those green cells can be regarded as pseudo – GFP positive cells and the most possible reason is the color from bisindolylmaleimide I. Hence, it could be inferred that bisindolylmaleimide I functions as a fluorochrome, which might also be excited with green laser, or energy transfer might occur between bisindolylmaleimide I and GFP since they are associated closely and both of two fluorochromes are detected. Currently, we are working with other colorless PKC inhibitors to test its roles in the regulation of HR repair.

Reference

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