Role of Akt signaling in homologous recombination-dependent repair of radiation-induced DNA double strand breaks

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Dedicated to my beloved parents and lovely husband

for their support and sacrifices.

Contents

List of Abbreviations:

A-NHEJ	Alternative NHEJ
ATR	Ataxia Telangiectasia and Rad3-Related
ATM	Ataxia Telangiectasia Mutated
ACLY	ATP-Citrate Lyase
BRCA1	Breast-Cancer Susceptibility gene 1
BRCA2	Breast-Cancer Susceptibility gene 2
Chk1	Checkpoint Kinase 1
Chk2	Checkpoint Kinase 2
C-NHEJ	Classical Non-Homologous End Joining
HCT116	Colon Carcinoma Cell Line
DMSO	Dimethyl Sulfoxide
DDR	DNA Damage Response
DNA	Deoxyribonucleic Acid
DSBs	DNA Double Strand Breaks
DNA-PKcs	DNA-Dependent Protein Kinase Catalytic Subunit
Gy	Gray
H2AX	Histon 2AX
HDR	Homology Directed Recombination
ILK	Integrin-Linked Kinase
mTORC1	mammalian Target of Rapamycin-Complex 1
MRE11	Meiotic Recombination 11
MMEJ	Microhomology-Mediated End-Joining
MRN	Mre11/Rad50/Nbs1
NCS	Neocarzinostatin
NFBD1	Nuclear Factor with BRCT Domains 1
NBS	Nijmegen Breakage Syndrome
53BP1	P53 binding protein 1

PBS	Phosphate Buffer Saline
PI3K	phosphatidylinositol 3-Kinase
PARP1	Poly ADP-Ribose Polymerase 1
PNKP	Polynucleotide Kinase Phosphatase
PTM	Post-Translational Modifications
PKB	Protein Kinase B
PLA	Proximity Ligation Assay
Rad51	Radiation Repair Protein 51
mTORC2	Rapamycin Complex 2
ROS	Reactive Oxygen Species
RTKs	Receptor Tyrosine Kinases
RPA	Replication Protein A
SSBs	Single Strand Breaks
siRNA	Small Interfering RNA
TBS	Tris Buffered Saline
TDT	Terminal Deoxynucleotidyl Transferase
UBE2S	Ubiquitin Conjugating Enzyme E2S
WRN	Werner's Syndrome Protein
XRCC4	X-ray Cross Complementing Protein 4

1) Introduction

1.1 Radiation therapy in cancer

After the discovery of X-rays by Wilhelm Conrad Röntgen in 1895, ionizing radiation has been developed as an effective treatment for a wide range of malignancies (Connell and Hellman 2009). Radiotherapy as a local treatment applies ions (electrically charged particles) and deposits high physical energy to kill tumor cells mostly in combination with surgery and systemic chemotherapy (Barton et al. 2014). It is estimated that approximately 50% of all cancer patients need to be treated by radiation therapy during the course of disease (Delaney et al. 2005, Begg et al. 2011).

Ionizing radiation including gamma rays, X-rays and radioactive particles acts through displacement of electrons and is able to break chemical bonds. This leads to disruption of the genetic integrity of cells by producing DNA double strand breaks (DSBs),single strand breaks (SSBs) as well as the generation of reactive oxygen species (ROS) (Borrego-Soto et al. 2015). The efficacy of ionizing radiation for killing cells depends on different factors including total dose, fractionation rate, linear energy transfer (LET) and sensitivity of cells (Baskar et al. 2014).

The main aim of radiation therapy is to prevent extension capability of cancer cells by inducing damages in DNA and eventually killing of cells through various cellular mechanism including apoptosis, mitotic cell death or mitotic catastrophe, necrosis, senescence and autophagy (Baskar et al. 2012). After radiation exposure cells try to activate repair pathway to protect the genome from damages. Therefore, resistance to radiation may develop and can finally result in failure of cancer treatment. In this context, different factors are involved in radio-resistance of cancer cells, e.g. DNA damage repair pathways, cell cycle arrest mechanisms, alterations in oncogene and tumor suppressor status, changes in tumor microenvironment, cell death, generation of cancer stem cells (CSCs) and tumor metabolism (Tang et al. 2018).

In spite of the successes that has been achieved over years to increase in the number of cancer survivors using radiation therapy, radio-resistance still remains a major obstacle to cancer treatment. Thus, further investigations are required to optimize the efficacy of radiation therapy alone or in combination with molecular targeted therapy approaches.

1.2 DNA double strand breaks repair

DNA damage can occur as a result of many endogenous and exogenous insults that are able to influence DNA replication and chromosome segregation. DNA is subject to various types of damages including base lesions, intra- and interstrand cross-links, DNA-protein cross-links, single strand breaks (SSBs) and double-strand breaks (DSBs) (Mehta and Haber 2014). Among all DNA damages types double strand breaks (DSBs) are the most dangerous ones as this kind of DNA lesion can lead to mutations, loss of heterozygosity, chromosome rearrangements, cancer and cell death (Cannan and Pederson 2016). DNA double strand breaks can take place during normal cellular process such as metabolic reactions, DNA replication and DNA repair or as a result of exposure to exogenous agents, e.g. radiation and certain chemicals (Lindahl 1993). One gray of IR results in approximately 2000 base modifications, 1000 DNA single-strand breaks, and 35 DSBs per cell (Rothkamm et al. 2003).

Presence of DSBs in the DNA results in activation of cell cycle checkpoint responses which block cell cycle progression to prevent transmission of damaged chromosomes and finally in stimulation of the DNA repair machinery (Petrini and Stracker 2003). Sensitivity to DSB-causing agents has been reported in different human syndromes such as Ataxia telangiectasia (A-T) and Nijmegen breakage syndrome (NBS) (Khanna et al., 1998).

DNA damage response (DDR) proteins are involved in the process of genome protecting against DNA damage. Assembly of multiple DDR proteins quickly happen

at the site of breaks which are visible as distinct foci by microscopy. DDR proteins can be classified in four groups: sensor, transducer, mediator and effector proteins (Coster and Goldberg 2010). Sensor proteins including ataxia telangiectasia mutated(ATM), ataxia telangiectasia and Rad3-related (ATR), Rad17-RFC complex and the 9-1-1 complex are important for damage detection and transporting the damage signal to transducer proteins such as Checkpoint kinase 1 (Chk1) and Checkpoint kinase 2 (Chk2) Ser/Thr kinases as well as Cdc25 phosphatases. This signal substantially amplified by mediator proteins and results in recruitment and activation of downstream DDR effector proteins (Dasika et al. 1999, Coster and Goldberg 2010). MDC1, also known as NFBD1 (Nuclear Factor with BRCT Domains 1) is a key member of the DDR network. Presence of MDC1 at the site of damage is important for recruitment of other proteins, such as MRN complex (Mre11-Rad50-NBS1), Breast-Cancer Susceptibility gene 1 (BRCA1) and P53 binding protein 1 (53BP1). H2AX, a variant of histone H2A and an integral part of the nucleosome is another important protein of DDR (Goldberg et al. 2003, Lou et al. 2003, Mochan et al. 2003). Histone H2AX has a C-terminal tail with a conserved SQEY-COOH motif. In this motif the serine residue-139 (Ser-139) can be phosphorylated within minutes after generation of DSB (Fernandez-Capetillo O2004, Gideon Coster2010). This phosphorylation known as gamma H2AX (γ-H2AX) is reported to be executed by one of three phospho-inositide-3-kinase-related protein kinases (PIKKs): ATM, ATR or DNA-PK (Burma et al. 2001, Soubeyrand et al. 2001, Stiff et al. 2004, Ward and Chen 2004). γ-H2AX foci formation at the site of damage is required for the accumulation of downstream DDR proteins, recruitment of repair factors and activation of cell cycle checkpoints in response to DNA-damage (Bassing et al. 2003, Celeste et al. 2003, Lou et al. 2006).

In eukaryotes DNA double-strand breaks are repaired through two main pathways, i.e. the classical non-homologous end joining (C-NHEJ) or homology directed recombination (HDR). Both pathways are regulated at different levels and sub-pathways through cooperation of several proteins and enzymes (Featherstone and

Jackson 1999). In addition to these two major pathways for DNA-DSB repair, in case of their malfunction or absence, a third pathway operates as an alternative form of C-NHEJ, named alternative NHEJ (A-NHEJ) (Mladenov and Iliakis 2011).

1.2.1 Classical-Non Homologous End Joining

C-NHEJ is a dominant pathway of DSBs repair and is active in all cell cycle phases. NHEJ is an error prone pathway which can results in insertions, deletions, substitutions and translocations at the site of damage. This pathway is conserved from bacteria to higher eukaryotes (Lieber 2010).

This pathway is initiated by binding of a double-stranded DNA (dsDNA) end-binding protein complex, i.e. the Ku70-Ku80 heterodimer (Ku) to the 5′ end of DNA. This protein is a platform for the combination of various factors involved in NHEJ (Marini et al. 2019). After binding of Ku70-Ku80 heterodimer to DNA, its conformation changes and it recruits DNA-dependent protein kinase catalytic subunit (DNA-PKcs). DNA-PKcs is a large serine/threonine kinase and its activation increases after binding to Ku70/80.(Meek 2004). DNA-PKcs kinase activity is needed for phosphorylating downstream proteins in NHEJ pathway including end processing or ligation factors. Polynucleotide kinase phosphatase (PNKP), terminal deoxynucleotidyl transferase (TDT), DNA polymerases λ and μ, and Artemis are important proteins which are required for processing of DNA break ends creating ligatable ends (Davis and Chen 2013). Lig4-XRCC4-XLF complex is needed for sealing DNA ends. X-ray cross complementing protein 4 (XRCC4) is able to bind to DNA-PK and Lig4, therefore it can stabilize and stimulate ligase4 activity. Binding of XLF to the XRCC4/Lig4 complex is the final step that leads to reclosing of DNA ends (Gottlieb and Jackson 1993, Riballo et al. 2004).

It has been shown that the absence of NHEJ factors are associated with different disorders. For instance, mutation in Artemis leads to progressive radiosensitive severe combined immunodeficiency (RS-SCID) (Hendrickson et al. 1991). Moreover, LIGIV gene mutations result in LigIV syndrome and severe problems such as immunodeficiency, radiosensitivity and developmental delay (Chistiakov et al. 2009).

1.2.2 Alternative-Non Homologous End Joining

Alternative non-homologous end-joining (alt-NHEJ) is a backup repair pathway. It is also known as microhomology-mediated end-joining (MMEJ), or KU-independent end-joining pathway. In A-NHEJ pathway microhomologies of DNA ends can be used for repairing. In comparison to C-NHEJ pathway the probability of translocation formation, deletions and other sequence alterations at the junction is higher in A-NHEJ pathway (Ferguson et al. 2000). Likewise, the repair velocity for DSBs by A-NHEJ is slower than for C-NHEJ (t_{50} 30 min to 20 h) (Dueva and Iliakis 2013).

Poly (ADP-ribose) polymerase 1 (PARP1) and LigIII/XRCC1 complex are the main players of the A-NHEJ pathway in vertebrates (Herceg and Wang 2001, Hassa et al. 2006). Similar to HRR, the end resection process in A-NHEJ is initiated by the Mre11/Rad50/Nbs1 (MRN) complex and CtIP. Poly (ADP-ribose) polymerase 1 (PARP1) is a sensor of DNA damage that binds to single strand breaks (SSBs) as well as double strand breaks. PARP can activate poly (ADP-ribosyl) ation of proteins and recruits MRE11/RAD50/NBS1 (MRN) complex to the damage sites. In subsequent steps Exo1 or DNA2 interact to produce long stretches of single stranded DNA. Ligation of DNA ends is terminated either by LIG3 or LIG1 and cooperation of XRCC1 complex (Iliakis 2009, Ali et al. 2012). Likewise, it is reported that binding of KU to DSBs leads to suppression of Exo1 and Dna2 function and reduced micro-homology mediated end joining (Symington 2016). There is evidence showing the association of A-NHEJ upregulation and expression of oncogenic BCR-

ABL gene fusion and chronic myelogenous leukemia (CML). It is shown that decreasing of C-NHEJ proteins such as Artemis and DNA LigIV and consequently increasing of A-NHEJ proteins, LigIII and Werner's syndrome protein (WRN) is associated with genomic instability in BCR-ABL–positive CML cells. (Sallmyr et al. 2008, Poplawski and Blasiak 2010)

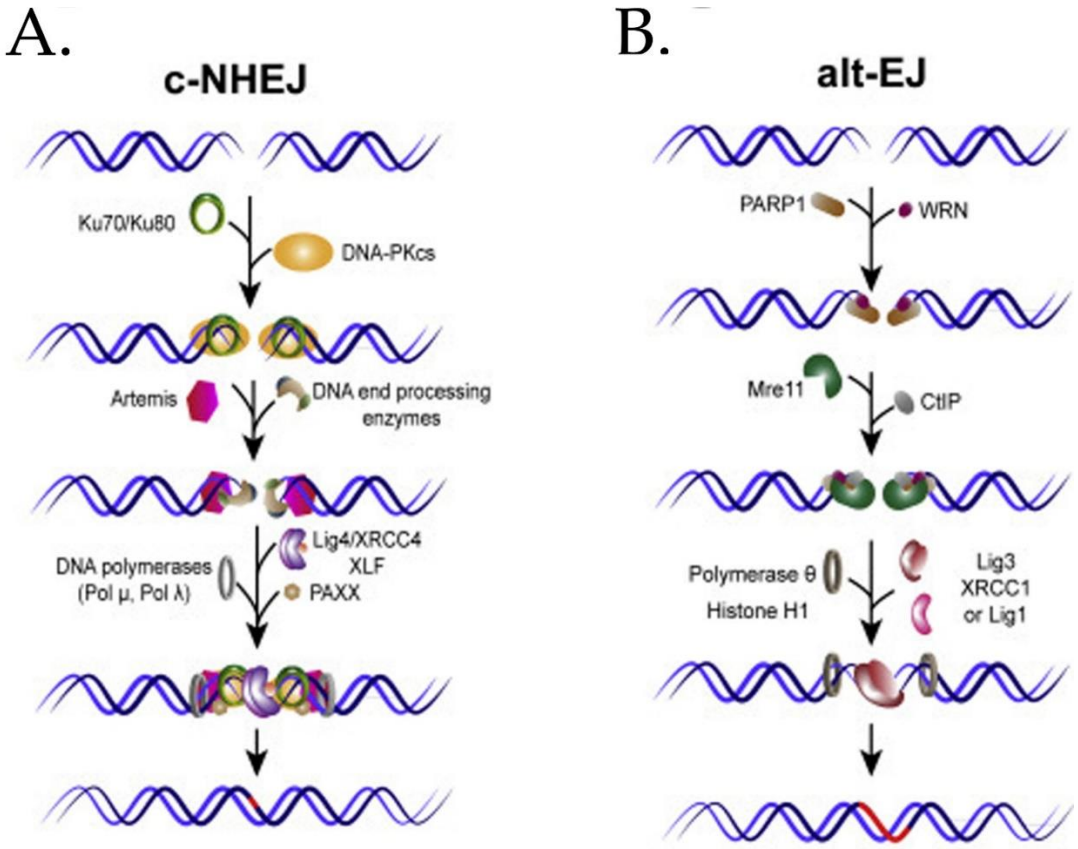

Figure 1.1. Schematic representation of DNA repair by Non-Homologous End Joining repair pathways. (A) C- NHEJ starts with binding of KU heterodimer to double-stranded DNA ends and recruitment of DNA-PKcs. Subsequent steps is DNA ends processing and recruitment of the DNA ligase IV (LIG4)-XRCC4 complex and DNA polymerases which results in ligation of DNA ends.(B) A- NHEJ starts after resection of damaged ends by CtIP and MRN complex. Binding of PARP1 and reqruitment of DNA repair factors promotes ligation of DNA ends through cooperation of Lig1/3 and XRCC1. modified from (Iliakis et al. 2015)

1.2.3 Homologous recombination repair

The first step in HR is DNA-end resection by means of 5' to 3' nucleolytic resection of the DSBs DNA-ends and producing of 3′-OH single-stranded DNA (ssDNA) (Her and Bunting 2018). This process is done by MRN complex including Mre11-Rad50-Xrs2. Meiotic recombination 11 (MRE11) acts as an endonuclease that nicks up to 300 nucleotides of 5′ terminus of DSB away from the break point and also has 3′−5′ exonuclease activity for extending of the nick to the DNA end (Nimonkar et al. 2012). Other proteins such as CtIP , Breast cancer type 1 susceptibility protein (BRCA1) , Exo1, BLM and DNA2 proteins also cooperate in this process which is followed by covering of 3' ss-DNA overhang by Replication Protein A (RPA) (Gravel et al. 2008, Mimitou and Symington 2009, Cannavo and Cejka 2014). RPA is the main eukaryote single-stranded DNA binding protein, consist of three subunits RPA70 (RPA1), RPA32 (RPA2), and RPA14 (RPA3). RPA resolves secondary structures and ssDNA bound by RPA cannot pair with other proteins (San Filippo et al. 2008).

Subsequently, RPA is replaced by the radiation repair protein 51 (Rad51) by means of mediator proteins such as Radiation repair protein 52 (Rad52), Rad51 paralogs including Rad55-Rad57 and Shu1-Psy3 in Saccharomyces cerevisiae and (RAD51B/C/D, XRCC2/3) in mammals. Breast cancer type 2 susceptibility protein (BRCA2) is another mediator proteins which cooperates in RAD51 filament nucleation to the dsDNA junction ((Sung and Robberson 1995, Robertson et al. 2009). BRCA2 has ssDNA binding motifs (OB-folds), a dsDNA binding motif (tower domain), and a number of Rad51 binding sites. (Yang et al. 2005). Rad51 is a recombination enzyme and acts as core enzymatic reaction in HR (West 2003). It is loaded to 3' ssDNA and forms pre-synaptic nucleoprotein filament for searching of homologous template and invading to the sister chromatin. BRCA1–BARD1 complex is also involve in RAD51-mediated homologous pairing (Tarsounas et al. 2003). Following the synapsis step, the post-synapsis homologous recombination can be completed through three different pathways. One of these pathways is break-induced replication (BIR) that acts when DNA breaks have only one end. This

pathway may cause translocations, chromosomal rearrangements, copy-number variations and complex chromosomal changes (Sakofsky and Malkova 2017). The second pathway is synthesis-dependent strand-annealing (SDSA) in which DNA synthesis starts from 3' at the 3′ termini of the invading single-strand tail and is followed by annealing of the newly synthesized strand with its complement (Miura et al. 2012). Double-strand break repair is the other pathway of Homologous recombination in which double Holliday junction forms and can lead to non-crossover or crossover products (Li and Heyer 2008).

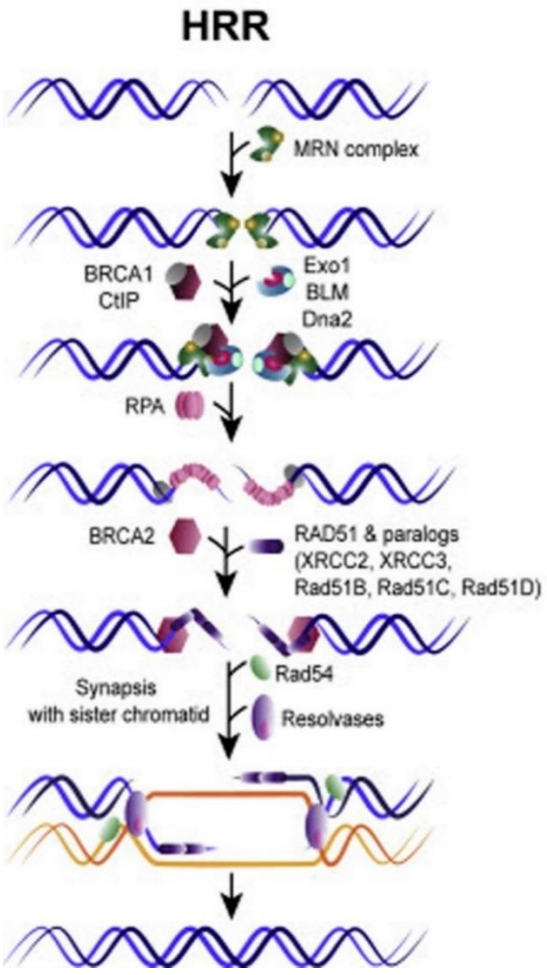

Figure 1.2 Model for HRR pathway. MRN complex recognizes DSBs. End resection process is done by CtIP and ssDNA is formed. ssDNA is covered with RPA. Replacement of Rad51 with RPA leads to formation of RAD51 nucleoprotein filament homology sequence searching and strand invasion. DNA synthesis, ligation, and resolution of Holliday junctions results in DSBs repair. Modified from (Iliakis et al. 2015).

1.3 PI3K/Akt signaling pathway

The phosphatidylinositol 3-kinase (PI3K)/Protein Kinase B (PKB, Akt) pathway is one of the most frequently mutated pathways in cancer. Mutations of the PI3K-pathway are associated with tumor growth, metastatic spread, and resistance to treatment (Fruman and Rommel 2014). PI3K is a member of lipid kinase family and acts as a critical signaling component of growth factor receptor tyrosine kinases (RTKs). This kinase can be activated by phosphorylating the 3-hydroxyl inositol phospholipids and producing $PI(3,4,5)P_3$ and $PI(3,4)P_2$ which leads to translocation and activation of Protein kinase B (PKB)/Akt to the inner membrane (Adimonye et al. 2018).

Akt is a serine/threonine kinase protein from the cAMP-dependent, cGMP-dependent, protein kinase C (AGC) kinase family with various important roles in cellular process. In 1977, this protein identified for first time in the transforming murine leukemia virus (AKT-8 provirus) and classified as an oncogene (Staal and Hartley 1988). The Akt structure consists of a three domains: 1) an amino terminal pleckstrin homology (PH) domain; 2) a central kinase domain containing a regulatory threonine residue (Thr308) 3); a carboxyl-terminal regulatory domain containing the serine regulatory residue (Ser473)(Song et al. 2005).

Although Phosphorylation at T308 by PDK1 increases the activity of Akt1, in order to full activation, Akt needs to be phosphorylated at Ser473s as well. It is suggested that phosphorylation at Ser473s is done by so-called PDK2 and is important for stabilizing T308 phosphorylation (Alessi et al. 1996). Phosphoinositide-dependent Mammalian Target of Rapamycin complex 2 (mTORC2), inhibitor of B-kinase (IKK), DNA-dependent protein kinase catalytic subunit (DNA-PKcs) and Integrin-linked kinase (ILK) or auto-phosphorylation of Akt are proposed to act as PDK2 (Yang et al. 2002).

Receptor tyrosine kinases(RTKs), integrins, B, T cell receptors and cytokine receptors are able to activate Akt signaling pathway (Nitulescu et al. 2018). Regarding termination of Akt activity, two pathway has been proposed: 1.removing

of activating lipid messengers PI(3,4,5)P3 and PI(3,4)P2 by 30-lipid phosphatase PTEN as well as the 40-lipid phosphatase inositol polyphosphate-4-phosphatase (INPP4B) and 2. Inhibition of Akt through dephosphorylating of S473 or T308 by different phosphatases (Manning and Toker 2017, Rodgers et al. 2017).

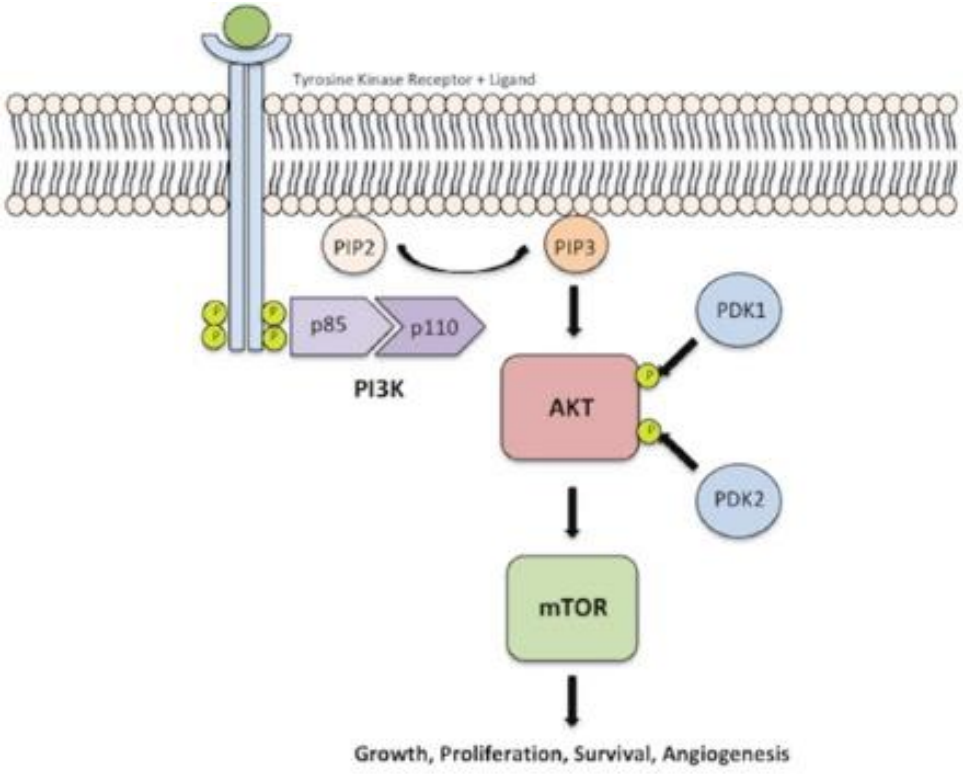

Figure 1.3. Activation of Akt. PI3K pathway is activated by growth factors or cytokines and subsequently Akt protein is activated by phosphorylation at T308 and S473 by PDK1 and PDK2. modified from (Adimonye et al. 2018).

Akt consist of three isoforms: AKT1 (PKBα) (including 3 splice variants), AKT2 (PKBβ), and AKT3 (PKBγ) (including 2 splice variants). These three isoforms are produced by different genes and have 80% similarity in amino acid identity (Ruan and Kazlauskas 2011) . Akt isoforms exert critical roles in cellular process through different downstream targets (Brown and Banerji 2017, Manning and Toker 2017). The main isoform in the majority of tissues is Akt1. However, Akt2 is reported as the predominant isoform in liver, skeletal muscle and adipose tissue and Akt3 expression is mainly in the brain and testes (Wang et al. 2017). Moreover, Cho et al (Cho et al. 2001) showed that depletion of the Akt2 isoform results in hyperinsulinaemia and insulin resistance in mice and Tschopp et al. (Tschopp et al. 2005) reported that *Akt3*$^{-/-}$ mice have smaller brains. Overexpression of AKT2 is also associated with invasion and metastasis in human breast and ovarian cancer cells (Arboleda et al. 2003).

In general, Akt is involved in the regulation of cell survival, cell growth and proliferation by affecting downstream proteins including BAD, FOXO, TSC2, PRAS40, GSK3β and CDKN (Hers et al. 2011) (Manning and Cantley 2007). Impairment of Akt signaling has been investigated in different cancers. For instance, Akt1 upregulation is reported for gastric carcinoma, glioblastomas and gliosarcomas while Akt2 amplification is shown in head and neck squamous cell carcinoma, pancreatic, ovarian and breast cancers (Manning and Cantley 2007) . Akt3 is also shown in androgen resistant prostate cancer cells, estrogen receptor-deficient breast cancer cells, and in primary ovarian cancers (Nakatani et al. 1999). Finally, an oncogenetic activation mutation (E17K) in the PH domain of Akt1 is reported in melanoma, breast, colorectal, lung and ovarian cancers (Shoji et al. 2009, Nitulescu et al. 2018).

With reference to the role of Akt signaling in cell survival, proliferation and invasion, this pathway has been considered as therapeutic target in cancer research. Inhibition of Akt signaling pathway could induce apoptosis and increase sensitivity of tumor cells to cytotoxic agents (Altomare and Testa 2005). Therefore, various Akt inhibitors

are developed in order to specifically target this protein. These inhibitors are either allosteric inhibitors of the AKT PH-domain that block localization of AKT to the plasma membrane or they are ATP-competitive inhibitors of Akt. Numerous studies have used different Akt inhibitors in combination with chemo- or radiotherapy in different cancers (Brown and Banerji 2017).

In this context, Page et al (Page et al. 2000) showed that ovarian cancer cell lines get more resistant to paclitaxel after constitutive AKT1 activity or *AKT2* gene amplification. Hu et al (Hu et al. 2002) reported that dual treatment of ovarian cancer models with the PI3K inhibitor LY294002 plus paclitaxel increased the efficiency of treatment. It is also shown that PI3K inhibition leads to apoptosis in tumor cells with high level of Akt (Brognard et al. 2001, Altomare et al. 2004). Finally, a phase I clinical study by Vink et al (Vink et al. 2006) showed that the Akt inhibitor Perifosine can be safely combined with fractionated radiotherapy in patients with advanced solid tumors.

1.4 The involvement of Akt in DSBs repair

In various studies it has been shown that Akt is phosphorylated following exposure of cells to IR (Li et al. 2009, Toulany and Rodemann 2013, Freudlsperger et al. 2015). After inducing DNA lesions, ataxia telangiectasia mutated (ATM), ataxia telangiectasia and Rad3-related (ATR), and DNA-dependent protein kinase catalytic subunit (DNA-PK) are able to activate Akt in the DNA damage response pathway (Liu et al. 2014). Szymonowicz et al (Szymonowicz et al. 2018) reported that overexpression of Akt mutated in both phosphorylation sites (T308A,S473A) induced radiosensitivity of prostate cancer cells. Several studies provided evidence that Akt is involved in the regulation of NHEJ pathway (Toulany et al. 2012, Sahlberg et al. 2014).

Holler et al (Holler et al. 2016) showed that dual inhibition of mammalian target of rapamycin-complex 1 (mTORC1) and Akt leads to blocking of NHEJ pathway and

increased DNA-DSBs. Fraser et al (Fraser et al. 2011) reported that pAKT (S473) colocalized with γ-H2AX and pATM (Ser1981) at the site of damages and involve in NHEJ repair of IR induced DSBs. Toulany et al (Toulany et al. 2012) showed that Akt forms a complex with DNA-PKcs to induce DNA-PKcs and Ku binding to DNA ends. Moreover, Akt can activate kinase activity of DNA-PKcs for efficient NHEJ and stimulates its autophosphorylation in order to induce removal of DNA-PKcs from DNA to facilitate the ligation process. Sahlberg et al (Häggblad Sahlberg et al. 2017) showed that AKT1 and AKT2 isoforms significantly increase the survival of colorectal cancer cells exposed to ionizing radiation.

Concerning the role of Akt in homologous recombination repair, conflicting data exist. For instance, Plo et al (Plo et al. 2008) reported that the presence of AKT1 in breast cancers cells resulted in a BRCA1-deficient–like phenotype via cytoplasmic retention of BRCA1 and RAD51 and Jia et al (Jia et al. 2013) showed that activation of AKT1 in BRCA1-deficient cells impacts the interaction of Chk1 and Rad51 to consequently reduce HR. On the other hand Mueck et al (Mueck et al. 2017) showed that downregulation of AKT1 resulted in reduced Rad51 protein expression and diminished Rad51-foci formation after radiation exposure of NSCL cancer cells. Moreover, data from Philip et al (Philip et al. 2017) indicated that PI3K inhibition reduces RAD51 foci formation and induces sensitivity of PTEN mutated cells to the PARP inhibitor treatment.

1.5 Aim of the study

Based on this knowledge and scientific background with respect to the addressed role of Akt isoforms in DNA-DSB repair processes and especially HR the study was designed to clarify the roles of Akt1 and Akt2 isoforms in regulation of homologous recombination repair of human colon carcinoma cells.

Furthermore, as our lab has identified the role of Akt in non-homologous end-joining (NHEJ) DNA-DSB repair, the study was also designed to shed further light onto the role of HR versus NHEJ in Akt-mediated radioresistance of cells from solid human tumors. With respect to this point that all studies so for only address the role of Akt1, this project investigated the role of the Akt isoforms Akt-1, Akt-2, in the regulating/modifying DNA double-strand breaks repair through the homologous recombination repair mechanism. .

Previous results using the HR reporter assay system demonstrated that Akt1-knockdown decreases the relative proportion of GFP-positive cells indicating an inhibition of HR-repair. Moreover, as the knock down approaches for Akt used did not allow complete knockdown of the Akt proteins of interest, in the present study knock-out cells for Akt1 and Akt2 isoforms were used. This approach did not only allow a verification of the results obtained so far but also provided clear cut answers to the importance of Akt-isoforms in the regulation of DNA double-strand break repair through the homologous recombination mechanism. Thus, the study aimed to provide detailed mechanistic evidence to show how and to what degree antagonistic strategies directed against Akt may be effective to overcome radioresistance of different tumor entities during radiotherapy.

2) Materials and Methods:

2.1 Materials

2.1.1 Cell lines:

Table 2.1. Cell line used in this project.

Cell line	Description	Cultured in
HCT116 Parental (Horizon, UK)	Colon carcinoma cell line	RPMI+10%FCS
HCT116 AKT1-KO (Horizon, UK)	Colon carcinoma cell line	RPMI+10%FCS+0.3mg/mL G148
HCT116 AKT2-KO (Horizon, UK)	Colon carcinoma cell line	RPMI+10%FCS+0.3mg/mL G148
HCT116 DNAPK-KO	Colon carcinoma cell line	RPMI+10%FCS

2.1.2 Antibodies:

Primary antibodies

Table 2.2.Primary antibodies.

Antibody	Species	Dilution(Application)	Company/Cat.N.
anti-γH2AX	Mouse	1:500(IF)	Merck/05-636
anti-P-Akt(S473)	Rabbit	1:1000 (WB)	Cell Signaling/4060
anti-Akt1	Mouse	1:1000 (WB), 1:100(IP)	Biosciences/ 610877
anti-Akt2	Mouse	1:1000 (WB), 1:100(IP)	Cell Signaling/2964
anti-Akt3	Mouse	1:1000 (WB)	Cell Signaling/8018
anti- Rad51	Mouse	1:1000 (WB)	Abcam/88572
anti- pRad51	Rabbit	1:1000 (WB)	Abcam/31769
anti-RPA2	Mouse	1:1000 (WB), 1:100(IP)	Abcam /2175
DNA-PKcs	Mouse	1:1000 (WB)	Abcam/1832

anti- CENP-F	Rabbit	1:700(IF)	LSBio/B276
anti- BRCA2	Rabbit	1:1000 (WB),1:100(IP)	Abcam /27976
anti-GAPDH	Mouse	1:1000 (WB)	Cell Signaling/2118
anti-Lamin A/C	Mouse	1:500 (WB)	Abcam/40567
anti-Lamin B	Mouse	1:1000 (WB)	Santa Cruz/74015
anti-Tubulin	Mouse	1:1000 (WB)	Calbiochem/ CP06
anti-IgG	Rabbit	1:1000 (IP)	Cell Signaling/7074

Secondary antibodies

Table 2.3. Secondary antibodies.

Antibody	Dilution(Application)	Company/Cat.N.
HRP-linked donkey anti rabbit	1:2000 (WB)	GE Healthcare/ NA934
HRP-linked sheep anti mouse	1:2000 (WB)	GE Healthcare/ NA931
Donkey anti-rabbit AlexaFluor 594	1:1000 (IF)	Thermofisher/ A32754
Goat anti-mouse AlexaFluor 488	1:1000 (IF)	Thermofisher/ A11001

2.1.3 Small molecule inhibitors:

2.4. List of inhibitors.

Inhibitor	target	Concentration(used)	Company
MK2206	Akt	5mg (1µM)	Selleckchem/ S1078
NU7441	DNA-PK	5mg (1µM)	Selleckchem/ S2638
Olaparib	PARP	5mg (1µM)	Selleckchem/ S1060
BO2	Rad51	5mg (1µM)	Sigma /0364

2.1.4 siRNA

Table 2.5 siRNA

siRNA	Catalogue Number	Concentration	Company
AKT1-siRNA	M-003000-03	5nM	Dharmacon
AKT2-siRNA	M-003001-02	5nM	Dharmacon
nontargeting siRNA	D-001810	5nM	Dharmacon

2.1.5 Chemicals

Table 2.6 Chemicals

Chemicals	Company
Acrylamide	Roth
Agarose	Sigma-Aldrich
Ammonium persulfate (APS)	Sigma-Aldrich
ß-Mercaptoethanol	Sigma-Aldrich
Boric acid	Sigma-Aldrich
Bovine serum albumin (BSA)	Roth
Bromophenol blue	Applichem
Crystal violet	Applichem
4',6-diamidino-2-phenylindole (DAPI)	Serva
DMEM	Gibco
Dimethylsulfoxide (DMSO)	Applichem
Dithiotreitol (DTT)	Sigma-Aldrich
EDTA	Sigma-Aldrich
Ethanol	Merck
Ethidium bromide	Roth
Formaldehyde	Merck

Glucose	Sigma-Aldrich
Glycerin	Applichem
Glycin	Roth
HCl	Roth
HEPES	Applichem
Isopropanol	Merck
KCl	Roth
KH2PO4	Applichem
Methanol	Merck
MgCl2	Applichem
Na2HPO4	Sigma-Aldrich
Na3VO4	Sigma-Aldrich
NaCl	Merck
NaF	Sigma-Aldrich
NaHCO3	Biochrom
NaOH	Roth
Nonidet P-40 (NP-40)	Sigma-Aldrich
Propidium iodide	Roth
RPMI-1640	Gibco
Sodium dodecylsulfate (SDS)	Serva
TEMED	Sigma-Aldrich
Tris-Base	Sigma-Aldrich
Tris-HCl	Sigma-Aldrich
Triton X-100	Sigma-Aldrich
Trypsin	Serva
Tween-20	Roth
Vectashield Mounting Medium with DAPI	Vector Laboratories

2.1.6 Buffers, solutions and growth media

Table 2.7 Buffers, solutions and growth media

Anode buffer	3.10 g boricacid 4 ml 10% SDS 200 ml methanol ad 1 l ddH2O pH 9.0
Cathode buffer	3.10 g boric acid 4 ml 10% SDS 50 ml methanol ad 1 l ddH2O pH 9.0
Lysis buffer A (Cytoplasmic buffer)	10 mM HEPES, pH 7.9 10 mM KCl 0.1 mM EDTA phosphatase and protease inhibitors
Lysis buffer C (Nuclear buffer)	20 mM HEPES, pH 7.9 400 mM KCl 1 mM EDTA 10 ml glycerol phosphatase, and protease inhibitors
Lysis buffer, whole cell	3.94 g Tris-HCl 5.40 g ß-glycerol phosphate 4.38 g NaCl 0.09 g Na3VO4 50 ml glycerol 5 ml Tween-20

	0.02 g NaF in 500 ml ddH2O pH 7.5 1 M DTT tablet protease inhibitor (complete mini, Sigma) phosphatase inhibitor cocktail 2 (Sigma) phosphatase inhibitor cocktail 3 ((Sigma)
Protein loading buffer	20 ml glycerin 20 ml 10% SDS 2.50 mg bromophenol blue 25 ml stacking gel buffer (4x) ad 95 ml ddH2O 56.2 µl β-mercaptoethanol per 1000 µl
SDS running buffer (5x)	144.10 g glycin 30.30 g Tris-base 10.00 g SDS In 2 l ddH2O pH 8.6
Separation gel buffer (4x)	90.85 g Tris-base 20 ml 10% SDS ad 500 ml ddH2O pH 8.8
Stacking gel buffer (4x)	30.30 g Tris-base 20 ml 10% SDS ad 500 ml ddH2O pH 6.8

Staining solution (CFA assay)	0.50 g crystal violet 27 ml formaldehyde In 1liter PBS
Stripping buffer	4.50 g glycin 3 ml 10% SDS 3 ml Tween-20 in 300 ml ddH2O pH 2.2
DMEM medium	12.04 g DMEM 3.30 g NaHCO3 In 900 ml ddH2O pH 7.2
RPMI medium	9.38 g RPMI-1640 1.80 g NaHCO3 In 900 ml ddH2O pH 7.2
PBS	13.7 mM NaCl 2.7 mM KCl 80.9 mM Na2HPO4 1.5 mM KH2PO4 pH 7.4
Western blot washing buffer (TBST)	3.15 g Tris-HCl 11.70 g NaCl 2 ml Tween-20 ad 2 l ddH2O pH 7.5
Propidium iodide (PI) solution	0.1 mg/ml PI 10 mg/ml RNaseA In PBS

IP washing buffer	10mM Tris HCL 150Mm NACL 0.5Mm EDTA phosphatase and protease inhibitors

2.1.7 Kits

Table 2.8 Kits

Kit	Company
Duolink® PLA Multicolor Probemaker Kit	Sigma-Aldrich

2.1.8 Laboratory consumables

Table 2.9 Laboratory consumables

Laboratory consumables	Company
Cell culture flask (20 cm^2, 75 cm^2)	BD Falcon
Cell culture dishes (6 cm,10 cm,15 cm)	BD Falcon
Culture slides (4well)	BD Falcon
Cover slips	Roth
Centrifuge tubes (15 ml, 50 ml)	Greiner
FACS tubes	Beckman Coulter
Pasteur pipets	Wilhelm Ulbrich
Filter paper, Whatman	GE Healthcare
Nitrocellulose blotting membrane	GE Healthcare
Immersion oil	Zeiss
Micro tubes	Roth

2.1.9 Instruments

Table 2.10 Instruments

Equipment	Manufacturer
Binocular	Zeiss
Centrifuges	Roth Hettich
Clean bench	Thermo Fisher Scientific
Drying cabinet	Heraeus
Electrophoresis equipment	Hoefer
Flow cytometer	BD Biosciences
Fluorescence microscope	Zeiss
Incubators	Binder, Heraeus
Irradiation device	Gulmay
Light microscope	Leitz
Semidry blot equipment	Hoefer
Spectrophotometer	Thermo Fisher Scientific
Western blot scanner	LI-COR

2.2 Methods

2.2.1 Cultivation of human cell lines

The cells (HCT116 parental, AKT1-KO, AKT2-KO, and DNAPK-KO) were grown in RPMI, routinely supplemented with 10% fetal calf serum and 1% penicillin/streptomycin and under sterile condition. All cells were cultured in a humidified atmosphere of 7% CO2/93% air at 37° C. The HCT116 AKT1-KO and AKT2-KO cells were cultured with 0.3 mg/ml G418. To passage the cells, the old culture media was removed and cells were washed with PBS. Cells were incubated with 1ml Trypsin for 2 min and new media were added to cells to stop trypsinization. Cells were transferred to new culture flask and incubated.

Table 2.11 Cell number and culture media volume for different culture dishes

Culture dishes	Cell number
6 cm plate	300,000 cells per well
10 cm plate	420,000 cells per well
15 cm plate	900,000 cells per well
4 well slides	20,000 cells per well

2.2.2 Cell cycle analysis

The number of 300,000 cells were seeded in 6cm plates. Cell cycle distribution was analyzed 3, 4 and 5 days after seeding. The cells were harvested by trypsinization as well as collecting floating cells in the media. The cells were spun down (350 g, 6 min) and fixed in 70% ethanol. After washing with PBS, the cells were incubated with ribonuclease (100 µg/ml in PBS) for 10 min, washed again and the DNA was stained using propidium iodide. Cell cycle fractions were determined by flow cytometer.

2.2.3 Subcellular fractionation

Nuclear cytoplasmic fractionation was performed in order to determine post-irradiation localization of proteins of interest after irradiation. To this aim, cells were swollen in cytoplasmic lysis buffer for 15 min on ice. NP40 5% were added and after short vortex cell lysates were centrifuged at 10,000 rpm for 2 min at 4°C to sediment the nuclei. Following separation of Obtained supernatant as cytoplasmic fraction, nuclear pellet was washed. After two times washing in cytoplasmic lysis buffer they re-suspended in the nuclear lysis buffer and incubated on ice for 1 hour. After sonication and centrifugation (10,000 rpm, 10 min, and 4°C) nuclear fraction was extracted. Equal amounts of protein were resolved by SDS-PAGE and transferred to a nitrocellulose membrane.

2.2.4 Irradiation

A Gulmay RS225 X-ray machine (Gulmay Ltd., 293 Chertsey, UK) is used for irradiation of cells. Irradiation is performed at 200 kVp, 15 mA and with 0.5-mm copper filter. Cells were irradiated with single doses of 2 – 4 Gy.

2.2.5 siRNA Transfection

Silencing of AKT isoforms cells was transiently done by transfecting 50 nM of a pool of siRNAs against AKT1 and AKT2. Non-targeting siRNA is transfected in parallel to the control condition. Lipofectamine 2000 and Opti-MEM® serum free medium were used for optimizing transfection. The Day after transfection media containing siRNA was removed and fresh medium added to the cells. Knockdown efficiency were analyzed 72 h after transfection by western blotting.

2.2.6 Protein assay

To calculate the concentration of obtained protein needed for western blot and immunoprecipitation Biorad protein assay was used. 5 µl of protein solution were mixed with 25 µl of Biorad reagent A and 200 µl of Biorad reagent B and incubated for 7 min at RT. The absorption of light at 590 was measured by microplate reader.

2.2.7 Immunofluorescence Analysis

Immunofluorescence analyses were performed for detecting γ-H2AX foci in order to evaluate the efficiency of repair in cells after exposure to irradiation. Moreover, Rad 51 foci was detected for evaluating the functionality of HRR in cells. To specifically check the repair process in S and G2 phase of cell cycle, immunofluorescence analyses were performed for CENP-F protein (a marker for proliferating cells).
To perform these analysis, cells were plated on 4-well chamber slides. Cells were allowed to grow for 3 days to reach the highest percentage of G2 cells. Cells were exposed to a single dose of 4 Gy of ionizing irradiation either with or without inhibitor treatment for 2 hours. Thereafter, Cells were incubated for 6 and 24 hour post irradiation and fixed in 3.7% formaldehyde for 10 minutes. After permeabilizing with 0.1% Triton X-100 in PBS for 10 minutes, cells were incubated in blocking solution (5% bovine serum albumin in PBS) for 1 hour at room temperature. After overnight incubation with primary antibody at 4°C, cells were washed with PBS, and then incubated in the dark with secondary antibody for 1 hour at room temperature. At the last step, cells were washed with PBS and mounted in Vectashield mounting. Imaging was performed by fluorescence microscope (Axioplan 2, Zeiss,Jena, Germany). At least 100 nucleus was counted per for each experimental condition and graphed using the Sigma plot graphics software (SPSS).

2.2.8 Immunoprecipitation

To clarify if AKT is in the complex with BRCA2, RPA and Rad51, immunoprecipitation assay was performed as follows. Cells were washed with phosphate buffered saline and lysed with lysis buffer containing 50 mM HEPES, pH 7.4, 150 mM NaCl, 1% NP-40, 0.5%, 10% glycerol, 1mM NAF, 1 mM EDTA, 1 mM DTT, and phosphatase as well as protease inhibitors. After protein concentration analysis, 2.5 mg of obtained protein from cell lysate were incubated with 1 µg of antibody (RPA2, AKT1, AKT2, BRCA2 and normal mouse IgG) for 1 hour at 4°C. Thereafter, 50 µl A/G agarose beads were added and cell lysates were incubated overnight at 4°C. The immune-precipitates were washed three times with washing buffer and were centrifuged for 2min (2700 g). Immune-complexes received were then extracted by boiling in loading buffer for 5 minutes.

2.2.9 Colony Formation Assay

To check radiosensitivity of HCT116 cells colony forming assay were used in different condition. Sub confluent cells with a density of 350 cells/well were plated into 6well plates. After 48h, cells were treated with above mentioned inhibitors for 2 hours. Cells were irradiated with single doses of 0, 2 and 4 Gy and incubated for 12 days for colony formation. Cells fixed and stained with crystal violet and colonies with more than 50 cells were counted as survivors. The survival fractions (SF) were calculated by normalizing the plating efficiency of the treated cells to the plating efficiency of the untreated cells according to the formula published in (Mueck et al. 2017).

2.2.10 Proximity ligation assay

Proximity ligation assay were used to detect possible protein-protein interactions of Akt1 and Akt2 with RPA2 and BRCA2in HCT116 fixed cells following the manufacturer's protocol. Irradiated (4 Gy) and non-irradiated cells were fixed in 3.7% formaldehyde for 10 minutes. After permeabilizing with 0.1% Triton X-100 in PBS for 10 minutes, cells were incubated with blocking solution for 30 min at humidity chamber. For each condition the antibody of two proteins of interest (from different species) were added and cells were incubated overnight at 4°C. Next, following two times washing, PLA probes were added to slides and were incubated for 60 min at 37°C. PLA probes contain secondary antibodies against two different species with two oligonucleotides. Next, cells were again washed and ligation buffer were added to cells. Incubation with amplification stock solution for 100 min at 37°C resulted in rolling circle amplification. This structure is detectable through oligonucleotides which are labeled with fluorescence. Finally, the cells were washed with washing buffer and mounted for Vectashield mounting. Imaging was performed with a fluorescence microscope (Axioplan 2, Zeiss,Jena, Germany). For negative control condition, primary antibodies were not added to the cells. For positive control condition cells were treated with EGF for 10 min and fixed with 3.7% formaldehyde.

3) Result

3.1 The effect of Akt depletion on cell cycle distribution and cell proliferation

To address the question of role of Akt in homologous recombination repair pathway the human colorectal cancer cell line HCT116 parental cells as well as HCT116 cells with inactivated AKT1 and AKT2 genes were used. First of all, as shown in Figure 3.1 western blot analyses were performed to check the accuracy of knockout. As Ericson et al (Ericson et al. 2010) already reported it could be observed that AKT3 is inactive in HCT116 parental and HCT116 AKT-1- KO cells as. However, Akt3 is expressed in AKT2-KO cells indicating a potential compensatory effect in these cells.

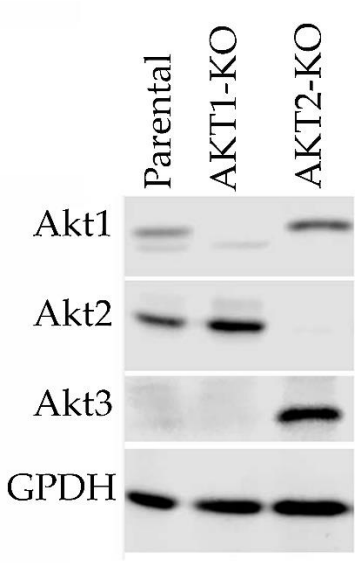

Figure 3.1 Protein levels of Akt isoforms Akt1, Akt2 and Akt3 in HCT116 parental and knockout cells were quantified by western blotting.

As it is already mentioned, HRR is only active in late S and G2 cells. In order to determine the percentage of cells in different cell cycle phases, cell cycle distribution analysis was performed by flowcytometry. Cell cycle distribution at different time points after seeding with a density of 300,000 cells showed that in HCT116 Parental, AKT1-KO and AKT2-KO cells, proportion of G2 and S phase cells reach the highest

amount three days after seeding of cells (Figure 3.2). Based on these results, all further experiments were performed on day 3 after seeding the cells.

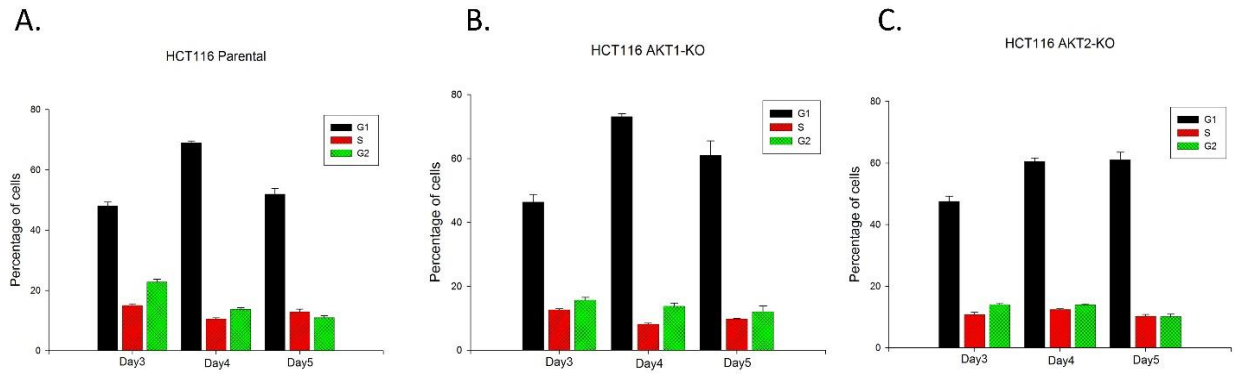

Figure 3.2. Cell cycle distribution analysis of HCT116 parental, AKT1-KO and AKT2-KO cells. The number of 300,000 cells were seeded in 6cm plates and percentage of cells in different cell cycle phases obtained by FACS analysis in different time points after seeding (Day 3, Day 4 and Day 5). Percentage of cells in G1, S and G2 phases at indicated time pints for (A) HCT116 parental, (B) AKT1-KO and (C) AKT2-KO cells. Error bars represent the SEM of two independent experiments with 3 parallel cultures each.

Centromeric protein F (CENP-F protein), also known as p330d, is a cell cycle regulated protein and is associated with centromeres and the nuclear matrix. CENP-F expression is very low in G0/G1 while it accumulates in the nucleus of cells in S-phase as well as G2/M (Landberg et al. 1996). Immunofluorescence analysis was performed for staining of CENP-F protein in HCT116 cells. Three days after seeding of cells in 4well chamber slides (20000 cells per well) it could be observed that the majority of cell population are in G0/G1 phase (CENP-F negative cells). Approximately 20 percent of cells were positive for CENP-F protein indicating cells

in S and G2/M phases. As it is shown in Figure 3.3 Akt isoforms depletion did not change the proportion of cells in S/G2 phases.

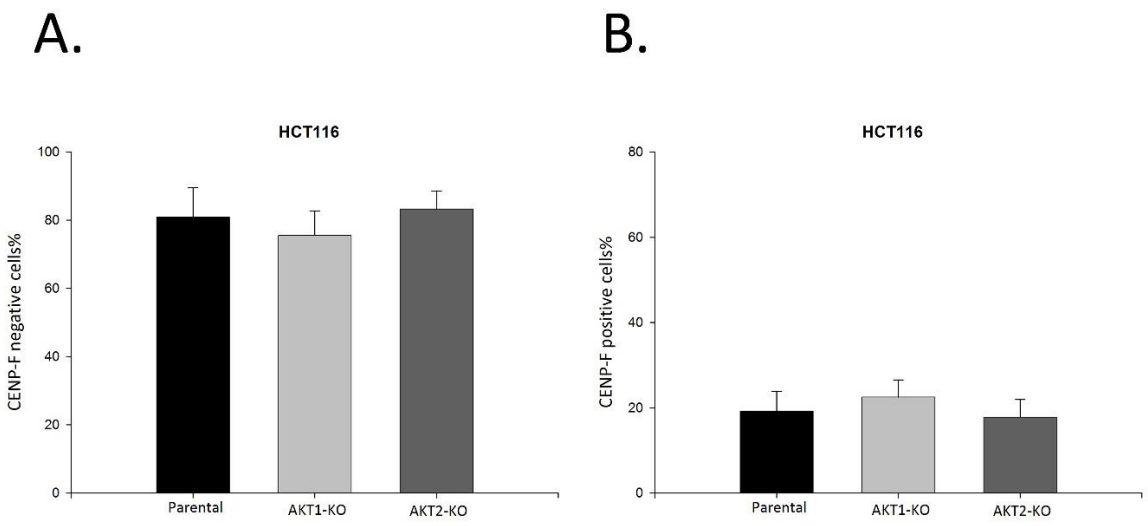

Figure 3.3. Determination of cells in S and G2 phase of cell cycle using immunostaining of CENP-F protein. (A) CENP-F negative cells. (B) CENP-F positive cells represent cells in S and G2 phase. Error bars represent the SEM of three independent experiments.

3.2 Increased residual γ-H2AX in AKT1-KO and AKT2-KO cells

To examine the influence of AKT isoforms on DSBs repair through homologous recombination, γ-H2AX foci assays for HCT116 parental and AKT1 and AKT2 knockout cells were performed. To determine specifically cells which are able to perform homologous recombination, Centromeric protein F (CENP-F) staining were applied. Cells were irradiated and 24 hours after 4Gy, number of γ-H2AX foci in CENP-F positive protein were monitored. As shown in Figure 3.4 AKT1-KO and AKT2-KO cells presented higher amount of damages in comparison to parental cells 24h after 4Gy irradiation. The result indicates that in f Akt1- and Akt2-knock out cells

repair of radiation induced DSBs in cells which are in S and G2 phases of the cell cycle is significantly reduced when compared to AKT wild type cells.

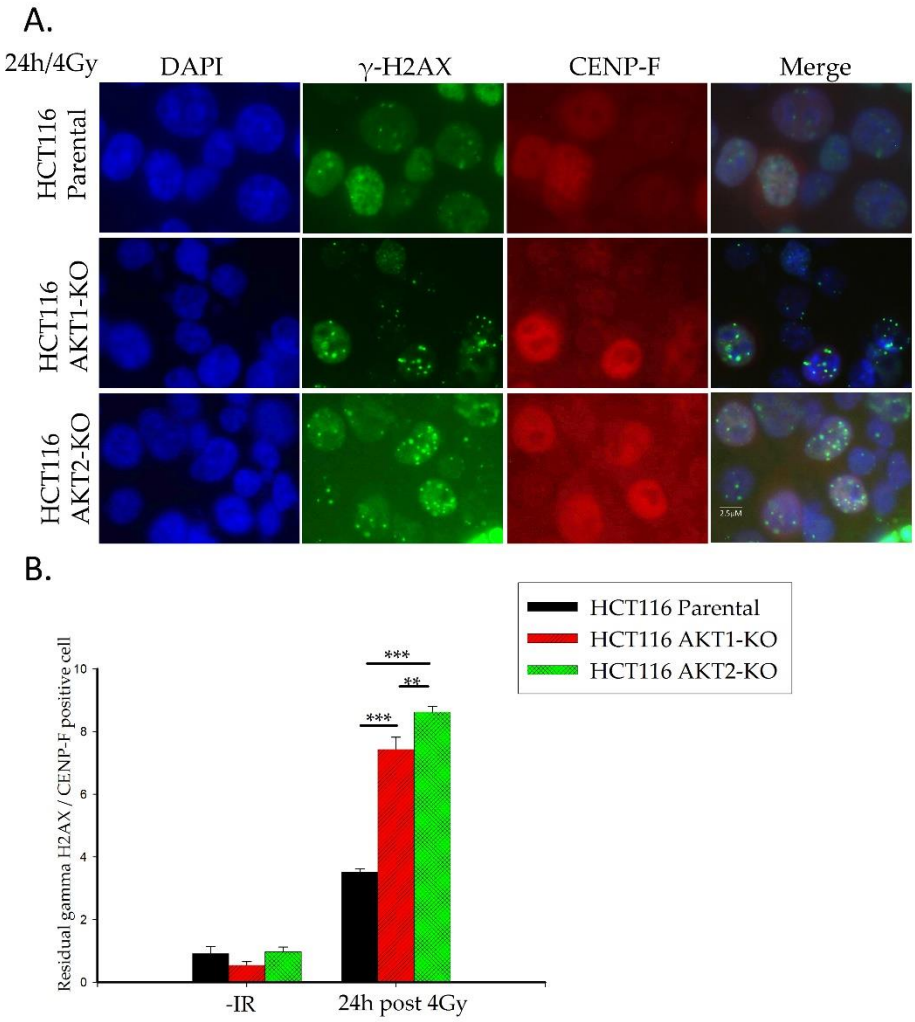

Figure 3.4. AKT depletion results in increased residual γ-H2AX in irradiated CENP-F positive cells. (A) Immunofluorescence analysis was performed as described in material and methods. The γ-H2AX foci (green) 24 hours after 4 Gy in CENP-F positive cells (red). (B) The number of γ-H2AX foci was analyzed 24 h after 4 Gy radiation. The data represent the mean ± SEM of three independent experiments and a total of at least 300 nuclei per condition (** $p < 0.01$, and *** $p < 0.001$, Student's t-test).(Mohammadian Gol et al. 2019)

Next to specifically check the effect of Akt on homologous recombination C-NHEJ and A-NHEJ were blocked using respectively DNA-PK inhibitor NU7441 and PARP-1 inhibitor Olaparib. Following 2hours treatment with inhibitors, cells were irradiated with 4Gy. HCT116 AKT1 and AKT2 knockout cells presented significantly increased number of residual γ-H2AX foci in compare to parental control cells. Yet, in AKT2-KO cells amount of damages were more than AKT1-KO (Figure 3.5). Furthermore, the effect of pan Akt inhibitor MK2206 on the DSBs repair was analyzed. MK2206 is an allosteric inhibitor of Akt which is able to block kinase activity of Akt at both phosphorylation sites (Akt T308 and S473) and that it does not change the basic protein level of Akt (Szymonowicz et al. 2018). In parallel with MK treatment, cells were treated with DNAPK inhibitor NU7441 and PARP-1 inhibitor Olaparib separately as well as combination treatment of three inhibitor. As it is shown in Figure 3.5B using MK, NU and Olaparib increased the number of γ-H2AX approximately to the same level. Combination treatment of MK, NU and Olaparib showed further increasing of damages. This result indicates that Akt is important for the repair of DSBs in S and G2 phases cells.

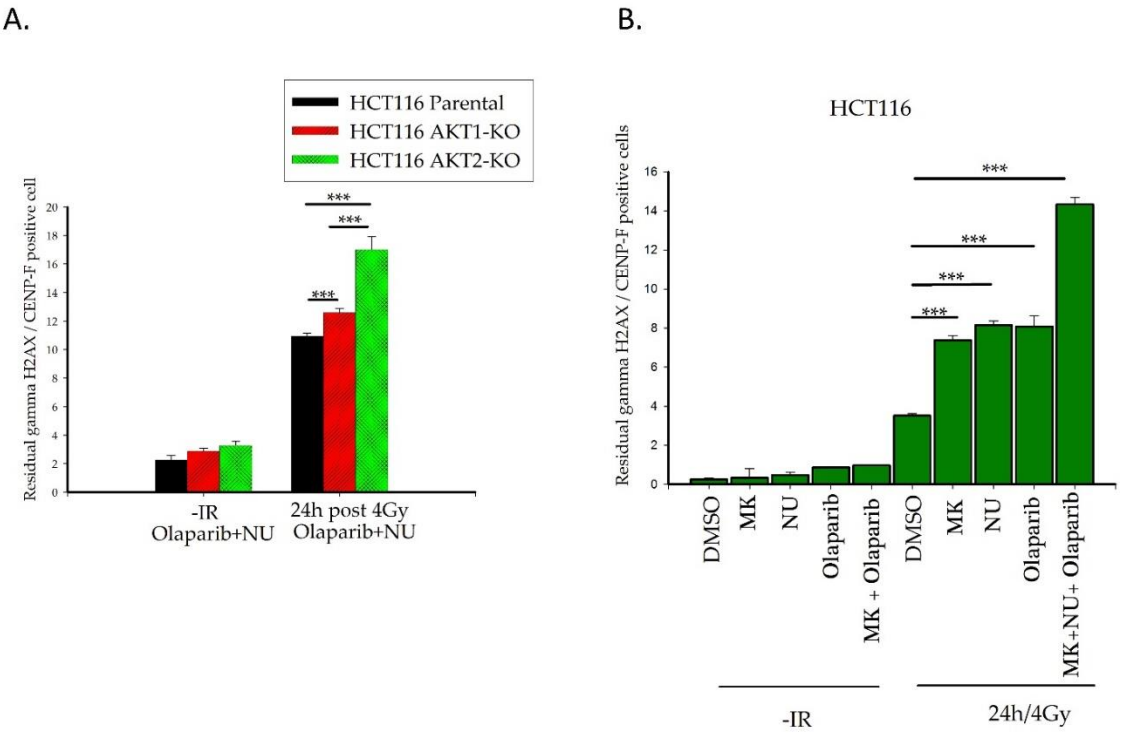

Figure 3.5. Blocking of c-NHEJ and alt-NHEJ pathways (A) The HCT116 parental and knockout cells were irradiated 2 h after treatment with a combination of NU7441 and Olaparib (1 μM each). (B) The HCT116 parental cells were treated with 1 μM of inhibitors (MK2206, NU7441, and Olaparib) for 2 h and irradiated with 4 Gy. The number of γ-H2AX foci were counted 24 h after irradiation. The data represent the mean ± SEM of three independent experiments and a total of at least 300 nuclei per condition (*** $p < 0.001$, Student's t-test). (Mohammadian Gol et al. 2019)

3.3 Blocking of HRR through inhibiting Rad51 binding to the damage site.

It has been reported that BO2 can inhibit Rad51 foci formation in different cell line (Algotar et al. 2014, Huang and Mazin 2014).To examine the inhibitory effect of BO2 on Rad51 foci formation of HCT116 cells, cells were treated with 1μM of this inhibitor 2h before irradiation. The number of Rad51 foci in the nucleus of cells were monitored 6 hours after 4 Gy Irradiation. As it is shown in Figure 3.6 BO2 treatment significantly reduced number of Rad51 foci in HCT116 parental cells. Next, DSBs

repair efficiency of parental and knockout cells under BO2 treatment after irradiation was analyzed. As shown in Figure 3.6 inhibition of Rad51 binding to the DNA using BO2 inhibitor resulted in increased γ-H2AX in compare to DMSO treated cells. Moreover, enhancement of residual DSBs is stronger in AKT1 and AKT2 knout cells.

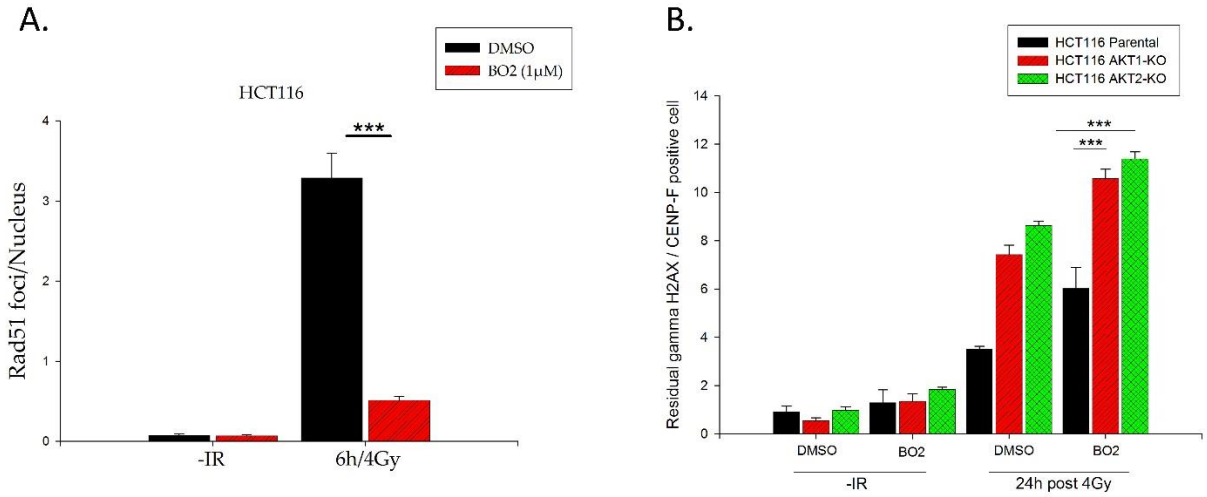

Figure 3.6. Blocking of Rad51 focus formation at the site of damage leads to increased γ-H2AX. (A) Number of Rad51 foci 6 hours after 4Gy irradiation in HCT116 BO2 treated cells. (B) The number of γ-H2AX foci in CENP-F positive HCT116 parental, AKT1-KO and AKT2-KO cells after BO2 treatment and 24 hour post irradiation. The data represent the mean ± SEM of three independent experiments and a total of at least 300 nuclei per condition (*** $p < 0.001$, Student's t-test).

3.4 The role of Akt in post irradiation clonogenic survival capacity of HCT116

Post-irradiation cell survival of HCT116 Parental, AKT1-KO and AKT2-KO was investigated using a standard colony formation assay. This assay is testing the ability of single cells to proliferate into a large colony, i.e. more than 50 cells. To this aim proliferating cells were trypsinized and seeded in 6well plates. Cells were irradiated 48 hours after seeding with indicated doses and incubated for 13 days. As demonstrated in Figure 3.7 AKT1-KO and AKT2-KO cells are significantly more

sensitive to irradiation in comparison to parental cells. Interestingly, among the Akt-KO cells AKT2-KO cells showed a significantly stronger effect than AKT1-KO cells.

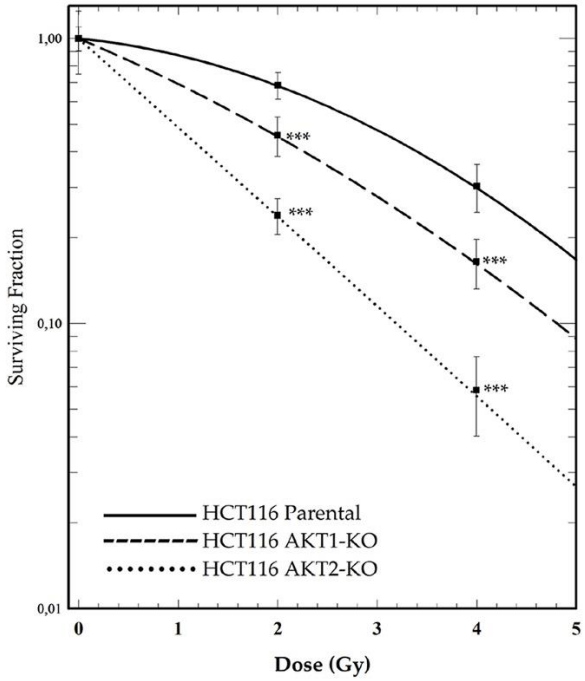

Figure 3.7. Clonogenic cell survival of irradiated HTC116 parental and AKT1-KO and AKT2-KO Cells. Clonogenic assays were performed as described in the Materials and Methods. (A) HCT116 parental, AKT1-KO, and AKT2-KO cells irradiated with 0, 2, and 4 Gy. Data points represent the mean surviving fractions (SF) ± the standard deviation (SD) of three independent experiments (n = 18; *** $p < 0.001$, Student's t-test). (Mohammadian Gol et al. 2019)

Next, the colony formation ability was tested after treatment of cells with DNA-PK inhibitor NU7441 or Rad51 inhibitor BO2. As shown in Figure 3.8 inhibiting of DNA-PK resulted in decreased colony survival in all three cell line (HCT116 parental and AKT-KO cells) but this decreasing is more pronounced in AKT1-KO cells.

Furthermore, blocking of Rad51 showed a stronger effect on AKT2-KO cells in compare to parental and AKT1-KO cells.

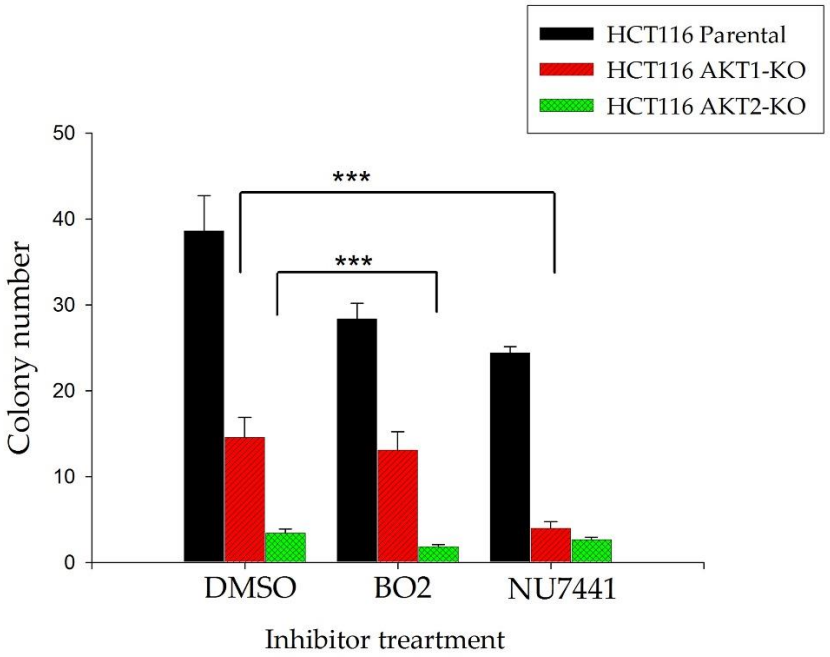

Figure 3.8 Inhibition of HR and C-NHEJ. All three cell lines were treated with 1 µM of the indicated inhibitors 2 h before radiation exposure 2 Gy. Bars represent the average number of colonies formed when 350 cells were seeded for the different treatment conditions (n = 18;*** $p < 0.001$, Student's t-test).(Mohammadian Gol et al. 2019)

3.5 The effect of Akt on HRR through regulation of Rad51

Rad51 is main player of homologous recombination repair of DSBs as well as normal meiotic recombination (Tashiro et al. 2000). It has been shown that downregulation of Akt1 in the non-small cell lung cancer cell line A549 leads to reduced Rad51 foci formation (Mueck et al. 2017). To asses HR dependent repair in HCT116 AKT wild type and knockout cells Rad51 foci formation in these cells was analyzed after 6 and 24h exposure to irradiation. As shown in Figure 3.9 the number of Rad51 foci was

significantly reduced in AKT1-KO and AKT2-KO cells 6h after 4Gy IR indicating an impaired homologous recombination in the knockout cells.

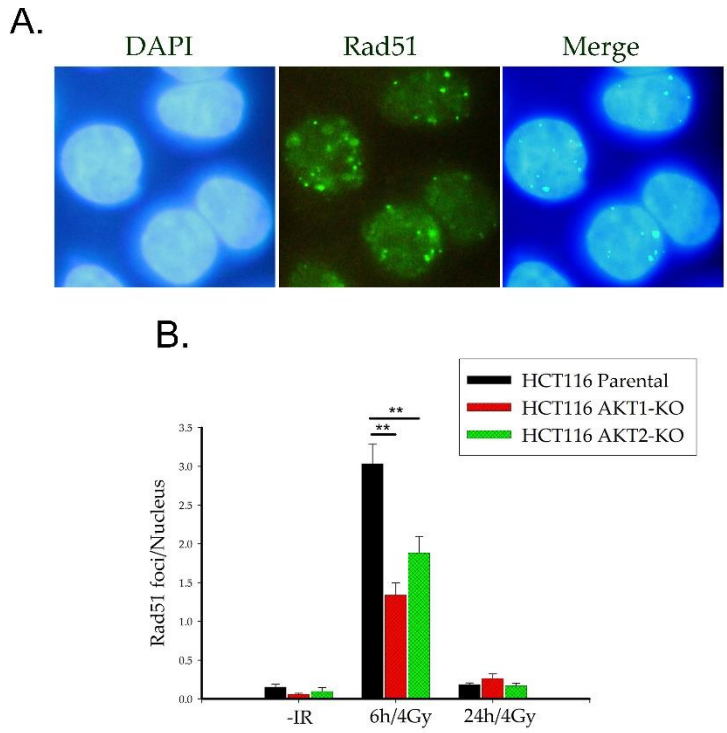

Figure 3.9 Rad51 foci formation in HCT116 parental and AKT1/AKT2 depleted cells. (A) Immunofluorescence analysis was performed as described in the Materials and Methods. Rad51 foci (green) in sub-confluent cells (blue). (B) Rad51 foci number per nucleus were analyzed after immunofluorescence staining of HCT116 parental, AKT1-KO, and AKT2-KO irradiated cells. (Mohammadian Gol et al. 2019).

To test whether the effect of AKT depletion on Rad51 foci formation could be observed in the absence of functional NHEJ, siRNA mediated knockdown of AKT1 and AKT2 was performed in HCT116 DNAPK-KO cells. Quantification of Rad51 foci in HCT116 DNAPK-KO cells 6 hours post IR showed that in the absence of NHEJ, Rad51 foci formation increases significantly in comparison to DNAPK wild type cells Figure 3.10 A and B. This effect which is also clearly demonstrated by western blot analysis for Rad51 expression (Figure 3.10 C) might be due to a compensatory strategy of cells in response to the loss of NHEJ pathway and its repair capacity.

Moreover, Akt1 and Akt2 isoforms single knockdown as well as dual knockdown presented a significant decrease of Rad51 foci in both HCT116 Parental and DNAPK-KO cells. Interestingly, the reduction of Rad51 foci was stronger after downregulation of Akt2 isoform (Figure 3.10 B). This result indicates that both Akt1 and Akt2 are involved in the recruitment of Rad51 to the site of damage after inducing DSBs.

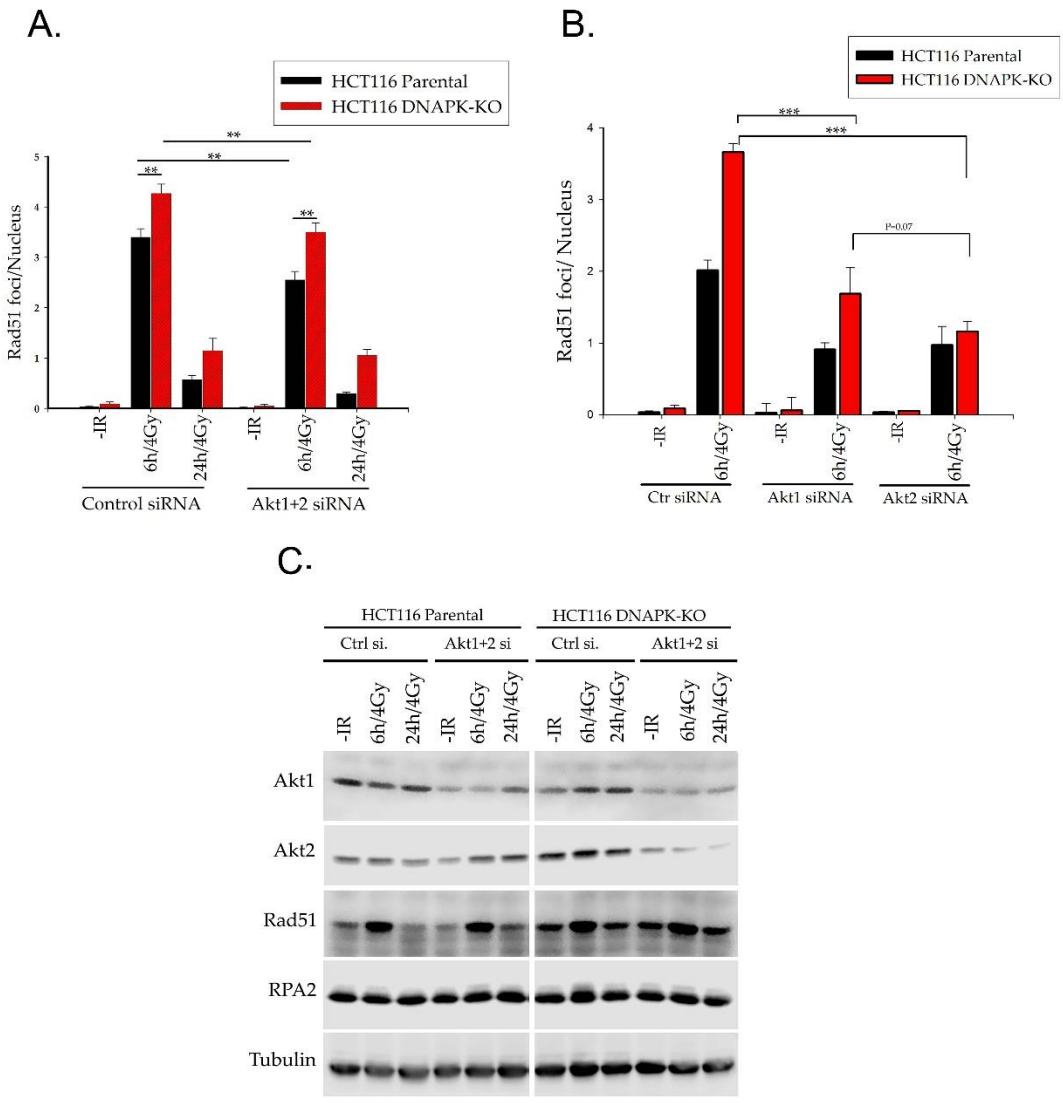

Figure 3.10. Rad51 foci formation in HCT116 Parental and DNAPK-KO cells. HCT116 parental and DNAPK-KO cells were transfected with AKT1-siRNA, AKT2-siRNA, and control-siRNA. (C) Rad 51 foci number per nucleus after dual knockdown of Akt1 and Akt2 and (D) single knockdown of Akt1 and Akt2. The number of Rad51 foci were counted at the indicated time points after 4 Gy. Bars represent the mean number of foci/cell ± SEM from at least three independent experiments. (E) siRNA transfection efficacy was analyzed via a western blot (** $p < 0.01$, *** $p < 0.001$, Student's t-test). (Mohammadian Gol et al. 2019)

To confirm these data, western blot analysis were done to check Rad51 protein level after irradiation in HCT116 Parental and AKT1-KO and AKT2-KO cells. To this aim, nuclear cytoplasmic fractionation was performed 6h and 24h after irradiating the cells with 4 Gy as well as for nonirradiated cells. As it is shown in Figure 3.11 Rad51 protein level in the nucleus of AKT1-KO and AKT2-KO cells is less than in parental cells after irradiation. The reduction of Rad51 translocation to the nucleus is stronger in AKT2-KO cells. This result is another proof for the involvement of Akt1 and Akt2 in Rad51 nuclear translocation following radiation induced DNA damage. Moreover, RPA2 protein level was also reduced in knockout cells versus parental cells. As displacement of RPA and Rad51 is a pre-requisite for RAD51 loading to the damage site during homologous recombination (Stauffer and Chazin 2004) these data indicate the importance of Akt in the translocation and loading process of Rad51 to the DNA-DSB.

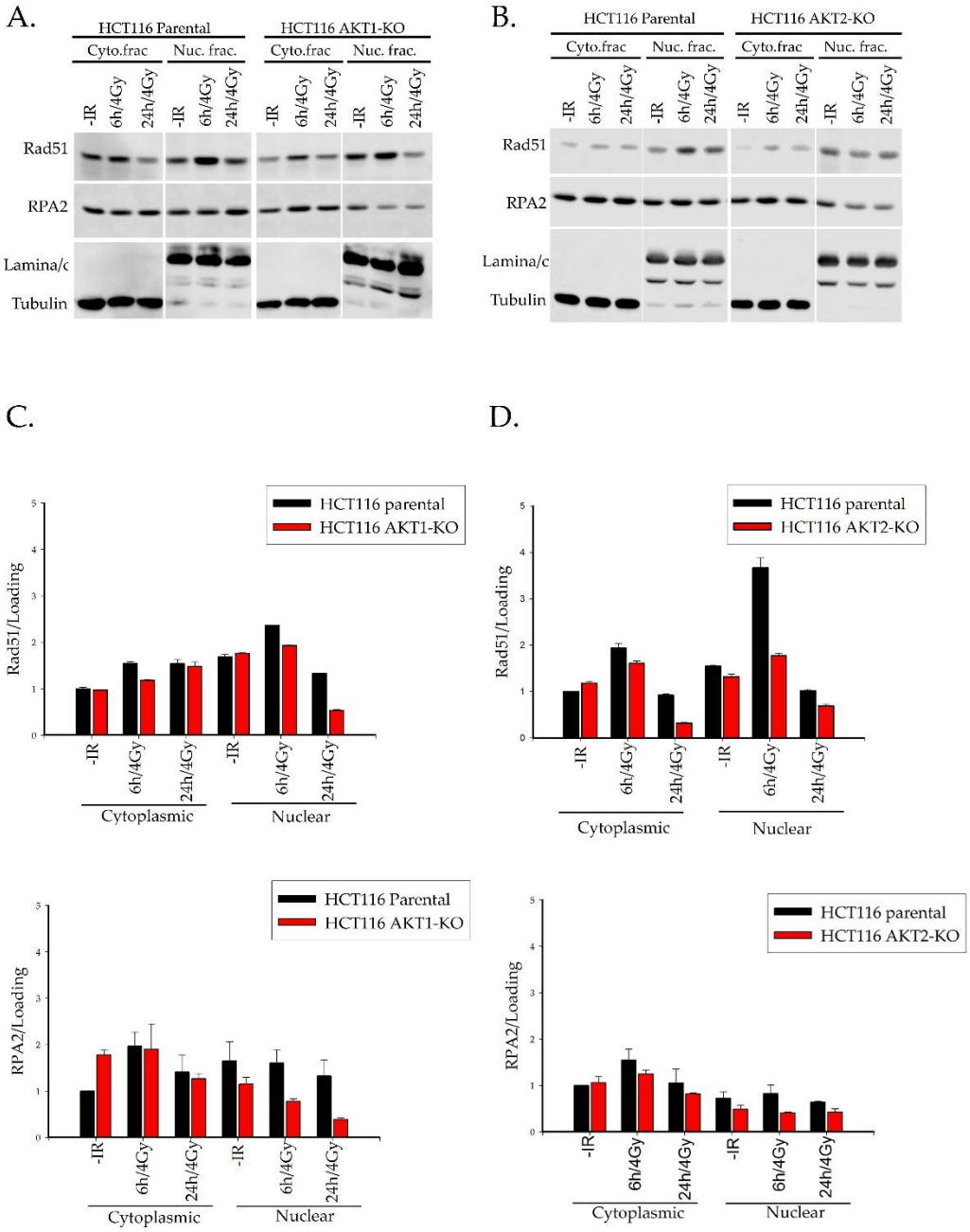

Figure 3.11. Nuclear translocation of Rad51 in HCT116 parental, AKT1 and AKT2 knockout cells. (A,B) Cytoplasmic and nuclear fractions were prepared for HCT116 parental, AKT1-KO, and AKT2-KO cells Six and 24 hours after irradiation. (C,D) Rad51 and RPA2 protein levels were determined by western blotting. Tubulin and lamin were used as cytoplasmic and nuclear markers, respectively. Densitometry is based on the mean ± SEM of three independent experiments. (Mohammadian Gol et al. 2019).

3.6 AKT knockout cells are sensitive to Olaparib

It has been reported that HR deficiency leads to strong sensitivity to PARP inhibition (Kötter et al. 2014, Philip et al. 2017). Therefore, the PARP inhibitor Olaparib was applied to HCT116 parental and AKT1 and AKT2 knock out cells in order to test the efficiency of HRR after radiation exposure of these cells. In comparison to parental HCT116 cells Olaparib treatment of HCT116-AKT-KO cells induced a significantly decreased clonogenic activity (Figure 3.12). Interestingly, in the absence of AKT2, the sensitivity of HCT116 cells to Olaparib was extremely elevated. As it is shown in Figure 3.12 AKT2-KO cells were not able to form colony after Olaparib treatment.

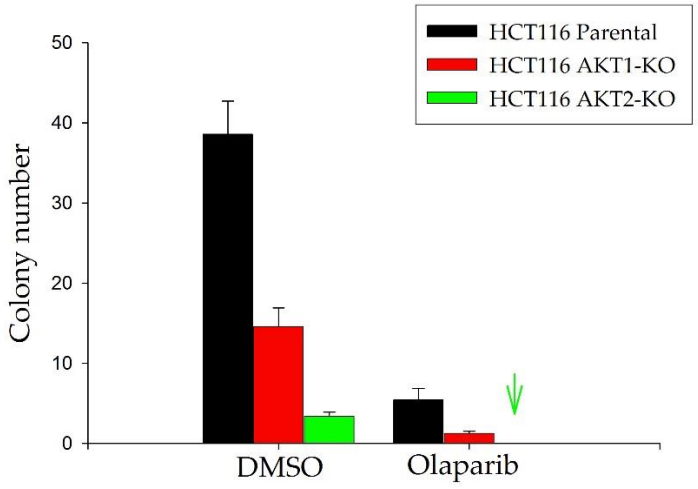

Figure 3.12 inhibition of PARP increase radiosensitivity of HCT116 cells. All three cell lines were treated with 1 µM of the Olaparib 2 h before radiation exposure. Clonogenic assays were performed as described in the Materials and Methods. Bars represent the average number of colonies formed after 2Gy Irradiation when 350 cells were seeded (n=3).(Mohammadian Gol et al. 2019)

Data reported by Mukhopadhyay et al indicate that PARP inhibition is also associated with Rad51 foci formation. Inhibition of PARP results in increased Rad51 foci while in HR deficient cells PARP inhibition has no effect on Rad51 foci formation (Mukhopadhyay 2010). In this context, the effect of Olaparib on Rad51 and γ-H2AX foci formation was analysed. The results of these experiments showed that although inhibition of PARP enhanced the number of residual γ-H2AX foci in all three cell lines, i.e. AKT-KO and parental cells, Rad51 foci formation is only increased in HCT116 parental cells. Thus, based on these results AKT1-KO and AKT2-KO cells are sensitive to Olaparib and this sensitivity may be an indicator for a deficiency in HRR of AKT1- and AKT2 knock out cells.

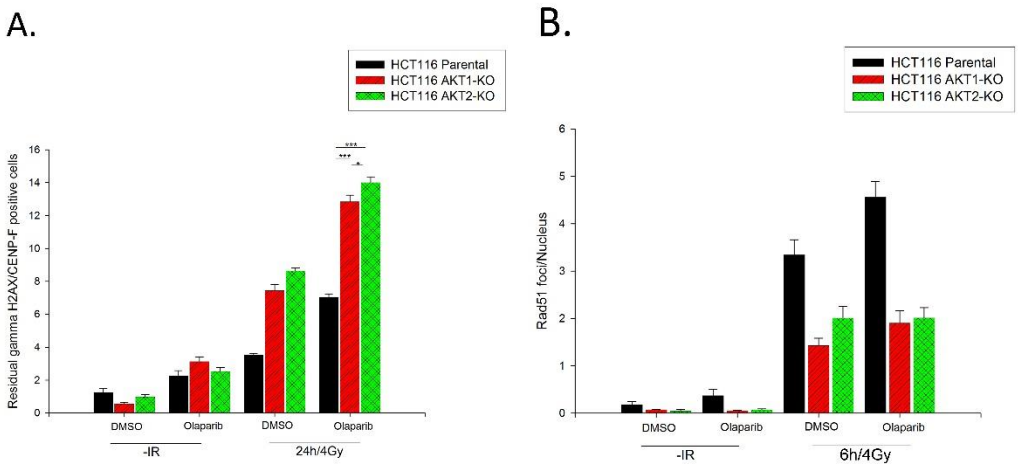

Figure 3.13.Inhibition of PARP increase residual γ-H2AX foci as well as Rad51 foci in HCT116 cells. All three cell lines were treated with 1 µM of the Olaparib 2 h before radiation exposure. (A) Residual γ-H2AX number 24h after 4Gy irradiation. (B) Number of Rad51 foci 6h after 4Gy Irradiation. Bars represent the mean number of foci/cell ± SEM from at least three independent experiments. (* $p < 0.05$, *** $p < 0.001$, Student's t-test).

3.7 Kinase activity of Akt is not involved in translocation of Rad51.

Next, the efficacy of Rad51 nuclear foci formation was analyzed in cells treated with the Akt-inhibitor MK2206. Blocking of Akt-kinase activity with this inhibitor in HCT116 parental cells resulted in a slight reduction of Rad51 foci formation, however, this effect was not significant indicating that nuclear translocation of Rad51 in Akt-wild type cells is not dependent on kinase activity of Akt.

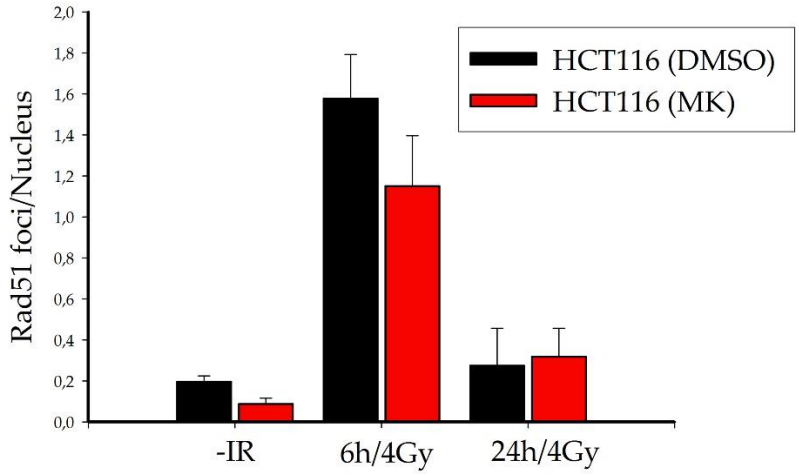

Figure 3.14 Blocking of kinase activity of Akt. (A) Rad51 foci number per nucleus were analyzed after immunofluorescence staining of HCT116 parental following 2 h MK2206 (1 µM) treatment as well as 6 h after irradiation.(Mohammadian Gol et al. 2019)

In further analyses the translocation of Rad51 under MK2206 treatment was tested in HCT116 parental and HCT116-DNAPK-KO cells. After nuclear/cytoplasmic fractionation of MK2206 treated cells revealed no alteration of Rad51 protein levels in the nucleus when compare to DMSO treated control cells neither for HCT116 Parental nor HCT116 DNAPK-KO cells (Figure 3.15).

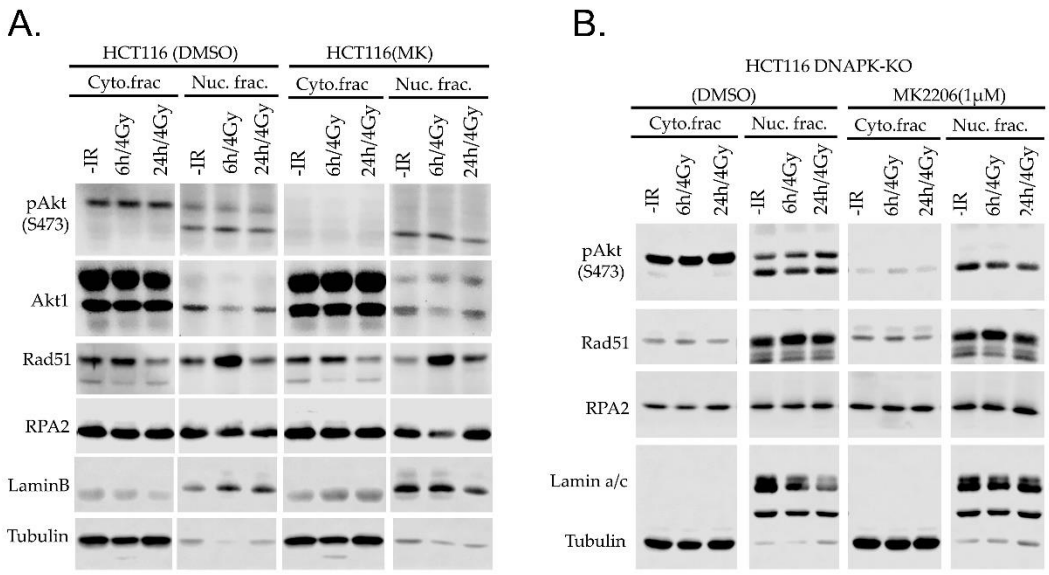

Figure 3.15. Rad51 translocation after MK2206 treatment. MK2206 (1 μM)-treated and nontreated cell were irradiated, and the cytoplasmic and nuclear fractions were prepared 6 and 24 h later for (A) HCT116 parental and(B) DNAPK-KO cells cells.

3.8 Phosphorylation of Rad51.

It is reported by Sørensen et al (Sørensen et al. 2005) that Chk1 mediated phosphorylation of Rad51 at amino acid residue T309 is required for the formation of nuclear RAD51 foci at the DNA damage site. Thus, the phosphorylation of Rad51 (T309) after radiation in HCT116 parental and AKT1/2-knockout cells was analysed. To this aim, nuclear/cytoplasmic fractionation of HCT116 cells at different time points post IR was performed. As shown in Figure 3.16 phosphorylation of Rad51 increased in a time-dependent manner after radiation exposure. In comparison to parental cells, AKT1 and AKT2 knock out cells presented elevated phosphorylation. However, Rad51 and RPA total protein level is decreased in the nucleus of AKT1-KO and AKT2-KO cell as it was already shown in previous results, see Figure 3.4. Moreover, at early time points after irradiation (i.e. from 1h to 6h) MK2206 treatment

of parental HCT116 cells did not affect the phosphorylation of Rad51 at T309. Nevertheless a small increase is apparent at 24h after IR in MK2206 treated cells.

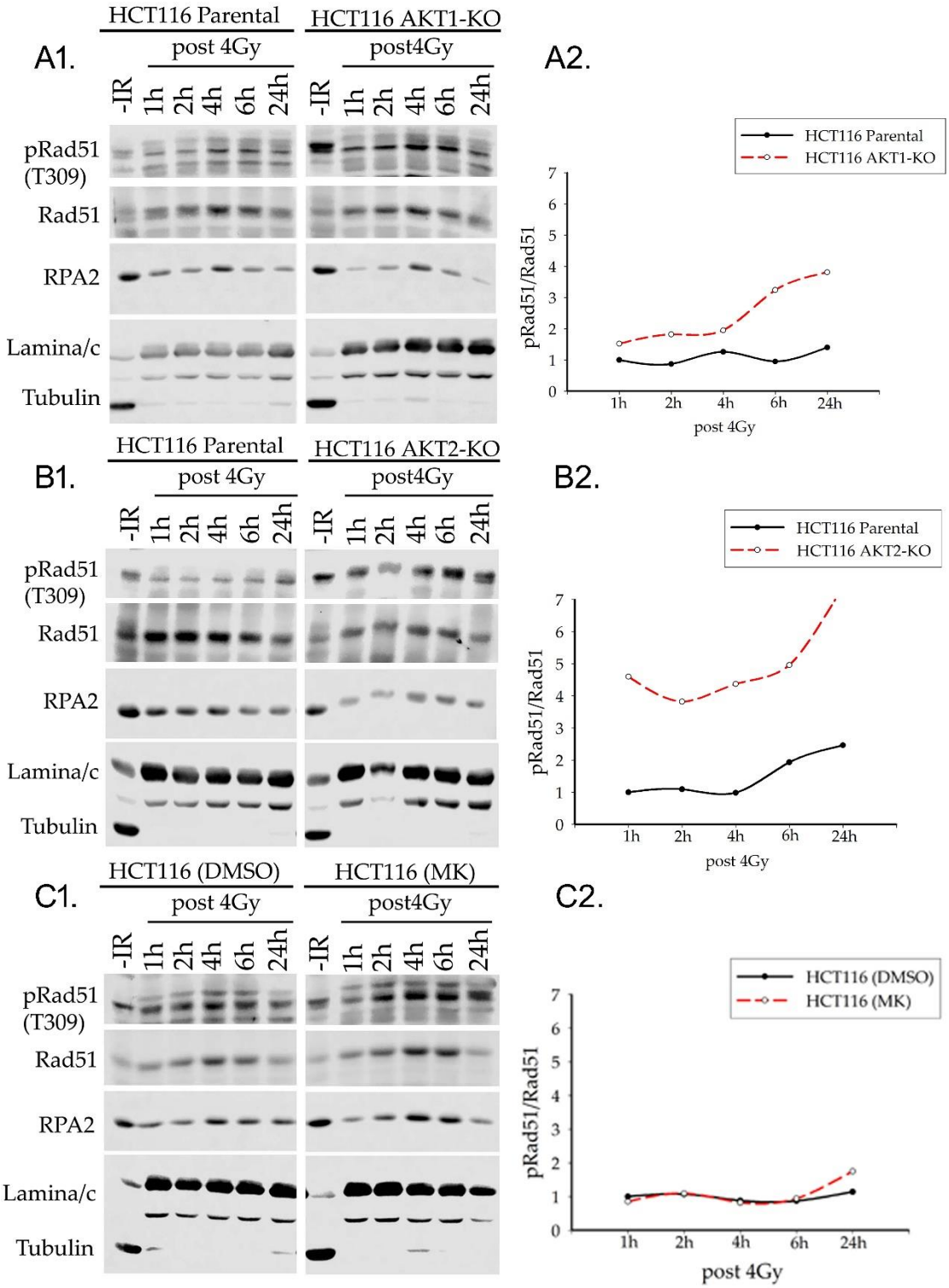

Figure 3.16.Phosphorylation of Rad51 at T309 is higher in the absence of Akt1 and Akt2. Nuclear cytoplasmic fractionations were performed 1, 2, 4, 6, and 24 h after irradiation with 4 Gy as well as non-irradiated HCT116 cells. The level of pRad51, Rad51 and RPA2 in nuclear fraction of (A1) HCT116 parental and AKT1-KO, (B1) parental and AKT2-KO cells; (C1) MK- and DMSO-treated parental HCT116 cells. (A2, B2, C2) Ratios of nuclear pRad51 (T309)/Rad51 total protein were determined by western blotting and normalized to the level of pRad51 at 1h. The non-irradiated condition was excluded for normalization because of contamination with cytoplasmic fraction (*n* = 2).(Mohammadian Gol et al. 2019).

3.9 Akt and HRR protein interaction:

As it was observed that kinase activity of Akt is not involved in translocation and foci formation of Rad51, potential protein-protein interaction between Akt and Rad51 as well as other HR proteins including RPA and BRCA2 was examined. Immunoprecipitation experiments showed that neither Akt1 nor Akt2 do not directly interact with Rad51 and RPA2.

Regarding AKT-BRCA2 interaction, although immunoprecipitation of Akt1 did not show any interaction of Akt1 and BRCA2, a slight band of Akt1 and Akt2 was to be detected after co-immunoprecipitation of BRCA2 (Figure 3.17). Together, these results indicate that there is no clear and stable protein interaction of Akt1 and Akt2 with Rad51 and RPA as well as with BRCA2.

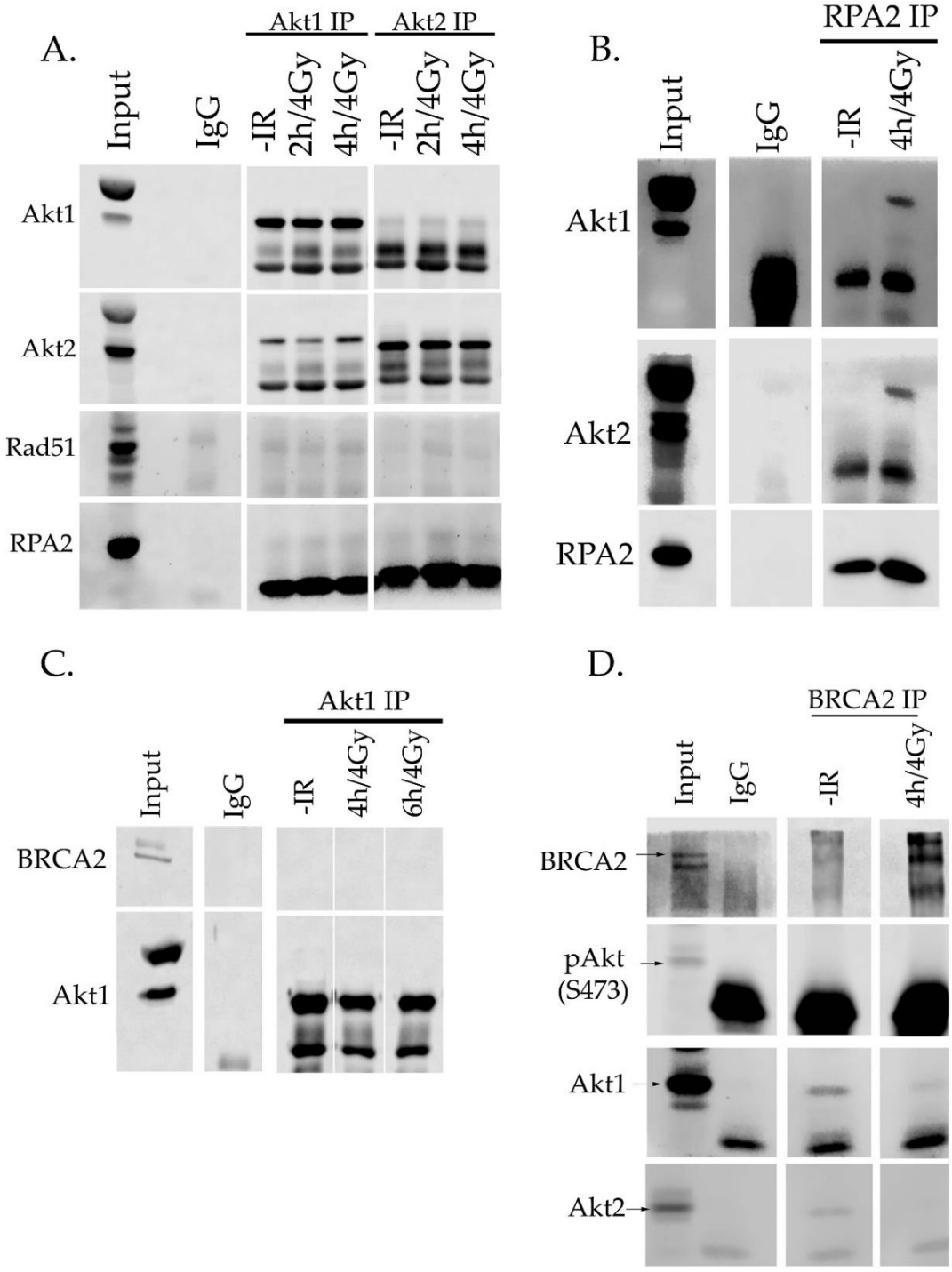

Figure 3.17. Akt is not in a complex with BRCA2, RPA2, and Rad51. Immunoprecipitation and co- immunoprecipitation analysis for (A) Akt1 and Akt2 (B) RPA2 (C) Akt1 (D) BRCA2 were performed as described in the Materials and methods. IgG protein immunoprecipitation was used as a control.(Mohammadian Gol et al. 2019)

To identify potential transient or indirect interactions between Akt1 and AKT2 with BRCA2 and RPA a proximity ligation assay (PLA) was performed. PLA assay is a method for detection of protein–protein interactions (PPI) in tissue section or cell cultures. In this technique two applied antibodies conjugates to complementary oligonucleotides. When the proteins of interest are in close distance (<40 nm) subsequent hybridization and amplification steps produce fluorescent signal. The generated fluorescent signal is visualized as fluorescent dot by microscopy. As it is shown in Figure 3.18 the PLA assay showed an interaction of Akt1 and Akt2 with BRCA2 in non-irradiated HCT116 cells. Fluorescent dots reflecting Akt1/2 and BRCA2 interaction were mostly outside of the nucleus and reduced after irradiation. As BRCA2 is one of the potential mediator proteins for translocation of Rad51, these transient interaction of Akt1/2 and BRCA2 outside of the nucleus might be associated with the role of Akt in translocation of Rad51 to the nucleus. On the other hand a reduced interaction after irradiation might be due to a radiation independent role of Akt in other cell processes.

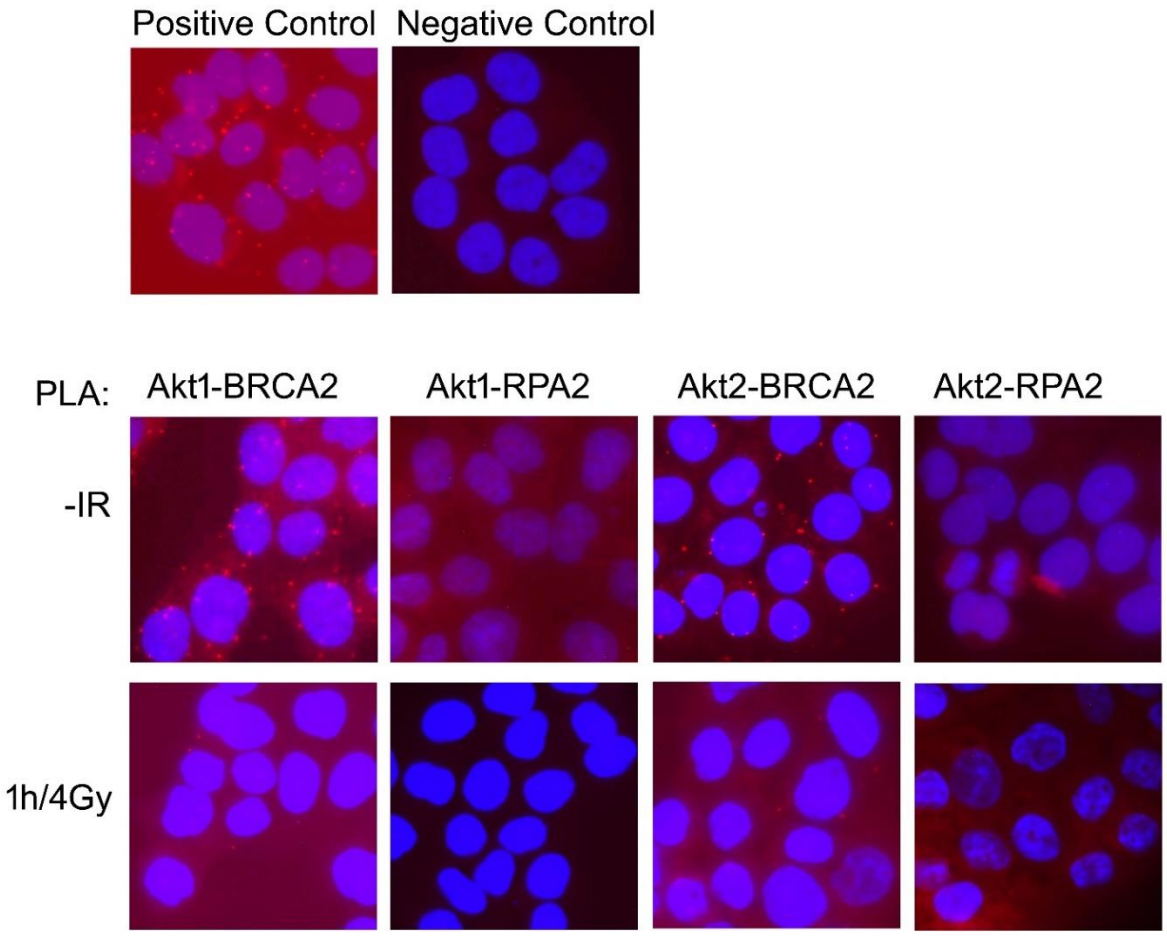

Figure 3.18. Proximity ligation assay in HCT116 parental cells. PLA assay is performed as described in the Materials and methods. EGF treated cells were used as positive control for detection of EGFR-HER2 dimerization. For negative control condition primary antibodies were not added to the cells.

4) Discussion

4.1 The role of Akt in regulating DSBs repair

DNA DSBs in mammalian cell are repaired by three pathways including classical non-homologous end joining (C-NHEJ) that acts throughout the cell cycle, homologous recombination repair (HRR) that operates exclusively in G2/S phase, and alternative NHEJ (A-NHEJ) that is a backup pathway in the absence of functional C-NHEJ. The role of Akt in the context of DSBs repair is linked to both major pathways, i.e. C-NHEJ and HRR. Various studies have investigated the importance of Akt with respect to either stimulatory or inhibitory effects on DSBs repair. It has been shown that after irradiation P-AKT (Ser-473) is colocalized with γ-H2AX at the sites of DSBs and promotes DSB repair and thus mediates cellular radioresistance (Sørensen et al. 2005, Toulany and Rodemann 2015, Holler et al. 2016, Szymonowicz et al. 2018).

Different preclinical investigations have reported that genetic or pharmacologic inhibition of Akt is associated with decreased DNA-PKcs-dependent DSB repair and induced cytotoxicity to chemo- and radiotheraphy. Toulany et al (Toulany et al 2012) demonstrated that AKT1 directly interacts with DNA-PK through its C-terminal domain and induces accumulation of DNA-PKcs at the sites of DNA-DSBs. Moreover, they reported that Akt1 promotes DNA-PKcs kinase activity. Therefore, Akt1 is needed for efficient NHEJ DNA-DSB repair. Interaction of DNA-PKcs is also reported for the Akt-isoform Akt3 in Non-small-cell lung carcinoma (NSCLC) cell line (Mueck et al. 2017). Sahlberg et al. (Sahlberg et al. 2014) reported that single or dual depletion of AKT1 or AKT2 impairs the process of DNA-rejoining of DSBs and increases radiosensitivity. Moreover, Turner et al. (Turner et al. 2015) showed an enhanced activation of DNA repair proteins in Akt3-expressing human Glioblastoma (GBM) cells and as consequence an increased resistance to radiation and Temozolomide. Investigations by Oeck et al. (Oeck et al. 2017) showed that activated mutant form of Akt1 (Akt1-E17K) in murine prostate cancer cells stimulates radiation-induced DNA damage repair and mediates cellular radioresistance . It is

also shown that phosphorylation of Cernunnos/XRCC4-Like Factor (XLF) by Akt leads to its dissociation with DNA ligase IV/XRCC4- complex and impairs C-NHEJ (Liu et al. 2015). Ubiquitin-conjugating enzyme E2S (UBE2S) is another target protein of Akt which exerts an important role in DSBs repair. UBE2S phosphorylation by Akt1 as well as UBE2S/Akt1 interaction stimulates Ku70-UBE2S binding and subsequently the accumulation of UBE2S-Ku70 at the site of damages which leads to an improved C-NHEJ (Hu et al. 2017)

4.2 Akt influence HR repair pathway through regulating HRR proteins

BRCA1 (breast-cancer susceptibility gene 1) exerts different regulatory functions including processes of DNA damage repair and checkpoint activation. BRCA1 is frequently mutated in breast and ovarian cancer. Akt is known to phosphorylate BRCA1 at two amino acid residues, i.e. S694 and T509. Plo et al (Plo et al. 2008) showed that constitutive activation of Akt1 can result in decreased BRCA1-foci formation, cytoplasmic retention of BRCA1 and Rad51, and impaired HRR.

MERIT40 is a member of RAP80 ubiquitin recognition complex which is important for assembly of BRCA1 complexes at DNA damage sites. MERIT40 is also able to cooperate with BRCA2 in order to resolve interstrand cross-links (Jiang et al. 2015). Akt has been described to phosphorylate MERIT40 at S29 (Brown et al. 2015). Brown et al (Brown et al. 2015) showed that treatment of breast cancer cell lines with doxorubicin results in phosphorylation of MERIT40 by Akt and facilitates the assembly of BRCA1 complexes in response to DNA damage through HRR.

Xiang T et al (Xiang et al. 2011) based on investigation on BRCA1-deficient cells demonstrated that reduced BRCA1 expression seems to be associated with an increased phosphorylation of Akt. Another study reported by Jia et al (Jia et al. 2013) indicated that in BRCA deficient cells, activation of Akt leads to blocking of Chk1-Rad51 interaction and consequently impairs HRR. This group suggested that BRCA1 deficiency is associated with activate Akt1 which contributes to tumorigenesis by influencing Chk1-Rad51 signaling pathway.

In contrast to the results reported by Jia et al (Jia et al. 2013) and Plo et al.(Plo et al. 2008) a study published by Mueck et al (Mueck et al. 2017) indicated that Akt1 functions as a promoter of homologous recombination repair. In detail Mueck et al presented data that downregulation of Akt1 in non-small cell lung cancer cells (NSCLC) mediated a decreased clonogenicity after treatment with Mitomycin C. Especially and based on a HR-reporter assay system the authors reported that Akt1-knock down significantly impaired HR. Moreover, the study by Mueck et al indicated that siRNA mediated downregulation of Akt1 led to an increased number of residual DSBs after irradiation which is partially independent of the DNA-PKcs. Mueck et al. also claimed that the demonstrated and significantly increased number of residual BRCA1 foci after Akt1-knock down serves as an indicator of unsuccessful HR in irradiated cells (Mueck et al. 2017).

In line with this study of Mueck et al. , the results presented in the present study indicate that the number of residual γ-H2AX foci 24 hour after 4 Gy irradiation in HCT116 AKT1- and AKT2-knock out cells is significantly higher than that of parental wild type cells. In contrast to all studies done and discussed above concerning the role of Akt in HRR, in the present study centromeric protein F (CENP-F) staining was used to specifically analyze cells in late S- as well as G2-phase, i.e. HR-competent cells. Moreover, by blocking other pathways of DSBs repair, i.e. C-NHEJ and A-NHEJ, the ability of cells for repairing of DSBs preferentially through HRR was analyzed. Applying this approach it could be demonstrated that non-repaired DNA-DSB in CENP-F positive cells is increased in irradiated HCT116 AKT1- and AKT2-knock out cells (see Figure 3.5). Furthermore, blockage of homologous recombination using the Rad51 inhibitor BO2, indicated that AKT1 and AKT2 knock out cells are more sensitive to this inhibitor than the parental cells as the number of unrepaired DNA-DSB 24 hour after irradiation was significantly higher in BO2 treated Akt knock out cells (see Figure 3.6).

The results of post-irradiation clonogenic cell survival is also another proof for the involvement of Akt1 and Akt2 in repair pathways. Consistent with the results obtained through γ-H2AX analysis, post-irradiation cell survival capacity of AKT1 and

AKT2 knock out cells was significantly lower in comparison to parental cells. As described in the introduction above it is proven by many studies that Akt1 plays a prominent role in the regulation of the NHEJ pathway (e.g. see (Sahlberg et al. 2014, Toulany et al. 2017)). Using DNAPK inhibitor strongly increased the radiosensitivity of AKT1-KO cells due to their impaired NHEJ capacity. Yet, in AKT2-KO cells Akt1 is functional. Thus, these cells have proficient NHEJ and consequently treatment with the DNAPK inhibitor results only in a slight effect in cells irradiated with 2 Gy. Thus, the data presented suggest that knock out of AKT2 affects HRR more than knock out of AKT1 (see Figure 3.8). Yet, as AKT2-KO cells presented a significantly increased radiosensitivity after BO2 treatment when compared to AKT1-KO cells, it can be concluded that both Akt1 as well as Akt2 are involved in the regulation of homologous recombination repair process of DNA-DSBs.

4.3 Inhibition of Kinase activity of Akt

Activation of the PI3K/Akt/mTOR pathway in tumor cells is an important factor involved in sensitivity to Akt-specific inhibitors (She et al. 2008). For instance, catalytic Akt inhibitors e.g. GDC-0068 and AZD5363 induce inhibitory effects in breast tumor cell lines with Akt mutations while allosteric inhibitors like MK-2206 do not exert inhibitory effect on breast, ovarian and colon cancer cells (Carpten et al. 2007, Banerji et al. 2012). On the other side, MK2206 in combination with MEK1/2 inhibitor AZD6244 can stimulate apoptosis and inhibition of cell growth in A549 and H157 lung cancer cells (Meng et al. 2010).

It has been demonstrated that MK2206 increases effect of chemotherapeutic agents such as carboplatin, docetaxel, gemcitabine, and doxorubicin and molecularly targeted drugs (erlotinib or lapatinib) in different cancer cell line including lung, breast, ovarian, gastric or hepatocellular carcinoma (Simioni et al. 2013).

An antitumor effect of MK2206 is also reported in combination with radiation in various studies (Li et al. 2009, Holler et al. 2016). In contrast to these reports,

Djuzenova et al (Djuzenova et al. 2019) showed that blocking of Akt in glioblastoma cell line does not enhance IR-induced DNA damage and has no effect on cell cycle distribution, apoptosis or autophagy.

MK2206 is able to block kinase activity of Akt at both phosphorylation sites (Akt T308 and S473) but does not change the basic protein level of Akt (Szymonowicz et al. 2018). In the present study, treatment of HCT116 cells with MK2206 led to increased residual γ-H2AX foci indicating a specific effect of kinase activity on the repair of radiation induced DNA-DSB-repair (see also Figure 3.14). However, as presented herein (see Figure 3.15) the results indicate that Akt kinase activity has no effect on nuclear translocation of RPA and Rad51 protein. Therefore, based on our results, the effect of MK2206 on increased γ-H2AX seems to be independent of the role of Akt on Rad51 and RPA translocation. Interestingly, the application of MK2206 inhibitor on HCT116 DNAPK-KO cells also did not change Rad51 translocation. Thus, the increased amount of residual γ-H2AX-foci after MK2206 treatment of irradiated cells might be due to the role of Akt kinase activity in phosphorylation of other target proteins in DSBs repair pathway. For instance, Akt can phosphorylate MERIT40 in HRR pathway and XLF or UBE2S in NHEJ pathway (Liu et al. 2015, Brown and Banerji 2017).

4.4 Rad51, main player of HRR

Rad51 is a key enzymes required for pairing and exchange of strands between homologous DNA molecules in HRR (Vispé et al. 1998). Rad51 is a conserved protein in eukaryotes. Rad51 of vertebrates and fungi have about 74% protein sequence similarity. Human and mouse Rad51 homologs are 99% identical. This level of similarity indicates that the function of Rad51 is also conserved. The human Rad51 (HsRad51) has also structural and functional homologies with the bacterial recombinase RecA (Stassen et al. 1997). Rad51 is a monomer when it is in a solution but it can polymerase and form a nucleoprotein filament on both double-stranded

DNA (dsDNA) and single strand DNA (ssDNA). Moreover, Rad51 is able to bind two DNA molecules simultaneously through its primary and secondary DNA binding sites to do recombination (McEntee et al. 1981, Shinohara et al. 1992, Baumann et al. 1996). Rad51 activity also needs DNA dependent manner hydrolase of ATP (Sung1994). Following DNA damage Rad51 translocates to the nucleus (Gildemeister et al. 2009) and a clear correlation of Rad51 nuclear accumulation with PI3K pathway and Akt protein has been reported in different studies (Plo et al. 2008, Juvekar et al. 2012, Mueck et al. 2017, Philip et al. 2017).

In this context, Philip et al (Philip et al. 2017) showed that inhibition of PI3K pathway using BKM-120 inhibitor leads to reduction of Rad51 foci after inducing damages in DNA by doxorubicin. Plo et al (Plo et al. 2008) demonstrated correlation of Akt1 with BRCA1 and RAD51 cytoplasmic localization analyzing biopsies from breast cancer patients. They showed that in AKT-low breast cancers, BRCA1 and Rad51 mostly localized in the nucleus whereas in AKT-high tumors, there is mainly cytoplasmic localization of both BRCA1 and RAD51. Thus, Plo et al concluded that Akt activation results in cytoplasmic retention of BRCA1 and Rad51 in breast cancer cell. Xu et al. (Xu et al. 2010) showed that inhibition of Akt by AKT1/2I restored accumulation of CtIP, RPA, and Rad51 in RO-3306–arrested Cdk1AS and HCT116 cells in G2 phase.

In contrast to these studies the report from Mueck et al (Mueck et al. 2017) demonstrated that siRNA mediated knockdown of Akt1 led to a significant decrease of Rad51 protein amount in the nucleus of the non-small cell lung cancer (NSCLC) cell line A549. Moreover, Muek et al showed that colocalization of γ-H2AX and Rad51 foci is significantly reduced after downregulation of Akt1 in A549 as well as H460 cells. In agreement with the study from Mueck et al (Mueck et al. 2017) but in contrast to Xu et al (Xu et al. 2010, Mueck et al. 2017) the data presented herein indicate that in the absence of the Akt isoforms Akt1 and Akt2 the nuclear accumulation of Rad51 in irradiated cells is markedly reduced (see again Figure 3.9). This reduction might be an indication of impaired homologous recombination in

AKT1/2 knockout cells which leads to increased residual damages in CENP-F positive cells following irradiation (see again Figure 3.4). Furthermore, the presented western blot analysis clearly indicate a reduced Rad51 nuclear translocation in knockout cells when compared to parental cells. Therefore, the study presented provides clear evidence that both the Akt isoforms Akt1 and Akt2 are involved in Rad51 nuclear translocation and foci formation after radiation induced DNA-DSB.

Post-translational modifications (PTM) of Rad51 are important for its activity through DNA damages response (Chabot et al. 2019). RAD51 has different phosphorylation sites on threonine, tyrosine and serine residues although the exact function of these phosphorylations is still not clearly understood. It has been reported that phosphorylation of Rad51 by CK2 on S14 and phosphorylation by Plk1 on T13 are important for the interaction of Rad51 with NBS1 (Yata et al. 2012). c-ABL is a tyrosine kinase which is able to phosphorylates Y315 and Y54 residues of Rad51. These phosphorylation sites are important for the recombinase activity of Rad51 (Shimizu et al. 2009, Alligand et al. 2017). Chabot et al (Chabot et al. 2019) demonstrated Rad51 phosphorylation by c-MET and its importance for the regulation of the BRCA2-RAD51 interaction.

Sørensen et al. (Sørensen et al. 2005) described an interaction of Chk1 and RAD51 via phosphorylation of Rad51 on T309 by Chk1. They showed that Chk1 depletion results in impairment of RAD51 nuclear foci formation after hydroxyurea treatment. They also provided evidence that mutation on Rad51 at the T309 phosphorylation site mediates sensitivity to hydroxyurea. In agreement to this report a study by Marzio et al 2019 (Marzio et al. 2019) showed that in neocarzinostatin (NCS) treated U2OS cells mutant Rad51(T309A) is not able to load on the chromatin as wild type Rad51. Based on their result Rad51 phosphorylation on T309 induces binding of Rad51 to BRCA2 and leads subsequently to an increased Rad51 stability. Nevertheless, Marzio et al 2019 pointed out that phosphorylation of Rad51 in T309 is not the only involved factor in Rad51 binding to BRCA2 and Rad51 stability at

damage site. Thus, other modifications in binding site of Rad51 and BRCA2 should be investigated.

In present study, when compared to wild type parental cells AKT1 and AKT2 depleted HCT116 cells showed elevated levels of pRad51 (T309) in the nucleus (see again Figure 3.16). However, this elevated phosphorylation cannot be correlated with an improved homologous recombination capacity as proposed by Marzio et al (Marzio et al. 2019) and Sørensen et al (Sørensen et al. 2005) because the data presented in this study, i.e. the repair efficiency analyzed by the number of γ-H2AX foci in CENP-F positive cells, indicates an opposite effect. Likewise, the reports from Sørensen et al. do not provide an adequate explanation regarding the effect of Rad51 mutation (T309A) on accumulation of γ-H2AX foci on the site of damage and homologous recombination repair. Marzio et al 2019. applied NCS as inducer of a variety of DNA-damages in U2OS cells while in this project ionizing radiation was applied to induce specifically DNA-DSBs in HCT116 colon cancer cells, which were then specifically analysed by the γ-H2AX focus assay. Therefore, phosphorylation status might be different in these different cell systems. Moreover, Marzio et al. showed only Rad51 presence in chromatin fraction after using lentiviruses expressing mutant Rad51 (T309A) in cells presenting a knockdown phenotype for Rad51. Likewise, in the Marzi et al. study the authors did not show Rad51 focus formation in mutant cells nor the phosphorylation status of normal and mutant cells after NCS treatment. Taken together, at present there is not sufficient evidence for explaining the specific function of Rad51T309 phosphorylation especially following radiation exposure of cells.

4.5 Inhibition of Poly-(adenosine diphosphate-ribose)-polymerase (PARP).

Poly-(adenosine diphosphate-ribose)-polymerases (PARPs) are a family of enzymes with at least 18 members which are able to transfer ADP-ribose to different target proteins involved in various cellular processes. PARPs family members exert roles in DNA replication, modulation of chromatin structure, transcription, DNA repair and cell death (Morales et al. 2014, Lesueur et al. 2017). In this context PARP1 is the first characterized and active member of the PARP family.

PARP activity increases after exposure of DNA to DNA damaging agents or ionizing irradiation (SKIDMORE et al. 1979). PARP is involved in base excision repair (BER), SSB and DSBs repair(Lesueur et al. 2017). Due to the importance of PARPs proteins in DNA damages responses, therapeutic inhibition of PARP in combination with chemotherapy or radiotherapy has been considered for various cancers. Yet, inhibition of PARP can increase the sensitivity of tumors to chemotherapeutic DNA damaging drugs. Moreover, blocking of PARP results in impairment of SSB and BER repair and consequently persistence of DNA damages which under normal conditions would be repaired via homologous recombination. In cells lacking proficient homologous recombination repair (e.g., BRCA1 and BRCA2 deficient cells), PARP inhibition leads to synthetic lethality effect due to the accumulation of DSBs (Kyle et al. 2008).

The main mechanism of PARP inhibitors is trapping of PARP1/2 on DNA which results in a toxic PARP–DNA complex (PARP trapping) (Boussios et al. 2019). It has been suggested that PARP inhibitors act synergistically with other factors involved in cell signaling pathways and these synergistic effect may be useful in cancer therapy. For instance, based on reports from Ibrahim et al (Ibrahim et al. 2012) combined inhibition of PI3K pathway and PARP resulted in an increased accumulation of γ-H2AX foci in triple-negative breast cancers. Likewise, Ibrahim et al provided evidence that blocking of PIK3CA by siRNA or pan-PI3K inhibitor

(BKM120), is associated with a reduced expression of BRCA1/2 and consequently impaired HRR.

Furthermore, Juvekar et al (Juvekar et al. 2012) demonstrated that inhibition of PI3K led to a decreased Akt phosphorylation and reduced Rad51 focus formation which in combination with PARP inhibitor led to increased γ-H2AX. In vivo studies by this group (Juvekar et al. 2012) revealed that treatment with the PARP inhibitor Olaparib diminishes tumor growth. Likewise, combined treatment with Olaparib and the pan-class IA PI3K inhibitor NVP-BKM120 resulted in increasing of tumor doubling time in the mouse model and xenotransplants from human BRCA1-related tumors. Furthermore, Mukhopadhyay et al (Mukhopadhyay et al. 2010) developed an assay for detecting HR status through Rad51 focus formation after PARP inhibitor AG014699 treatment in epithelial ovarian cancers (EOC). Mukhopadhyay et al showed that AG014699 treatment of HR-competent cell lines significantly increase Rad51 foci formation while AG014699 treatment has no effect on Rad51 foci formation in HR-deficient cell lines when compared to untreated controls.

Olaparib is first FDA approved and orally active PARP inhibitor presenting antitumor activity especially in tumors with deficient BRCA function (Jones 2010, Westin et al. 2018). In the present study, Olaparib treatment in HCT116 AKT1 depleted cells resulted in reduced clonogenic activity of cells after irradiation (see again Figure *3.12*). Interestingly, Olaparib treatment of HCT116 AKT2-KO cells completely impaired colonogenic survival indicating a non-functional homologous recombination repair mechanism. These results are in agreement with the findings presented in Figure 3.4 indicating the involvement of Akt1 and Akt2 in the repair of DSBs through HRR. Moreover, and in the context of the results reported by Mukhopadhyay et al (Mukhopadhyay et al. 2010), the stable level of Rad51 foci after Olaparib treatment in AKT1- and AKT2-knock out cells (see again Figure 3.13), is another indicator of nonfunctional homologous recombination in Akt depleted cells. Figure 4.1 proposes a possible model for the effect of Olaparib treatment on HRR in Akt proficient cells in comparison to Akt deficient cells.

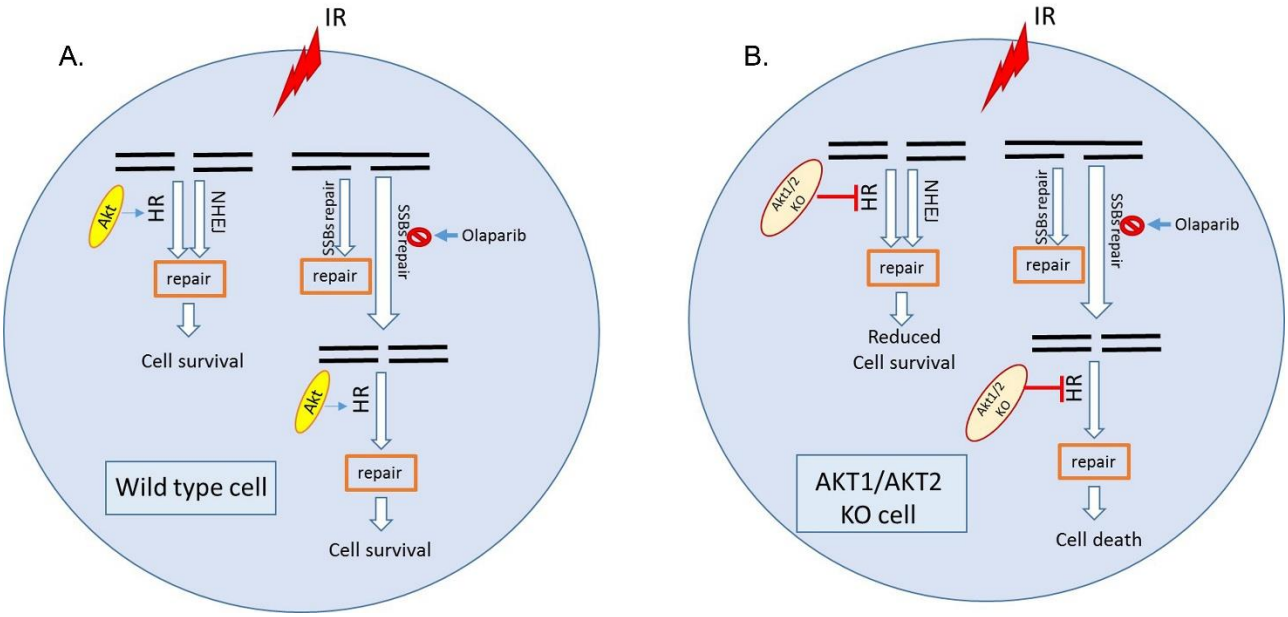

Figure 4.1.Model proposed for the effect of PARP inhibition by Olaparib in (A) wild type cell (B) AKT1-KO and AKT2-KO cells. Akt promotes homologous recombination after Olaparib treatment. In Akt deficient cells, HR is not function and cannot repair DSBs produced by irradiation and Olaparib treatment.

4.6 Interaction of Akt with homologous recombination proteins

BRCA proteins including BRCA1 and BRCA2 (Breast-Cancer susceptibility gene 1 and 2) are tumor suppressor genes involved in various cellular processes. Both BRCA1 and BRCA2 proteins have specific roles in DNA repair and transcriptional regulation after DNA damage as well as maintenance of chromosomal stability, cell cycle regulation and apoptosis (Yoshida and Miki 2004).

In response to DNA damage BRCA1 is phosphorylated by different kinases, e.g. Ataxia telangiectasia mutated kinase (ATM) and G2/M control kinase (Bell et al. 1999, Gatei et al. 2001). It has been shown that after DNA damaging insults, both BRCA1 and BRCA2 colocalize with Rad51 at the sites of recombination (Scully et al. 1997). Although Rad51 focus formation is reduced in BRCA1-deficient cells, direct interaction of BRCA1 and Rad51 has not been reported (Venkitaraman 2001).

Due to the lack of nuclear localization signals (NLSs), Rad51 is not able to enter to the nucleus by itself. Thus, Rad51 needs to interact with other proteins presenting a functional NLS (Gildemeister et al. 2009). Specific mediator proteins are reported for nuclear assembly of RAD51 such as BRCA2, Rad52, and Rad51 paralogues (Rad51B, C, D and XRCC 2, 3) (Suwaki et al. 2011). Yet, the results presented in this thesis did not show any direct and stable interaction of BRCA2 with Akt1 and Akt2 (see again Figure 3.17). However, result obtained by the PLA assay showed an interaction between both Akt1 and Akt2 with BRCA2 at least in nonirradiated cells (see Figure 3.18).

Replication protein A (RPA) is the other protein involved in HRR which binds to Rad51 directly and promotes recombination (Liu and Huang 2016). RPA has three subunits including RPA1 (70 kDa), RPA2 (32 kDa), and RPA3 (14 kDa). In our study, western blot analysis showed that RPA2 protein level in the nucleus of Akt1 and Akt2 knockout cells is less than wild type cells. However, in the present study neither immunoprecipitation analysis nor PLA assay did indicate any interaction of Akt1 and Akt2 with RPA. Thus, these results suggest that the effect of Akt on Rad51 translocation is not through direct interaction with BRCA2 and RPA. To this topic further studies should analyze in detail the role of other mediator proteins.

4.7 The specific role of Akt2 in homologous recombination

Regarding the role of Akt in homologous recombination repair process most of the existing data focused so far on the isoform Akt1. The present study is the first that provided evidence for the impact of Akt2 in the regulation of HRR. It has been proven that Akt isoforms have distinct functions and different expressions in different tissues (Thimmaiah et al. 2005). For instance, Matheny Jr et al. (Matheny Jr et al. 2018) showed that AKT2 is the predominant AKT isoform in human skeletal muscle. Wang et al (Wang et al. 2017) reported that AKT1 and AKT2 expression and phosphorylation is different in different cancer lineages. Moreover, a report from Rychahou at al. (Rychahou et al. 2008) indicated an AKT2 involvement in the metastatic process of colorectal cancer and Roy et al. demonstrated the upregulation of Akt2 in colon cancer. Concerning the specific role of Akt2 in repair of DSBs, Toulany et al. (Toulany et al. 2017) showed that knockdown of Akt2 isoform in A549 cell line reduced the number of residual γ-H2AX foci and leads to a slight radioprotection. In contrast to these reports, Sahlberg et al (Sahlberg et al. 2014) showed that deficiency of Akt1 as well as Akt2 inhibit the repair of radiation-induced DNA double strand breaks through NHEJ in colon carcinoma HCT116 cell line. Interestingly, results presented in the present thesis demonstrate that Akt2 has even stronger effect on HRR than Akt1 in the same cell line (HCT116). As it could not finally be demonstrated that the effect of Akt2 on homologous recombination repair results from the influence of Akt2 on Rad51 translocation and loading to the site of DNA-DSB, the exact molecular mechanism of this phenomenon has still to be resolved. One explanation to this topic could potentially be related to the function of Akt2 in glucose metabolism. The important role of Akt2 in the regulation of of glucose metabolism has clearly been proven (Bouzakri et al. 2006). It has also been demonstrated that ATM and AKT are able to regulate the ATP-citrate lyase (ACLY) in response to DNA-damage (Sivanand et al. 2017). Ampferl et al.(Ampferl et al. 2018) reported that nuclear ACLY activity is important for production of nuclear Acetyl-CoA as well as TIP60-driven histone H3 acetylation which is essential for

DNA repair. For activation, ACLY is phosphorylated by Akt at S455 (Berwick et al. 2002) and consequently acetylation of histone H3 and H4 increases. The enzymatic activity of ACLY results in recruitment of BRCA1 to the damage site and initiation of HR. The role of this pathway in regulation of homologous recombination by Akt2 needs to be investigated in more details and may shed further light on the function of Akt2.

4.8 Conclusion and outlook

Taken together, based on the data obtained, the presented study provides specified evidence that both Akt isoforms Akt1 and Akt2 have a role in the regulation of the homologous recombination repair mechanism. Furthermore, in the context of all studies performed to this topic the data presented clearly demonstrate especially the importance of Akt2 in the HRR process for the first time.

On the basis of the data presented in this thesis it can be proposed that depletion of Akt1 and especially Akt2 in combination with PARP inhibition induces a strong radiosensitization of human colon cancer cells. This inhibitory effect could be considered as a potential target for the future study in the context of radiation oncology research. Moreover, the investigation of specific role of different Akt isoforms in different tissue and various cancer cell lines in the future studies is recommended.

5) Summary

DNA double-strand breaks (DSBs) are genotoxic DNA lesions caused by ionizing radiation (IR) or mutagenic chemicals and potentially lead to chromosomal breakage, fragmentation, and translocation. Homologous recombination repair (HRR), classical non-homologous end-joining (C-NHEJ), and alternative non-homologous end-joining (Alt-NHEJ) are the major pathways employed by cells for repairing of DSBs. Therefore, their function is critical for protecting the genome stability in general but also for the development of radiation resistance e.g. in tumor cells.

The phosphatidylinositol-3-kinase (PI3K)/protein kinase B (AKT)/mTOR signaling pathway functions at different levels and mechanisms of cell survival and apoptosis. Stimulated PI3K/AKT pathway activated by receptor tyrosine kinases or mutational hyperactivation has been reported in different human cancer entities. Akt/PKB is a serine/threonine kinase and exists in three isoforms known as Akt1 (PKBα), Akt2 (PKBβ) and Akt3 (PKBγ). In human malignancies, Akt activity plays a major role in tumor cell survival. Accumulating evidence exists with respect to the regulatory role of Akt isoforms in repair of DSBs through NHEJ pathway. Akt1 stimulates DNA-PKcs kinase activity, which is a necessary step for progression of DSB repair through NHEJ. Potential involvement of Akt in HRR has been also reported however, existing data are conflicting and need to be investigated in more details.

The main aim of this thesis was to investigate the role of Akt isoforms in double strand breaks repair via homologous recombination repair. To achieve this, siRNA mediated knockdown as well as stable knock out approaches were applied to characterize the importance of Akt isoforms in the repair of DSBs executed by homologous recombination. Analyzing DSBs repair in human colorectal cancer cells after exposure to 4Gy irradiation indicated that HCT116 AKT1-KO and AKT2-KO cells present significantly enhanced levels of residual γ-H2AX foci in CENP-F positive cells (representing the S and G2 phase cells, i.e. cells which are competent for HRR). In comparison to wild type control cells inhibition of C-NHEJ and Alt-NHEJ

using DNA-PK and PARP inhibitors resulted in elevated non repaired residual DNA-DSB in HCT116 AKT1- and especially AKT2-knockout cells.

Moreover, Immunofluorescence analyses were performed to evaluate the regulation of Rad51 as a major protein of HRR by Akt. Analyses of Rad51 nuclear translocation and foci formation indicated that in the absence of Akt1 and Akt2 isoforms Rad51 nuclear accumulation is markedly reduced after radiation exposure. Furthermore, the results of colonogenic survival assays demonstrated that AKT1-KO and AKT2-KO cells in comparison to parental cells are significantly more sensitive to irradiation. It has been proven that deficiency of homologous recombination repair results in synthetic lethality after inhibition of poly-(adenosine diphosphate-ribose)-polymerase (PARP). In this project, PARP inhibitor Olaparib as a FDA approved drug for BRCA deficient tumors was used to evaluate the sensitivity of AKT depleted cells to inhibition of PARP. PARP inhibition in HCT116 cells by Olaparib showed that the clonogenic activity of cells is significantly reduced in AKT1-KO cells following irradiation. Interestingly, AKT2-KO were not able to form colonies after Olaparib treatment. These results indicate deficiency of HRR in AKT1-KO and AKT2-KO cells. The data obtained collectively suggest that both Akt isoforms Akt1 and Akt2 are important regulatory components in processes of DSB-repair via the homologous recombination mechanism.

6) Zusammenfassung

Nicht- oder falsch-reparierte Doppelstrangbrüche als Konsequenz einer Strahlen- bzw. Mutagenexposition können potentiell genotoxisch wirken und sich in Form von Chromosomenbrüchen und Chromosomentranslokationen im Genom manifestieren. Die wichtigsten Reparaturmechanismen für die Beseitigung von Doppelstrangbrüchen sind: die homologe Rekombination (HRR), das klassische nicht-homologe „end-joining“ (C-NHEJ) und das alternative nicht-homologe „end-joining“ (A-NHEJ). Das Funktionieren dieser drei Reparaturmechanismen ist essentiell für den Erhalt der genomischen Integrität der Zelle, spielt aber auch eine Rolle bei der Entstehung einer Strahlenresistenz in Tumorzellen.

Der Proteinkinase B (Akt/PKB) Signalweg spielt bei der Regulation von Zellüberleben und Apoptose nach Bestrahlung eine wichtige Rolle. So beobachtet man bei verschiedenen menschlichen Tumorentitäten eine durch Tyrosinkinase-Rezeptoren oder durch Mutationen getriebene hyperaktivierte PI3K/Akt-Signaltransduktion. Funktionell agiert Akt/PKB als Serin/Threonin-Kinase und tritt in den Isoformen Akt1 (PKBα), Akt2 (PKBβ) und Akt3 (PKBγ) auf. Die Akt Kinaseaktivität spielt besonders bei der Regulation des Zellüberlebens von Tumorzellen eine Rolle. Besonders wichtig scheint dabei der Effekt auf die Reparatur von Doppelstrangbrüchen durch das C-NHEJ zu sein. Dabei stimuliert die Akt1 die Kinaseaktivität der DNA-abhängigen Proteinkinase (DNA-PK) dem Schlüsselenzym des C-NHEJ. Aber auch für die HRR wird eine Beteiligung der Akt diskutiert. Aktuell widersprechen sich jedoch einige Daten und es besteht weiterer Forschungsbedarf in der Frage der Bedeutung der Akt.

So ist es das Ziel der vorliegenden Arbeit die Rolle der verschiedenen Akt-Isoformen bei der Doppelstrangbruchreparatur durch die HRR näher zu beleuchten. Dazu wurden, mit Hilfe von spezifischen siRNAs temporär bzw. mit spezifischen shRNAs stabil die Expressionen der Akt-Isoformen in der kolorektalen Tumorzellinie HCT116 blockiert. Es zeigte sich, dass in CENP-F Antigen positiven Zellen (d.h. in Zellen in der S- bzw. G2-Zellzyklusphase, die HRR-kompetent sind) nach Blockade von Akt1 oder Akt2 die Menge an nicht reparierten Doppelstrangbrüchen (γ-H2AX Foci, 24 h

nach einer Bestrahlung mit 4 Gy) signifikant erhöht waren. Die Blockade des C-NHEJ durch Einsatz von DNA-PK Inhibitoren, bzw. die Blockade des A-NHEJ durch poly-(adenosine diphosphate-ribose)-polymerase (PARP)-Inhibitoren zeigte für Zellen mit Akt1- und insbesondere aber für Zellen mit Akt2 Blockade im Vergleich zu den parentalen Kontrollzellen mit Akt-Wildtyp, eine deutliche Erhöhung der nichtreparierten Doppelstrangbrüche.

In Ergänzung dazu, wurden Immunfluoreszenzuntersuchungen durchgeführt, um den Einfluss von Akt auf die Regulation von RAD51, dem wichtigsten Protein bei der HRR, zu untersuchen. Untersuchungen zur Kerntranslokation von RAD51 und zur Foci-Bildung von RAD51 im Kern, zeigten, dass das Fehlen der Isoformen Akt1 und Akt2 mit einer deutlich geringeren nukleären Akkumulation von RAD51 nach einer Strahlenexposition assoziiert war. Diese Reduktion führte zu einer deutlichen Radiosensitivierung im Vergleich zu den Parentalzellen mit Wildtyp Akt.

Es ist bekannt, dass eine Blockade der HR zur sogenannten “synthetic lethality” in Gegenwart von PARP-Inhibitoren führt. Um die Sensitivität von Akt-defizienten Zellen auf eine PARP Inhibition zu testen, wurde der PARP-Inhibitor Olaparib eingesetzt, der von der FDA für die Behandlung von BRACA defizientem Brustkrebs zugelassen ist. In Akt1-defizienten Zellen führte die PARP-Inhibition zu einer signifikanten Radiosensitivierung, in Akt2-defizienten Zellen führte die Behandlung mit Olaparib sogar zum kompletten Verlust der Klonbildungsfähigkeit. Diese Ergebnisse zeigen, dass es in Akt1- und Akt2-defizienten Zellen zu einer starken Beeinträchtigung der HRR kommt, die die Zellen besonders sensitiv für eine Blockade der PARP und damit für eine Blockade des A-NHEJ macht.

Zusammenfassend lässt sich das Resümee ziehen, dass die beiden Isoformen Akt1 und Akt2 wichtige regulatorische Funktionen haben beim Vorgang der HRR, ohne die es bei der Reparatur von Doppelstrangbrüchen in der DNA zu einer massiven Beeinträchtigung kommt.

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8) Publications

Parts of this thesis have been published in form of the following scientific article:

Tahereh Mohammadian Gol,H. Peter Rodemann, Klaus Dittmann. Depletion of Akt1 and Akt2 Impairs the Repair of Radiation-Induced DNA Double Strand Breaks via Homologous Recombination. Int. J. Mol. Sci. 2019, 20(24), 6316.

Declaration of Contributions to the Dissertation

The dissertation work was carried out at the Division of Radiobiology and Molecular Environmental Research, Department of Radiation Oncology, University of Tübingen, under the supervision of Prof. Dr. H. Peter Rodemann and Prof. Dr. Klaus Dittmann.

The study was designed in collaboration with Prof. Dr. H. Peter Rodemann. I carried out all experiments independently. Statistical analysis was carried out by myself after a consultation with Prof. Dr. Klaus Dittmann.

I confirm that I wrote the dissertation myself (under the supervision of Prof. Dr. H. Peter Rodemann and Prof. Dr. Klaus Dittmann) and that any additional sources of information have been duly cited.

I confirm that this thesis presented for the degree of Doctor of Philosophy (Ph.D.), has not been submitted for any other degree or professional qualification. Parts of this work have been published in International Journal of Molecular Science as a research article.

Signed_____

on 26.02.2020 in Tübingen

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