Analysis of molecular components, causing Fanconi Anemia-like phenotype in HPV-16 infected Head and Neck Squamous Cell Carcinomas

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Dedicated to my parents Hermann and Anita Leick
for your support always
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<tr>
<td>5-FU</td>
<td>5-fluorouracil</td>
</tr>
<tr>
<td>53BP1</td>
<td>p53 binding protein 1</td>
</tr>
<tr>
<td>Ad5</td>
<td>Adenovirus 5</td>
</tr>
<tr>
<td>ADP</td>
<td>Adenosine Diphosphate</td>
</tr>
<tr>
<td>APS</td>
<td>Ammonium persulfate</td>
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<tr>
<td>ATM</td>
<td>Ataxia Telangiectasia Mutated</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine Triphosphate</td>
</tr>
<tr>
<td>ATR</td>
<td>ATM-Related</td>
</tr>
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<td>ATRIP</td>
<td>ATR-Interacting Protein</td>
</tr>
<tr>
<td>BACH1</td>
<td>BTB and CNC Homology 1</td>
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<tr>
<td>BCA</td>
<td>Bicinchoninic acid</td>
</tr>
<tr>
<td>BER</td>
<td>Base Excision Repair</td>
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<tr>
<td>BLM</td>
<td>Bloom syndrome protein</td>
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<tr>
<td>BRCA</td>
<td>Breast Cancer susceptibility protein</td>
</tr>
<tr>
<td>BRIP1</td>
<td>BRCA1 interacting protein C-terminal helicase 1</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine Serum Albumin</td>
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<tr>
<td>Cdc</td>
<td>cell division cycle</td>
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<tr>
<td>CDK</td>
<td>Cyclin-Dependent Kinase</td>
</tr>
<tr>
<td>CDKN2A</td>
<td>Cyclin-Dependent Kinase Inhibitor 2A</td>
</tr>
<tr>
<td>Chk</td>
<td>checkpoint kinase</td>
</tr>
<tr>
<td>CO</td>
<td>Hydrocortison (in Media)</td>
</tr>
<tr>
<td>CT</td>
<td>Computer tomography</td>
</tr>
<tr>
<td>CtIP</td>
<td>C-terminal-binding protein interacting protein</td>
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<tr>
<td>DAPI</td>
<td>4’,6-Diamidino-2-phenyindole dihydrochloride</td>
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<td>DDR</td>
<td>DNA Damage Response</td>
</tr>
<tr>
<td>DEB</td>
<td>Diepoxbutane</td>
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<tr>
<td>dH₂O</td>
<td>distilled H₂O</td>
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<tr>
<td>dHJ</td>
<td>double Holliday-Junctions</td>
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<td>D-loop</td>
<td>Displacement strand</td>
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<td>DMEM</td>
<td>Dubecco’s Modified Eagles Medium</td>
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<td>DMSO</td>
<td>Dimethylsulfoxide</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic Acid</td>
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<td>Description</td>
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<tr>
<td>DNA-PKcs</td>
<td>DNA-dependent Protein Kinase, catalytic subunit</td>
</tr>
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<td>DSBs</td>
<td>Double-Strand Breaks</td>
</tr>
<tr>
<td>E</td>
<td>Early region gene</td>
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<td>E6AP</td>
<td>E6 associated protein</td>
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<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
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<tr>
<td>EGFP</td>
<td>Enhanced Green Fluorescent Protein</td>
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<tr>
<td>EGFR</td>
<td>Epidermal Growth Factor Receptor</td>
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<td>ERCC4 (XPF)</td>
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<td>FA</td>
<td>Fanconi Anemia</td>
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<td>FAAP</td>
<td>Fanconi Anemia Associated Protein</td>
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<tr>
<td>FACS</td>
<td>Fluorescence-Activated Cell Sorting</td>
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<tr>
<td>FANC</td>
<td>Fanconi Anemia Complementation group</td>
</tr>
<tr>
<td>FBS</td>
<td>Fetal Bovine Serum</td>
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<tr>
<td>FNAC</td>
<td>Fine needle aspiration cytology</td>
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<td>FOXM1</td>
<td>Forkhead box M1</td>
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<tr>
<td>FSC</td>
<td>Forward scatter channel</td>
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<tr>
<td>h</td>
<td>hour</td>
</tr>
<tr>
<td>HCMV</td>
<td>Human Cytomegalovirus</td>
</tr>
<tr>
<td>HKC</td>
<td>Human keratinocytes</td>
</tr>
<tr>
<td>HMEC</td>
<td>Human mammary epithelial cells</td>
</tr>
<tr>
<td>HN</td>
<td>Head and neck cancers</td>
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<td>HPV</td>
<td>Human Papilloma Virus</td>
</tr>
<tr>
<td>HR</td>
<td>Homologous Recombination</td>
</tr>
<tr>
<td>ICLs</td>
<td>Interstrand Crosslinker</td>
</tr>
<tr>
<td>ID2</td>
<td>FANCI-FANCD2 complex</td>
</tr>
<tr>
<td>IFAR</td>
<td>International Fanconi Anemia Registry</td>
</tr>
<tr>
<td>INK4a</td>
<td>Cyclin-dependent Kinase Inhibitor p16</td>
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<tr>
<td>IQD</td>
<td>1,5-Isoquinolinediol</td>
</tr>
<tr>
<td>IR</td>
<td>Ionizing Radiation</td>
</tr>
<tr>
<td>kD</td>
<td>kiloDalton</td>
</tr>
<tr>
<td>L1</td>
<td>Late region major capsid protein</td>
</tr>
<tr>
<td>L2</td>
<td>Late region minor capsid protein</td>
</tr>
<tr>
<td>LCL</td>
<td>Lymphoblast cell lines</td>
</tr>
<tr>
<td>Acronym</td>
<td>Description</td>
</tr>
<tr>
<td>-------------</td>
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</tr>
<tr>
<td>LCR</td>
<td>Long control region</td>
</tr>
<tr>
<td>min</td>
<td>minute</td>
</tr>
<tr>
<td>MMC</td>
<td>Mitomycin C</td>
</tr>
<tr>
<td>MMEJ</td>
<td>Microhomology-Mediated End Joining</td>
</tr>
<tr>
<td>MMR</td>
<td>Mismatch Repair</td>
</tr>
<tr>
<td>MRE11</td>
<td>Meiotic recombination 11 homolog</td>
</tr>
<tr>
<td>MRN</td>
<td>Mre11-Rad50-NBS1-Complex</td>
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<td>MTT</td>
<td>3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium bromide</td>
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<tr>
<td>NBS1</td>
<td>Nijmegen Breakage syndrome 1</td>
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<tr>
<td>NER</td>
<td>Nucleotide Excision Repair</td>
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<td>NHEJ</td>
<td>Non-Homologous End Joining</td>
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<tr>
<td>cNHEJ</td>
<td>Canonical Non-Homologous End Joining</td>
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<tr>
<td>OPSCC</td>
<td>Oropharyngeal squamous cell carcinoma</td>
</tr>
<tr>
<td>ORF</td>
<td>Open reading frame</td>
</tr>
<tr>
<td>PALB2</td>
<td>Partner And Localizer of BRCA2</td>
</tr>
<tr>
<td>PAR</td>
<td>Poly(ADP-ribose)</td>
</tr>
<tr>
<td>PARP1</td>
<td>Poly(ADP-ribose) Polymerase 1</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate-Buffered Saline</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>PEES</td>
<td>Penicillin/Steptomycin</td>
</tr>
<tr>
<td>PI</td>
<td>Propidium Iodide</td>
</tr>
<tr>
<td>PTGES</td>
<td>Prostaglandin E Synthase</td>
</tr>
<tr>
<td>Rb/pRb</td>
<td>Retinoblastoma protein/phosphorylated Retinoblastoma protein</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive Oxygen Species</td>
</tr>
<tr>
<td>RPA</td>
<td>Replication Protein A</td>
</tr>
<tr>
<td>rpm</td>
<td>rounds per minute</td>
</tr>
<tr>
<td>SCC</td>
<td>Squamous cell carcinoma</td>
</tr>
<tr>
<td>SDSA</td>
<td>Synthesis dependent strand annealing</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis</td>
</tr>
<tr>
<td>SEM</td>
<td>Standard Error of Mean</td>
</tr>
<tr>
<td>shRNA</td>
<td>small hairpin RNA</td>
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<tr>
<td>SSA</td>
<td>Single-Strand Annealing</td>
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<tr>
<td>SSB</td>
<td>Single-Strand Break</td>
</tr>
<tr>
<td>SSC</td>
<td>Side scatter channel</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<td>--------------</td>
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</tr>
<tr>
<td>ssDNA</td>
<td>single-strand DNA</td>
</tr>
<tr>
<td>SV40</td>
<td>Simian Virus 40</td>
</tr>
<tr>
<td>TEMED</td>
<td>N,N,N’,N’-tetra-methyl-ethylene-diamine</td>
</tr>
<tr>
<td>TLS</td>
<td>Translesion bypass repair</td>
</tr>
<tr>
<td>TopBP1</td>
<td>DNA Topoisomerase 2-Binding Protein 1</td>
</tr>
<tr>
<td>TOP2A</td>
<td>Topoisomerase-II alpha</td>
</tr>
<tr>
<td>Ub</td>
<td>Ubiquitin</td>
</tr>
<tr>
<td>UMSCC</td>
<td>University of Michigan Squamous Cell Carcinoma</td>
</tr>
<tr>
<td>UV</td>
<td>Ultraviolet Light</td>
</tr>
<tr>
<td>XRCC4</td>
<td>X-ray cross-complementing protein 4</td>
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1 INTRODUCTION

1.1 DNA Damage

DNA encodes the genetic information necessary for survival and functioning of all living organisms and several viruses. Cells are continuously exposed to endogenous and exogenous stresses that impair DNA replication, transcription and recombination so they must be managed to preserve genomic integrity. Endogenous damage sources include reactive oxygen species (ROS) such as superoxide anions or hydroxyl radicals arising from cellular metabolism and resulting in lipid peroxidation, protein damage and single (SSB) as well as DNA double-strand breaks (DSB). Endogenous damage also arises during DNA replication where DNA-base alternations such as depurination or methylation lead to DNA instability (De Bont and van Larebeke 2004). Exogenous stresses arise from physical and chemical sources. Physical sources include ultraviolet (UV) light from the sun or ionizing radiation (IR) from medical diagnostic use of X-rays or radiotherapy for cancer treatment. UV light causes DNA lesions such as formation of pyrimidine dimers and IR causes lesions through base oxidation with subsequent formation of single-strand (SSB) and DSB (Hoeijmakers 2009). Chemical sources not only arise from environmental exposure but are also the basis of chemotherapy and include alkylating agents that add alkyl groups to DNA bases and crosslinking agents such as mitomycin C (MMC) that lead to covalent bond formation between bases of the same DNA strand or the opposite DNA strand forming intrastrand and more critical interstrand crosslinks (ICLs) (Ciccia and Elledge 2010). These ICLs are highly toxic lesions because they prevent DNA strand separation necessary for transcription and replication, leading to replication fork stalling and ultimately DSBs (Deans and West 2011). These DSBs are considered the most cytotoxic of DNA lesions as there is no complementary strand for repair and left unrepaired this damage can lead to chromosome breaks and translocations that predispose to cancer (Jackson and Bartek 2009).

1.2 DNA Repair

Failure to repair DSB leads to genome mutations that can be passed on, chromosome aberrations and cell death (Kasparek and Humphrey 2011). In order to prevent and regulate these outcomes, cells have developed complex mechanisms by which to detect different types of DNA damage and either initiate appropriate repair pathways or in case of
severe damage initiate apoptosis (FIGURE 1). This process of DNA damage detection by sensors, signal transduction by transducers, transmission to effectors to initiate cellular response and subsequent feedback to the sensors is known as DNA damage response (DDR) (Elledge 1996). DDR is initiated by sensing stalled replication forks or other DNA damage that then leads to cell cycle checkpoint activation which halts the cell cycle and gives the cell time to resolve and manage the situation. Further DDR achieves this by activation of transcriptional programs, initiation of apoptosis or, if possible, the implication of various DNA pathways to repair the damage and restore cellular function (Zhou and Elledge 2000).

**Figure 1 DNA damage response.** Exogenous or endogenous stresses generate various types of DNA damage that are dealt with by DNA damage responses that trigger cell cycle checkpoint activation, activate transcription, induce apoptosis or initiate one of several DNA repair pathways. The choice of DNA repair pathway is dependent on lesion type and cell cycle stages.

Lesions arising from modified DNA bases through small chemical changes are repaired by base excision repair (BER) and more complex base modifications such as pyrimidine dimers are repaired by nucleotide excision repair (NER) while bases paired with incorrect partners are dealt with by mismatch repair (MMR). These three repair mechanisms involve incision of a single-strand, removal of nucleotides and eventual single-strand ligation (Yang 2008). Another DNA repair mechanism essential to the maintenance of genomic stability and prevention of cancer is the Fanconi Anemia (FA) pathway, an essential tumor suppressive pathway responsible specifically for repair of ICLs (Kee and
D’Andrea 2010). This pathway will be discussed further in Section 1.5.2. In the case of DSB, the main pathways of repair are homologous recombination (HR) and non-homologous end joining (NHEJ) as well as microhomology-mediated end joining (MMEJ) and single-strand annealing (SSA).

Persistent DNA damage and faulty repair are the underlying causes of malignancy associated with Fanconi Anemia, a type of chromosome instability syndrome. Human papillomavirus infection (HPV) may resemble features of Fanconi Anemia associated DNA repair and DDR defects.

1.3 DSB Pathways and Repair Choice

Several pathways compete for the repair of DSB, but ultimately the DSB repair choice depends on various factors including cell cycle phases, expression and phosphorylation of repair proteins and availability of homologous repair templates (Shrivastav et al. 2008). External influences including certain diseases or viral infection may alter these factors and thereby influence repair choice.

1.3.1 Homologous Recombination

Homologous recombination (HR) requires the presence of homologous DNA sequences that serve as a template for repair at the damage site. These sequences are found, for example, on sister chromatids where the DNA sequence is perfectly homologous and therefore assures that HR repair takes place in an error-free manner (Wyman and Kanaar 2006). The availability of sister chromatids is restricted to and hence takes place in the S and G2 phases of the cell cycle (Saleh-Gohari and Helleday 2004). HR initiates with 5’ to 3’ DNA end processing of the broken DNA ends which requires the MRE11-RAD50-NBS1-complex (MRN). The three proteins comprising this complex are meiotic recombination 11 homolog (MRE11), RAD50 and Nijmegen breakage syndrome 1 (NBS1). The MRN recruitment to the DNA ends is crucial to ataxia telangiectasia mutated (ATM) activation that in turn binds to the DNA and initiates further DNA damage responses also activating HR (Paull and Lee 2005). Another protein, C-terminal-binding protein interacting protein (CtIP) cooperates with MRN to promote HR by DNA end resection. After end resection replication protein A (RPA) is recruited to the single-stranded DNA (ssDNA) ends and phosphorylated which activates ataxia telangiectasia and
Rad3-related protein (ATR) particularly at stalled replication forks (Wang et al. 2012). The resulting RPA-ssDNA entity serves not only to recruit the ATR checkpoint kinase, but also the associated ATR-Interacting Protein (ATRIP) (Zou and Elledge 2003; Namiki and Zou 2006). Together ATM and ATR activate further effectors of repair. RPA covers the ssDNA 3’ overhangs generated by DNA end processing to protect them from degradation (Zou et al. 2006). Other proteins including BRCA1 and BRCA2 then help recruit RAD51 that assembles into a nucleofilament and replaces RPA. This nucleofilament then invades the sister chromatid in its search for homology (Sung and Robberson 1995, Davies et al. 2001, West 2003). The invasion of the donor double-strand DNA, results in the formation of a displaced strand (D-loop). Repair continues with the extension of the 3’ end through DNA polymerases which use the invaded donor strand as a template. Finally, the formed D-loop is resolved either by synthesis-dependent strand annealing (SDSA) or homologous DSB repair. In SDSA the invaded strand flips back and re-anneals to the complementary DNA sequence of the original molecule. In DSB repair, second end capture of the other DSB end takes place in which the second 5’ overhang of the DSB is captured by the D-loop. This results in the formation of a double holiday junction (dHJ). Resolvases including GEN1 and SLX1/SLX4 finally resolve the dHJs in two possible ways, resulting in either crossover or non-crossover outcomes (Boddy et al. 2001; Li and Heyer 2008; San Filippo, Sung, and Klein 2008; Fekairi et al. 2009; Chapman, Taylor, and Boulton 2012). Cells defective in HR repair, through missing proteins of this machinery for example, are very susceptible to cancer, given that they cannot make use of this error-free repair.

1.3.2 Non-Homologous End Joining (NHEJ)

Non-homologous end joining (NHEJ) is active throughout the cell cycle as there is no need for template DNA. However, this pathway is more error-prone than HR due to occurrence of mutagenic deletions or insertions (Lieber and Wilson 2010). NHEJ is subdivided into canonical (c-NHEJ) and alternative NHEJ of which microhomology-mediated end joining (MMEJ) is a major mechanism. In this investigation NHEJ refers to all NHEJ mechanisms, predominantly c-NHEJ, which mediates ligation of broken DNA ends with only minimal end processing (Polo and Jackson 2011). Initially the heterodimer KU70/KU80 binds the DNA ends and recruits DNA-dependent protein kinase catalytic subunit (DNA-PKcs) to form a DNA-PKcs holoenzyme that stabilizes the DNA ends (Gottlieb and Jackson 1993). Factors including the nuclease Artemis then also bind to the
complex, leading to auto-phosphorylation of the DNA-PKcs that in turn stabilizes DNA and regulates end accessibility (Uematsu et al. 2007). Next, ligation occurs either directly or indirectly with extra processing steps. Direct ligation without end processing occurs via DNA ligase IV, whose activity is stimulated by complex formation with the X-ray cross-complementing protein 4 (XRCC4) that, at the same time, stabilizes DNA ligase IV (Grawunder et al. 1997). Indirect ligation occurs when removal of damaged ssDNA by Artemis takes place and DNA polymerases such as POLµ and POLλ bind to the complex and catalyze the synthesis of nucleotides to prepare the ends for ligation by Ligase4/XRCC4 (Ma et al. 2002, Mahajan et al. 2002). During NHEJ further processing units including MRN and CtIP are activated as well as the DDR protein 53 binding protein 1 (53BP1). 53BP1 plays a critical role in NHEJ repair in that it blocks DNA ends from excessive resection and thereby promotes NHEJ repair and impedes HR repair (Callen et al. 2013). The repair by NHEJ through ligation, either directly or indirectly, without a DNA template, as in HR, is potentially mutagenic or hence described as error-prone since non-compatible ends may be combined and genetic information lost.

1.3.3 Microhomology-mediated End Joining (MMEJ)

Microhomolgy-mediated end joining (MMEJ) is considered an alternative NHEJ form as it is KU70/80 and DNA ligase IV independent. The pathway was reported to be Poly(ADP-ribose) Polymerase 1 (PARP1) dependent, which is recruited in the absence of c-NHEJ factors like KU, and binds to the DNA ends to then recruit the MRN complex for end-processing (Wang et al. 2006). It repairs DSB using microhomologies consisting of 1 to 16 nucleotides distal to the DNA ends for repair that are exposed by nucleolytic processing of the 5’ proximated ends. These microhomologies are then annealed, strand overhangs removed nucleolytically and flanking single-stranded regions subjected to fill-in synthesis and finally ligation, resulting in loss of information adjacent to the annealed DSB site (Sfeir and Symington 2015).

1.3.4 Single-Strand Annealing (SSA)

Single-strand annealing (SSA) occurs when the DSB occurs between two repeated sequences. The exposed DNA ends are resected by MRE11 of the MRN complex leaving ssDNA overhangs (Valerie and Povirk 2003). RPA covers the ssDNA and RAD52 binds
to the resected DNA ends as a heptameric ring structure, facilitating annealing of the complementary DNA (Van Dyck et al. 2001). The protruding ends are removed by the nuclease excision repair cross-complementation group 1/excision repair cross-complementation group 4 (ERCC1/ERCC4 or XPF) (Adair 2000). DNA polymerases fill the gap and finally ligation occurs, again resulting in loss of information at the initial DSB site.

1.3.5 Repair Pathway Choice

The two major DSB repair pathways extensively studied are HR and c-NHEJ and they are also the two pathways mainly used by mammalian cells for repair (Chapman et al. 2012). The SSA and MMEJ pathways are usually the last option for repair given that they almost always result in DNA deletions. Pathway choice can be determined by DNA end processing. HR requires DSB end-processing as do the repair mechanisms MMEJ and SSA (Bennardo et al. 2008). The c-NHEJ pathway does not necessarily require end-processing but can comprise an end-processing step. The KU70/KU80 heterodimer blocks and holds dsDNA ends in close proximity. It promotes direct and error-free DSB ligation without end-processing but also following limited processing in an error-prone manner as small deletions, substitutions and even translocations can occur if DSBs are joined from other parts of the genome (Guirouilh-Barbat et al. 2007, Lieber 2010). This may not appear to be advantageous to the cell due to the error potential, but the ability of c-NHEJ to be active throughout the cell cycle and favorably in G1 poses an advantage to HR repair that is mostly prevalent after DNA replication (Guirouilh-Barbat et al. 2004, Kasperek and Humphrey 2011, Chapman et al. 2012). As a result the cell cycle phase determines the preferred pathway choice such that HR is the method of choice during S and G2 phase and c-NHEJ the preferred choice during G1. HR is considered error-free with RAD51 recombinase assembling as a nucleofilament and invading homologous duplex DNA to promote template oriented and thus homology-controlled DNA repair (San Filippo et al. 2008). This classification of error-free and error-prone should be used as an orientation given that HR may still produce errors through resolution into crossover products and c-NHEJ may operate error free by rejoining broken DSB ends without loss of information. Since availability and advantage of each type of repair are variable, cellular regulation of pathway choice is an important survival and quality-control mechanism. Thus, factors affecting DSB repair pathway choice are dependent on the presence and expression of
repair factors and proteins such as KU70/80, RAD51 and 53BP1. An example of this is the rapid binding of the abundant KU70/80 heterodimer to DNA ends which directs repair via c-NHEJ by its occupation of the DNA ends and activation of DNA-PKcs (Shibata et al. 2011). HR is inhibited by this as it requires end resection. A finding by Shirvastav et al. showed that DNA-PKcs null cells demonstrated elevated HR as a result of elimination of c-NHEJ competition (Shrivastav et al. 2008). Also the presence of key HR protein RAD51 plays a role in DSB repair pathway choice as RAD51 independent pathways NHEJ and SSA, are still active in the absence of RAD51 (Krejci et al. 2012). Repair protein 53BP1 displays inhibitory activity towards DSB resection necessary for HR and instead promotes NHEJ (Xie et al. 2007). It accomplishes this by inhibiting C-terminal binding, protein-interacting protein (CtIP)-mediated DNA end resection, leading to inhibition of DSB resection by HR (Bunting et al. 2010). These proteins and other DNA end processing factors contribute to pathway choice for DSBs normally regulated internally through cellular processes. Disruption especially of error-free DSB repair pathways from external sources such as viral infection, for example, can leave improperly repaired DNA, leading to genomic instability or genome rearrangements that promote malignant progression (Bennardo et al. 2009).

1.4 Head and Neck Cancer and HPV

1.4.1 Overview of Head and Neck Cancer

Head and neck cancers (HN) are histologically mostly squamous cell carcinomas (SCCs) arising from the mucosa lining the oral cavity, nasopharynx, oropharynx, hypopharynx, larynx and esophagus (Figure 2). HN cancers account for approximately 3-5% of malignant tumors worldwide, with higher prevalence in regions of Southeast Asia and Brazil (Ferlay et al. 2010, Georgopoulos et al. 2015). In developed regions the rate is lower, with HN cancers contributing approximately 4% of total malignancies in the United States and Europe (Siegel et al. 2012). Interestingly, a rise in cancer has been seen in a subset of HNSCCs, the oropharyngeal squamous cell carcinoma (OPSCC), in which a particular rise in incidence was observed for base of tongue and tonsillar cancer in the United States among young individuals between 40 and 55 years of age (Marur et al. 2010). The best established risk factors for HNSCCs has been the use of alcohol and tobacco (Argiris et al. 2008). However, in recent years HNSCCs attributed to oncogenic
forms of HPV have been on the rise, evident in an increase in OPSCCs where patient history is negative for alcohol and tobacco use but associated with persistent HPV infection (Sturgis and Cinciripini 2007). This persistent infection was shown in 95 percent of cases to be attributed to the high risk HPV-16 subtype (Kreimer et al. 2005). The role of HPV in cervical cancer has already been established decades ago and has since been extensively studied (Burd 2003).

![Anatomic sites of Head and Neck Carcinomas](image-url)

**Figure 2 Anatomic sites of Head and Neck Carcinomas.** The majority of carcinomas in this region are squamous cell carcinomas (HNSCCs) arising from the oral cavity, larynx, esophagus and pharynx including the nasopharynx, oropharynx, and hypopharynx (Zvonko 2012). Copyright license CCBY3.0 https://creativecommons.org/licenses/by/3.0/

It is now also recognized that persistent HPV-16 infection is a significant risk factor in the pathogenesis of HNSCCs, particularly OPSCCs (D’Souza et al. 2007, Gillison et al. 2008). Though HPV-positive HNSCCs may be on the rise compared to HPV-negative HNSCCs, attributed more to classical risk factors of tobacco and alcohol use, it is the more favorable clinical prognosis due to increased sensitivity to chemotherapy and radiation that make this
particular group of cancers an urgent point of study as the underlying mechanism of increased sensitivity remains poorly understood (Vidal and Gillison 2008, O’Rorke et al. 2012). More insight into these mechanisms may provide the basis for new multimodal treatments that improve quality of life and patient survival, or even lead to future prevention and screening strategies, a need that among the current often quite radical treatment options will certainly improve clinical treatment of patients affected by HPV positive HNSCCs.

1.4.2 HPV positive HNSCC: Clinic, Diagnosis and Current Therapy

Symptoms classical of HNSCC include pain, dysphagia, dysphonia, otalgia and persistent sore throat, but often the primary tumor site causes no symptoms at all leading to a late post-metastatic diagnosis (Price and Cohen 2012). Persistent cervical nodal hypertrophy is often the first and only clinical sign of HPV associated HNSCC with patients rarely reporting any throat or mouth problems. Cystic cervical lymph node metastasis, in particular, has been associated with HPV-related SCC in a study by Goldenberg et al. (Goldenberg et al. 2008). Focus on HPV-related HNSCC diagnosis is based on the presentation of cystic cervical nodal metastasis as well as demographic change from an older (45-75 years) HNSCC patient population with history of tobacco and alcohol use to a younger (35-55 years) group with attention placed on the more sensitive history of orogenital practices and number of partners (Koch 2012).

Diagnostic work-up of patient history and physical examination with special focus on occurrence of lateral cystic neck masses should be followed with diagnostic imaging including ultrasound and computer tomography (CT) as well as fine needle aspiration cytology (FNAC), checking the aspirate also for HPV DNA using either HPV-quantitative polymerase chain reaction (PCR) or in-situ hybridization. Ultimate diagnosis of HPV positive HNSCC is made through biopsies checking for pathological anatomical diagnosis and presence of HPV DNA (Sivars et al. 2014).

Treatment for HPV positive HNSCCs is, at this point, still similar to non-HPV positive HNSCCs where treatment in early stage is of single modality and involves either surgery or radiotherapy. Locally advanced cancer, however, is a multimodal treatment approach with surgery often involving neck dissection followed by adjuvant full-dose radiotherapy
to large areas of the head and neck (Sivars et al. 2016). More advanced cases are treated in an intensified manner often requiring 5-fluorouracil (5-FU) or cisplatin-based chemotherapy, which has been a cornerstone of recurrent and metastatic HNSCCs since the 1980s (Kish et al. 1982). Recurrent and metastatic disease is currently treated with third-generation chemotherapeutic agents such as taxol-based drugs and irinotecan that may also be given together with targeted biological therapy involving inhibitors of the epidermal growth factor receptor (EGFR), a receptor that is often over expressed in HNSCCs (Ang et al. 2002, Price and Cohen 2012). Cetuximab, a monoclonal antibody to EGFR, is an example of targeted clinical biological therapy currently in clinical use but tumor resistance is not uncommon (Bonner et al. 2006, Wise-Draper et al. 2012). This intense treatment regimen increases the adverse side effects not limited to mucositis, severe pain and dysphagia or surgical side effects including nerve damage, edema or functional shoulder and arm deficits. Molecular testing to date has not influenced treatment selection, a fact that, in light of these adverse side effects, especially for more treatment-sensitive, HPV-positive HNSCCs, places emphasis on the need for treatment de-escalation (Mirghani et al. 2015, Marur and Forastiere 2016).

1.4.3 UMSCC

In this investigation, University of Michigan squamous cell carcinoma (UMSCCs) cells from the oral cavity were used with cells originating either from the floor of mouth or lateral tongue. By definition they are an OPSCC, known to be particularly associated with HPV-positive HNSCCs and were, therefore, of interest to this investigation for analysis of the effects of HPV on the DNA repair mechanisms. The recognition of a Fanconi anemia-like behavior in the DNA repair mechanisms of these UMSCCs was a finding in which closer analysis of these molecular components could provide some insight into the underlying cause of increased sensitivity of HPV-positive HNSCCS to chemotherapy and radiation treatment. Such small advances in the molecular understanding of HPV’s influence on DNA repair mechanisms might provide opportunities to further targeted molecular therapies that can be the future of HPV-positive HNSCC treatment de-escalation.
1.4.3.1 Human Papillomavirus

The human papillomavirus (HPV) is a small, non-enveloped, double-stranded DNA virus that is capable of infecting mucosal epithelium and cutaneous epithelium. The virus belongs to one of the most common sexually transmitted diseases, but most cases of infection remain unnoticed due to the fact that infection is transient and often symptomless. Most individuals with sufficient immune response are able to clear the infection within 1 to 2 years but persistent infection with high-risk types is linked to malignant progression (Schiffman and Castle 2003). Approximately 150 types of HPV have been discovered and are classified based on oncogenic potential as either low-risk or high-risk types. Low-risk types include HPV-6 and -11 that are associated with benign low-grade cervical abnormalities, laryngeal papillomas or genital warts but seldom with cancer (Valentino and Poronsky 2015). High-risk subtypes include 16, 18, 31, 33, 35, 39, 45, 51, 52, 59, 68, 69, 73 and 82. Different than low-risk, high-risk HPV subtypes predominantly HPV-16 and -18, have been responsible for high-grade cervical abnormalities and development of several genital and oropharyngeal cancers (Markowitz et al. 2014). In particular HPV-16 infection has been the subtype associated with 60% of oropharyngeal cancers (Tommasino 2014).

1.4.3.2 HPV Life Cycle and Genome

The HPV viral life cycle begins with infection of undifferentiated cells at the basal epithelial layers, usually through small abrasions. This layer is essential to the viral ability to replicate as cell differentiation is necessary for virion production. The virus may also persist in a latent state in the basal layer and evade clearance, a characteristic often associated in particular with the high-risk HPV subtypes (Fernandes 2013). The HPV-16 virus consists of 7904 base pairs with early (E) region genes encoding 6 proteins, 2 late regions encoding major (L1) and minor (L2) capsid protein and a non-coding, long control region (LCR). The early genes’ regions encode non-structural proteins E1, E2, E4 and E5, all important proteins for viral replication, in addition to the oncoproteins E6 and E7. These two proteins are known for their ability to alter the cell cycle and promote persistence of an episomal HPV genome (Münger et al. 2004). The E6 and E7 proteins are especially overexpressed as the viral genome integrates in the host DNA since the open reading frame (ORF) of the gene encoding E2 protein is disrupted at this time, preventing
E2 from repressing E6 and E7. The resulting increased expression of E6 and E7 is associated with cellular proliferation and malignant progression (Hou et al. 2002).

1.4.3.3 E6 and E7 Role in Carcinogenesis

The role in cancer development attributed to E6 and E7 is the ability to inactivate two tumor suppressor proteins namely p53 by E6 and retinoblastoma protein (Rb) by E7. This inactivation leads to modification of the cell cycle in such a way that differentiating host cells are maintained in a state of favorable viral genome replication (Bodily and Laimins 2011). The tumor suppressor p53 is responsible for cell cycle arrest or apoptosis in response to DNA damage. E6 prevents this by binding p53 together with the E6-associated protein (E6AP), resulting in ubiquitination (Ub) and subsequent degradation of p53 in favor of cell cycle progression (Figure 3).

Figure 3 HPV E6 Oncoprotein. DNA damage normally leads to p53 activation and subsequent cell cycle arrest or apoptosis. The HPV E6 binds together with the E6-associated protein (E6AP) to p53, which results in E6-AP-mediated ubiquitination (Ub) and degradation of p53 thus allowing cell cycle progression.

Retinoblastoma protein (Rb) is activated by phosphorylation through cyclin D and E in complex with one of the cyclin-dependent kinases (CDKs) 4, 6 and 2. This results in the pRb hyperphosphorylated state, an oncoprotein that then liberates cellular transcription factor E2F that allows entry into S phase of the cell cycle. Dephosphorylation results in renewed binding of E2F and inhibition of cell cycle progression and cellular growth. E7 interferes with this regulated process (Figure 4) by binding Rb, freeing E2F and pushing the cell cycle forward (Duensing and Münger 2002). The liberation of E7 mediated E2F
results also in transcription of the cyclin-dependent kinase inhibitor 2A (CDKN2A) gene locus (Khleif et al. 1996). This gene encodes also cyclin-dependent kinase inhibitor p16 (INK4a), which is known to be highly overexpressed in HPV (+) cancers such that it is clinically used in cancer diagnosis as the surrogate marker for HPV positivity (Adams et al. 2014). These effects of E6 and E7 are examples of a few targets among many others yet to be more extensively studied. HPV is known to not only upregulate the CDKN2A gene but has also been found to upregulate topoisomerase II alpha (TOP2A), forkhead box M1 (FOXM1), prostaglandin E synthase (PTGES) and several other genes involved in cell cycle regulation (Santin et al. 2005).

Figure 4 HPV E7 Oncoprotein. Cell cycle progression normally occurs through initial phosphorylation of retinoblastoma protein (Rb) by CyclinD/CDK4,6 followed by additional phosphorylation by the CyclinE/CDK2 complex. This results in an Rb hyperphosphorylated (pRb) state that liberates cellular transcription factor E2F, and leads to S phase entry in the cell cycle. The pRb dephosphorylation events lead to renewed binding and inactivation of E2F and thus growth suppression in the Rb hypophosphorylated state. The HPV E7 binds to the hypophosphorylated form of the Rb proteins. This binding disrupts the complex between Rb and E2F, resulting in the Rb phosphorylation independent liberation and activation of E2F. This allows virally controlled entry into S phase leading to disruption of cell cycle arrest and apoptosis. White circles represent phosphorylation.
1.4.3.4 Cellular Repair Influence

The influence of HPV on HNSCC cancer development and treatment remains interesting in that improved tumor control and survival has been observed in response to chemoradiation therapy as demonstrated in a recent phase III clinical trial (Rischin et al. 2010). This is in line with previous studies suggesting that HPV (+) HNSCCs are more sensitive to radiation treatment (Gillison 2000). Still there is limited experimental data. Kimple et al. also could show that certain HPV (+) HNSCCs were more sensitive to chemoradiation but emphasized that the underlying mechanism remains unclear, discussing however that E6 and E7 are involved in DDR modulation for viral benefit (Kimple et al. 2013). Another group studying HPV-16/p16\textsuperscript{INK4a} -positive HNSCCs also reported increased radiosensitivity, not resulting from enhanced apoptosis or permanent G1 arrest, but reporting instead extensive G2 arrest and high residual DSB and postulating that a compromised DSB repair capacity may be the key component to this increased radiosensitivity (Rieckmann et al. 2013). As a more detailed characterization of DSB repair defects is necessary to better understand the cause of this increased radiosensitivity and generally better treatment outcomes of HPV (+) HNSCCs compared to HPV (-) HNSCCs, this investigation focused on the FA-like DSB repair defect seen in HPV (+) HNSCCs.

Current standard of care remains intense for all HNSCCs diagnosed at late stage with poor prognosis. Given the good prognosis at early diagnosis especially for HPV (+) HNSCCs, it is frustrating that clinically effective screening methods of early-stage disease, such as used for cervical cancer screening, are still lacking with only determination of surrogate markers such as p16 used for HPV status determination (Adams et al. 2014). Future cancer therapies should focus on targeting HPV as it is a prevalent virus and infection alone is not sufficient for malignant progression. Instead the effects it has on DNA stability and repair are central to understanding malignant potential as shown already several years ago by Cottage et al., demonstrating that destabilization of chromosome structure with deletions and translocations of chromosomes in immortalized keratinocytes was caused by HPV (Cottage et al. 2001). Additionally, E6 and E7 induced dysregulation of the cell cycle through pRb and p53 was also not sufficient for malignant transformation, instead other changes brought on by altered protein expression were shown by McLaughlin et al. to be partially responsible (McLaughlin-Drubin et al. 2011). Another example of
HPV E7 influence was seen in the upregulation of the human DEK proto-oncogene in HPV-16 positive cancer cell lines (Wise-Draper et al. 2005). DEK protein inhibits apoptosis, senescence and differentiation and was shown to act in DNA damage repair by promoting NHEJ (Kavanaugh et al. 2011, Wise-Draper et al. 2012). Depletion of DEK in human cancer cell lines was sufficient to induce DNA damage response after genotoxic treatment as evidenced by FANCD2 detection and apoptosis, with apoptosis specific to rapidly dividing cells (Wise-Draper et al. 2009). DEK is thus considered one possible target for cancer therapies. These investigations are the beginning of many more studies necessary to develop targeted therapy. As patients with HPV-positive disease have the potential to be treated with less intensive, individually adapted therapy, further study of HPV influence on DSB repair was of interest in this investigation to potentially provide a basis for such future target development that would improve clinical outcome for this patient group.

1.5 Fanconi Anemia

1.5.1 Clinic and Diagnosis

Fanconi Anemia (FA) is a genetic chromosomal instability disorder that is caused by mutations in at least 17 different genes and is characterized by multiple congenital abnormalities, progressive bone-marrow failure and cancer susceptibility (Joenje and Patel 2001, Kee and D’Andrea 2010, Kottemann and Smogorzewska 2013). It is an autosomal recessive disorder with an incidence of one to five per 1,000,000 births and is manifested by defects in DNA repair, hypersensitivity to DNA crosslinking agents such as MMC and a high degree of chromosomal aberrations (Auerbach 2009; Deakyne and Mazin 2011; Kim and D’Andrea 2012). Clinically FA is a heterogeneous disease with most patients displaying anemia and developing bone-marrow failure during childhood. These patients also have an increased risk of developing acute myelogenous leukemia and SCC, especially of the head and neck (Kutler et al. 2003; Rosenberg, Greene, and Alter 2003; D’Andrea 2010). The type of clinical manifestation is associated with where the mutation in one of the 17 FA genes lies; underlying the broad spectrum of clinical presentation and highlighting the difficulty to have a comprehensive disease diagnosis (Levitus et al. 2004; Wang and Smogorzewska 2015).
Fanconi Anemia patients show hypersensitivity to ICL damage, a type of DNA lesion known to be among the most cytotoxic ones due to its ability to block both DNA replication and transcription (Rothfuss and Grompe 2004, Deans and West 2011). This hypersensitivity arises from germline gene mutations occurring in a DNA repair pathway, the Fanconi Anemia pathway that specializes in repairing interstrand cross-links. Hence, FA patients display hypersensitivity to ICL-inducing agents such as MMC, cisplatin and photoactivated psoralens (Metzler 1986, Taniguchi and D’Andrea 2006, Moldovan and D’Andrea 2009). This principle of hypersensitivity to these agents causing chromosome breaks is used as the basis for the diagnostic tests where MMC or diepoxybutane (DEB) are added and the increase in number of chromosome breaks in FA cells is quantified (Auerbach 1993; Berger, Le Coniat, and Gendron 1993). These chromosome break studies are quite specific for FA and can be used for prenatal diagnostics by investigating amniotic cells, chorion villus cells or fetal blood (Tischkowitz and Hodgson 2003). Further cell cycle disturbances of FA patients with prolonged progression through and arrest in G2 can be measured by flow cytometry after MMC treatment to make the diagnosis (Schindler, Kubbies, Hoehn, Schinzel 1985).

A more specific diagnostic test for determining complementation group, is a screening test based on Western blot for FANCD2 in which the monoubiquinated and non-ubiquinated form of this key protein can be distinguished (Shimamura et al. 2002).

1.5.2 Pathway

To date there are 17 FA proteins or complementation groups known to be part of the FA repair pathway (Wang 2007; Sawyer et al. 2015; Wang and Smogorzewska 2015). These are divided into the core complex, the FANCI-FANCD2 (ID2) complex and the downstream proteins. The pathway is depicted in Figure 5. The core complex consists of eight FA proteins (FANCA, FANCB, FANCC, FANCE, FANCF, FANCG, FANCL, FANCM) and three FA-associated protein subunits (FAAP20, FAAP100 and FAAP24 (Moldovan and D’Andrea 2009). Of this core complex with associated protein, FANCM and FAAP24, belong to the anchoring complex (Walden and Deans 2014). DNA damage resulting from ICL induced stalled replication fork is recognized by FANCM, that in turn, helps load the core complex. The core complex in turn mediates the ubiquitination of the ID2 complex components. The activated ID2 complex then colocalizes with FA
downstream repair proteins (FANCD1, FANCJ, FANCN, FANCO, FANCP, FANCQ and FANCS) to initiate DNA damage repair by HR (Fei et al. 2005, Long et al. 2014). It is gene mutations in any of these 17 FANC genes that provide the basis for assignment to a complementation group designated FA-A to FA-S. Of these FANC genes several are better known by their hereditary breast and ovarian cancer risk gene names. FANCJ is also known as BACH1 or BRIP1 and is an essential tumor suppressor gene (Cantor and Guillemette 2011). FANCS is better known as BRCA1 and FANCD1 as BRCA2. Heterozygous carriers of mutations in hereditary breast and ovarian cancer genes, BRCA1 or BRCA2, have a 60–80% lifetime risk of breast cancer (Miki et al. 1994, Wooster et al. 1995, Antoniou et al. 2003). In the absence of FANCJ/BACH1/BRIP1 or its interaction with FANCS/BRCA1, cells have defects in several aspects of DNA damage response most notably in promoting HR repair, checkpoint control and in limiting DNA damage response. In contrast to breast or ovarian cancer patients, FA patients must inherit two mutated alleles (Liu and West 2002). For example, bi-allelic mutations or homozygous mutations in FANCD1/BRCA2, give rise to FA within the FA-D1 complementation group (Howlett et al. 2002). FANCN (also known as PALB2) is a nuclear binding partner of BRCA2, and bi-allelic mutations in FANCN/PALB2 are designated as FA-N and lead to high risk of childhood cancer (Reid et al. 2007). FANCO (also known as RAD51C) is another gene playing a vital role in HR-mediated repair of DNA lesions associated with replication. Deficiency in FANCO/RAD51C predisposes to ovarian cancer and results in hallmark FA phenotype - ICL sensitivity, chromatid-type error and G2 accumulation (Somyajit et al. 2012). The FA-M complementation group is also relevant in breast cancer predisposition. FANCM proteins support genome duplication and repair and function in ATR-mediated DNA damage checkpoint control. Recently, FANCM was also identified as a breast cancer susceptibility gene with mutations predisposing to triple-negative breast cancer (Kiiski et al. 2014, Peterlongo et al. 2015, Xue et al. 2015). Other FA genes known by another name include, FANCP also known as SLX4 and FANCQ also known as ERCC4(XPF) (Wang and Smogorzewska 2015). The clinical presentation of FA patients is related to the complementation group to which they belong with frequency and disease presentation dependent on the location of the FA pathway mutation. To better understand and develop more targeted treatment options a better understanding of the complementation group the patients are assigned to will be necessary.
1.6 FANCD2 and DSB Repair

Patients assigned to the complementation group FANCD2 only comprise about 1% to 3% of those diagnosed with FA and are far fewer than those assigned to complementation groups such as FANCA (64%) or FANCC (12%) (Tischkowitz and Dokal 2004; Wang and Smogorzewska 2015). FANCD2 patients display a severe phenotype having frequent malformations and hematological manifestations that present earlier and with rapid progression compared to other complementation groups (Kalb et al. 2007). Though much is known about the FA pathway, the extent of FANCD2 function remains a point of investigation. It is known that cells deficient in FANCD2 show defects in HR repair of DSB, translesion synthesis and gene conversion and therefore highlight its central role in genome stability (Thompson et al. 2005, Levitus et al. 2006). In addition Kalb et al., were able to show that the predominant mutation spectrum of FANCD2 involved splicing mutations which have become a growing target in the realm for experimental therapies.
They noted also that residual FANCD2 protein existed in the cell lines investigated from FANCD2 patients and suggested the lethality of biallelic null mutations (Kalb et al. 2007).

FA cells are known for not only insufficiency in homologous recombination, but also in DNA mismatch repair, nucleotide excision repair, translesion DNA synthesis, and other molecular defects, leading to genome instability (Shen et al. 2015). Though much about the role of FANCD2 function in DNA damage repair is still elusive, it was recently found that FANCD2 plays an important role in the early DNA damage response by recruiting a DNA translesion synthesis enzyme, the polymerase pol η, to the site of damage. A study by Fu et al. showed that monoubiquitination of FANCD2 has an anchor role in regulating pol η recruitment to sites of DNA repair (Fu et al. 2013). This role as an anchor allows the repair factors such as this polymerase to replicate past a damaged DNA base for example. That this is a critical function was indicated already by Knipsheer et al. who showed that lack of FANCD2 led to inhibition of both nucleolytic incision near the ICL and translesion DNA synthesis past the lesion (Knipscheer et al. 2009). An intact FA pathway protects a cell from oncogenesis by ability to repair and maintain genome stability. Sensitizing tumor cells to radiation or chemotherapy by FA pathway inhibition targeting FANCD2 could be of benefit regarding tumor therapy responsiveness.

1.7 Aim of Study

Knowledge that HPV (+) HNSCCs are a special tumor entity differing from HPV (-) HNSCCs, makes it essential to understand the molecular differences and explore new options capable to target the specific defects associated with HPV infection. In order to contribute to a better understanding of potential HPV-induced DNA repair changes, it was necessary to provide a better molecular basis. The discovery of an FA-like phenotype demonstrated for HPV (+) HNSCCs made it of interest to look at the molecular components of these HPV cells in the context of the FA pathway. The aim of my Dr. med. thesis was to analyze molecular components of DNA repair pathways that cause Fanconi Anemia-like phenotype in HPV-16 infected HNSCCs.

The therapies available for the HNSCC attributed to HPV infection are still limited clinically and do not differ much from non-HPV HNSCC. In both cases platinum-based chemoradiation with possible combination of cetuximab are the standard of care in locally
advanced HNSCC, while recurrent or metastatic disease is treated with chemotherapies based on cisplatin, in combination with 5-FU and cetuximab. In cases of low disease burden or patients who are asymptomatic, single agent treatment with taxanes such as docetaxel and paclitaxel are used to reduce treatment burden. Radio- and chemotherapy options together with potentially radical operations are used to potentially obtain a curative state or prolong median overall survival (Price and Cohen 2012, Hutchinson 2015, Nishimura et al. 2016). Given the specific nature of HPV (+) HNSCCs, there is much potential for alternative therapies targeting the effect of HPV on the molecular level. As HPV status has a prognostic role in disease progression, HPV subtypes are routinely diagnosed and thus identified (Sacco and Cohen 2015). However, an individual therapy concept for these HPV-positive HNSCCs has yet to be developed. Advances in cancers linked to HPV have been a subject of interest in the last years and focused largely on the effect of the virus’s E6 and E7 oncoproteins. These oncoproteins have been the targets of the cervical cancer vaccines developed in the last decade; however, beyond these targets further study of the virus and its effect on epithelial cells is necessary to better understand other potential molecular targets ranging from DNA repair mechanisms like HR to DNA repair components like RAD51 or RPA and proteins such as the core component FANCD2. These and potentially other proteins could serve as targets for development of small molecule therapies, which could be more beneficial regarding better treatment outcome in HPV-positive cancers, often demonstrating different resistance patterns compared with HPV-negative cancers. One such application of a small-molecule therapy approach is the use of PARP inhibitors, already in clinical use for certain ovarian cancer patients. PARP inhibitors show a promising strategy for targeting cancers with defective HR such as the BRCA1 and BRCA2 mutation-associated ovarian cancers (Livraghi and Garber 2015). Several PARP inhibitors are currently in trials in the adjuvant, neoadjuvant, and metastatic settings for the treatment of ovarian, BRCA-mutated breast, and other cancers. Recent approval of olaparib in BRCA deficient ovarian cancer patients has already provided an important new treatment option for ovarian cancer patients (Meehan and Chen 2016). As FA patients are defective in HR repair and highly susceptible to HPV (+) HNSCC, examining the FA pathway for potential targets could provide the basis for potential therapies of HPV (+) cancers in the future. Methods that enable this systematic analysis of such DNA repair pathways include, for example, the DSB repair assay system developed in the lab of Professor Wiesmüller, which will be further discussed in Section 2.2.3. In addition to this method, immunofluorescence analysis of critical repair
components assembly, together with Western Blot analysis of repair protein expression provide insight into specific aspects of the DNA repair process. Evaluating cell cycle distribution and apoptosis levels further indicate the stage of the cell cycle in which DDR is halted or damage deemed irreparable. Finally, exposure to drugs known to target HR pathway, can be used to assess cell survival and thus provide information on potential drug sensitivities that may lead to new targeted therapies. The aim of my thesis was therefore to analyze, using these methods, the molecular components of the DNA repair pathways that cause Fanconi Anemia-like phenotype in HPV-16 infected HNSCCs.
2 MATERIALS AND METHODS

2.1 Materials

All materials are listed with city of origin. For cities in Germany no country is listed.

2.1.1 Equipment

Amixa Nucleofector II Amixa GmbH, Köln

Analysis Balances:

P1200 Mettler, Gießen

Sartorius BP61 Sartorius, Göttingen

Autoklav, Varioklav 75S H+P, Oberschleißheim

Centrifuges:

Biofuge 13 Heraeus-Sepatech, Osterode

Biofuge pico Kendro, Osterode

Multifuge 1S-R Kendro, Osterode

Multifuge 3S-R Kendro, Osterode

Rotanta 96R Hettich, Tuttlingen

Concentrator 5301 Eppendorf, Hamburg

Flow cytometry apparatus:

FACSCalibur™ Becton Dickinson, Heidelberg

ChemiDoc™ MP Imaging System Bio-Rad Labs, München
## Incubators:

<table>
<thead>
<tr>
<th>Incubator</th>
<th>Manufacturer, Location</th>
</tr>
</thead>
<tbody>
<tr>
<td>Incubator B6760</td>
<td>Heraeus, Hanau</td>
</tr>
<tr>
<td>Incubator 311</td>
<td>Thermo, Egelsbach</td>
</tr>
<tr>
<td>Incubator 3862</td>
<td>Forma Scientific, Marietta, GA, USA</td>
</tr>
</tbody>
</table>

## Laminar flow:

<table>
<thead>
<tr>
<th>Laminar Flow</th>
<th>Manufacturer, Location</th>
</tr>
</thead>
<tbody>
<tr>
<td>Clean Air DLF/REC6</td>
<td>Clean Air Techniek, Woerden, Netherlands</td>
</tr>
<tr>
<td>Clean Air DLF/BSS6</td>
<td>Clean Air Techniek, Woerden, Netherlands</td>
</tr>
</tbody>
</table>

## NanoDrop® 2000 Spectrophotometer

<table>
<thead>
<tr>
<th>Spectrophotometer</th>
<th>Manufacturer, Location</th>
</tr>
</thead>
<tbody>
<tr>
<td>NanoDrop® 2000</td>
<td>Thermo Scientific, Wilmington, DE, USA</td>
</tr>
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</table>

## Microscopes:

<table>
<thead>
<tr>
<th>Microscope</th>
<th>Manufacturer, Location</th>
</tr>
</thead>
<tbody>
<tr>
<td>Axiovert 25</td>
<td>Zeiss, Jena</td>
</tr>
<tr>
<td>Olympus BX51</td>
<td>Olympus, Tokyo, Japan</td>
</tr>
<tr>
<td>Microtiterplate-Photometer MRX</td>
<td>Dynatech Laboratories, Chantilly, VA, USA</td>
</tr>
</tbody>
</table>

## Polyacrylamide-Gel electrophoresis system:

<table>
<thead>
<tr>
<th>System</th>
<th>Manufacturer, Location</th>
</tr>
</thead>
<tbody>
<tr>
<td>SE250</td>
<td>Amersham Pharmacia Biotech, Freiburg</td>
</tr>
<tr>
<td>Mighty Small II</td>
<td>Amersham Pharmacia Biotech, Freiburg</td>
</tr>
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</table>
Power Supply:

EPS 1000  Amersham Pharmacia Biotech, Freiburg
EPS 1001  Amersham Biosciences, Piscataway, NJ, USA
EPS 2A 200 Amersham Biosciences, Piscataway, NJ, USA

Shakers:

Certomat®R  B. Braun Biotech Int., Melsungen
Phero-Shaker  Biotec-Fischer, Reiskirchen
Variospeed  Biotec-Fischer, Reiskirchen
Easia®Shaker  Medgenix, Ratingen
VortexGenie 2  Bender und Hobein, Zürich, Switzerland

Western-Blot-Chamber (SDS-PAGE)  Amersham Pharmacia Biotech, Freiburg

2.1.2 Software

BDTM PlateManager 1.0.1  BD Biosciences, Heidelberg
BD Cell QuestTM Pro 5.2.1  BD Biosciences, Heidelberg
CellF 2.5  Soft Imaging System, Münster
Image Lab 4.1 software  Bio-Rad Laboratories, München
2.1.3 Chemicals and Biochemicals

The chemicals and reagents used originated mostly from Merck KGaA (Darmstadt), Serva Feinbiochemica GmbH & Co. KG (Heidelberg), Riedel de Häen AG (Seelze), Fluka and Sigma-Aldrich Chemie (Steinheim):

- 2-Mercaptoethanol: Bio-Rad Laboratories, Hercules, CA, USA
- Acetone: Sigma-Aldrich, Steinheim
- Acrylamide/Bisacrylamide (30:0.8) (w/v): National Diagnostics, Atlanta, GA, USA
- Agarose: Invitrogen, Karlsruhe
- Ammoniumperoxodisulfate (APS): Bio-Rad Laboratories, Hercules, CA, USA
- Ampicillin: Sigma-Aldrich, Steinheim
- Bacto Agar: Becton Dickinson, Sparks, MD, USA
- Bacto Yeast-extract: Becton Dickinson, Sparks, MD, USA
- Bacto Trypton: Becton Dickinson, Sparks, MD, USA
- Bromphenol Blue: Sigma-Aldrich, Steinheim
- Cryomax Solution: PAA, Pasching
3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium bromide (MTT)

Dimethylsulfoxide (DMSO)

DMEM High Glucose Medium

Dithiothreitol (DTT)

Dulbecco’s Phosphate Buffered Saline (DPBS)

Ethylene-diamine-tetra-acetic acid (EDTA)

Ethanol absolute

Ethylene glycol-bis(2-aminoethylether)-N,N,N′,N′ (EGTA)

FACS-Clean

FACS-Flow

FACS-Rinse

Fetal Bovine Serum (FBS)

Formaldehyde 37 %

Glycine

β-Glycerol phosphate

Goat serum

HCL (1M)

HEPES-Buffered Saline Solution (BSS)

1,5-Isoquinolinediol (IQD)

Sigma-Aldrich, Steinheim

Merck, Darmstadt

PAA, Pasching

Sigma-Aldrich, Steinheim

Gibco, Darmstadt

Applichem, Darmstadt

Sigma-Aldrich, Seelze

Roth, Karlsruhe

Becton-Dickinson, Heidelberg

Becton-Dickinson, Heidelberg

Becton-Dickinson, Heidelberg

Biochrom, Berlin

Sigma-Aldrich, Steinheim

Applichem, Darmstadt

Sigma-Aldrich, Steinheim

Sigma-Aldrich, Steinheim

Lonza, Köln

Enzo Life Science, Farmingdale, NY, USA
<table>
<thead>
<tr>
<th>Chemical</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hydrocortisone</td>
<td>Sigma Aldrich-Steinheim</td>
</tr>
<tr>
<td>Isopropanol</td>
<td>Sigma-Aldrich, Seelze</td>
</tr>
<tr>
<td>Isoseptol</td>
<td>University Ulm, Ulm</td>
</tr>
<tr>
<td>MgCl₂</td>
<td>Sigma-Aldrich, Seelze</td>
</tr>
<tr>
<td>MgSO₄</td>
<td>Sigma-Aldrich, Seelze</td>
</tr>
<tr>
<td>Milk powder Marvel</td>
<td>Premier Foods Ltd, Long Sutton, UK</td>
</tr>
<tr>
<td>Mitomycin C (MMC)</td>
<td>Sigma-Aldrich, Steinheim</td>
</tr>
<tr>
<td>N, N, N', N'-tetra-methyl-ethylene-diamine (TEMED)</td>
<td>Roth, Karlsruhe</td>
</tr>
<tr>
<td>Nonidet P40</td>
<td>Fluka, Neu-Ulm</td>
</tr>
<tr>
<td>PBS 10x</td>
<td>Gibco Life Technologies, Eggenstein</td>
</tr>
<tr>
<td>PBS 1x</td>
<td>PAA, Pasching</td>
</tr>
<tr>
<td>Penicillin/Streptomycin</td>
<td>Invitrogen, Karlsruhe</td>
</tr>
<tr>
<td>Poly-L-Lysine</td>
<td>Sigma-Aldrich, Steinheim</td>
</tr>
<tr>
<td>Ponceau Red</td>
<td>Sigma-Aldrich, Steinheim</td>
</tr>
<tr>
<td>Propidium iodide (PI)</td>
<td>Sigma-Aldrich, Steinheim</td>
</tr>
<tr>
<td>Protease Inhibitor Cocktail tablets</td>
<td>Roche, Mannheim</td>
</tr>
<tr>
<td>Puromycin</td>
<td>Sigma-Aldrich, Steinheim</td>
</tr>
<tr>
<td>RNase A</td>
<td>Marligen, Ijamsville, MD, USA</td>
</tr>
<tr>
<td>Rotiphorese 10 x SDS Page-Puffer</td>
<td>Roth, Karlsruhe</td>
</tr>
<tr>
<td>RPMI 1640 (without Phenol Red)</td>
<td>Gibco, Darmstadt</td>
</tr>
<tr>
<td>Material</td>
<td>Supplier</td>
</tr>
<tr>
<td>-----------------------------------------------</td>
<td>-----------------------------------</td>
</tr>
<tr>
<td>Sodium chloride</td>
<td>Applichem, Darmstadt</td>
</tr>
<tr>
<td>Dosium dodecyl sulfate (SDS)</td>
<td>Merck, Darmstadt</td>
</tr>
<tr>
<td>Sodium fluoride</td>
<td>Merck, Darmstadt</td>
</tr>
<tr>
<td>Sodium vanadate (NAV)</td>
<td>Sigma-Aldrich, Steinheim</td>
</tr>
<tr>
<td>Sucrose</td>
<td>Sigma-Aldrich, Steinheim</td>
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<tr>
<td>TE-Buffer</td>
<td>Origene, Rockville, MD, USA</td>
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<tr>
<td>Tris/HCl</td>
<td>Roth, Karlsruhe</td>
</tr>
<tr>
<td>Trizma Base</td>
<td>Sigma-Aldrich, Steinheim</td>
</tr>
<tr>
<td>Triton-X-100</td>
<td>Sigma-Aldrich, Steinheim</td>
</tr>
<tr>
<td>Trypan blue solution</td>
<td>Sigma-Aldrich, Steinheim</td>
</tr>
<tr>
<td>Trypsin-EDTA (1x)</td>
<td>PAA, Pasching</td>
</tr>
<tr>
<td>Trypsin Neutralising Solution (TNS)</td>
<td>Lonza, Köln</td>
</tr>
<tr>
<td>Tween 20</td>
<td>Merck, Darmstadt</td>
</tr>
<tr>
<td>Vectashield containing DAPI</td>
<td>Vector Laboratories, Burlingame, CA, USA</td>
</tr>
</tbody>
</table>

### 2.1.4 Kits and other Materials

<table>
<thead>
<tr>
<th>Kit</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>BCA Protein Assay Kit</td>
<td>Thermo Scientific, Rockford, IL, USA</td>
</tr>
<tr>
<td>Cell Line Amaxa Nucleofector Kit V</td>
<td>Lonza, Köln</td>
</tr>
<tr>
<td>Cover Slips (24x40mm)</td>
<td>Menzel-Gläser, Braunschweig</td>
</tr>
<tr>
<td>DetachKit</td>
<td>Promocell, Heidelberg</td>
</tr>
</tbody>
</table>
Chemiluminescence Substrates:

Clarity Western ECL
Bio-Rad Laboratories, Hercules, CA, USA

Super Signal® West Pico
Thermo Scientific, Rockford, IL, USA

Super Signal® West Dura Extended
Thermo Scientific, Rockford, IL, USA

Cryotubes (1.5 mL)
Greiner-Bio-One, Frickenhausen

Electroporation cuvettes (0.4 mm)
Bio-Rad Laboratories, Hercules, CA, USA

FACS tubes (5mL)
Becton-Dickinson, Heidelberg

Filter paper Whatman 3 MM
Schleicher and Schüll, Dassel

High Purity Plasmid Maxiprep System
Marligen Biosciences Inc., Ijamsville, MD, USA

Hybond-C-Extra, Nitrocellulose
Amersham Biosciences, Freiburg

Page Ruler™ Pre-Stained Protein Ladder
MBI Fermentas, St. Leon-Rot

Page Ruler™ Prest Plus Pre-Stained Protein
MBI Fermentas, St. Leon-Rot

Pipettes, glass disposable and plastic (µL-mL)
Eppendorf, AG, Hamburg

Safe-lock tubes (0.5 mL, 2 mL)
Eppendorf, AG, Hamburg

Surgical disposable scalpels
B. Braun, Melsungen

Tissue Culture Dish (100x20mm, 150x20mm)
Sarstedt, Nümbrecht

Tissue Culture Plate (6 well, 96 well)
Sarstedt, Nümbrecht

Tube PP (15 mL, 50 mL)
Sarstedt, Nümbrecht
## 2.1.5 Buffers and Solutions

<table>
<thead>
<tr>
<th>Buffer Type</th>
<th>Composition</th>
</tr>
</thead>
</table>
| Cell lysis buffer for protein extraction | 50 mM Tris-Base, pH 7.4  
150 mM NaCl  
2 mM EGTA  
2 mM EDTA  
25 mM Sodium fluoride  
25 mM β-Glycerol phosphate  
0.1 mM Sodium vanadate  
0.2 % Triton X-100  
0.3 % Nonidet P40  
1 Protease inhibitor cocktail tablet |
| 4x SDS Loading gel buffer | 0.5 M Tris/HCl, pH 6.8  
0.4 % SDS |
| 4x SDS Separating gel buffer | 1.5 M Tris/HCl, pH 8.8  
0.4 % SDS |
| 6 x SDS-buffer | 350 mM Tris/HCl pH 6.8  
9.3 % (w/v) DTT  
10 % (w/v) SDS  
36 % (v/v) Glycerin  
0.6 % (w/v) Bromophenol Blue  
10 % β-Mercaptoethanol (freshly added) |
| Fixing solution | 40 % (v/v) Ethanol  
50 % (v/v) Aceton  
10 % (v/v) ddH₂O |
| 10 x High-molecular-weight (HMW) buffer | 495 mM Tris-Base  
400 mM Glycine |
| LB-medium (Luria-Bertani) (Sambrook et al. 2001) | 10g Trypton  
10g NaCl  
5g Yeast extract  
Fill till 1L H₂O |
| 1 x DPBS-EDTA | 0.2 % EDTA in 1 x DPBS |
1 x PI-solution for cell cycle analysis 50 µg/mL RNAse A
PI diluted in PBS

Pre-extraction buffer for immunofluorescence
20 mM HEPES
50 mM NaCl
1 mM EDTA
3 mM MgCl₂
300 mM Sucrose
0.5% Triton
pH 7.4

1 x TBS-T 20 mM Tris/HCl, pH 7.6
137 mM Sodium chloride
0.2 % Tween 20

TBS-T 0.1 % Tween 20 in TBS (1x)

2.1.6 Cell Culture Media

Adherently growing cells DMem (+) CO 10 % FBS
1 % Penicillin/Streptomycin (PEES)
1.25 µg/mL Puromycin
0.4 µg/mL Hydrocortisone
In DMem High Glucose-Medium

DMem (-) CO 10 % FBS
1 % Penicillin/Streptomycin (PEES)
1.25 µg/mL Puromycin
In DMem High Glucose-Medium

2.1.7 Bacterial Media

10 x LB-Medium 100 g Bacto-Tryptone
50 g Yeast Extract
50 g NaCl in 1 L ddH₂O
pH 7.4

LB-Agar 15 g Bacto-Agar in 1 l LB-Medium
(Autoclaved, cooled and supplemented with antibiotic)

Ψ-Broth-Medium 10 mM MgSO₄
10 mM MgCl₂
in LB-Medium
pH 7.6
Table 1. Investigated Cell Lines

<table>
<thead>
<tr>
<th>Cell lines</th>
<th>Description</th>
<th>Cultivation</th>
</tr>
</thead>
<tbody>
<tr>
<td>UMSCC1-NTsh</td>
<td>Human squamous cell carcinoma cell line of the head and neck, floor of mouth; non-targeting short hairpin sequence (NTsh), HPV (-)</td>
<td>DMEM with Hydrocortisone</td>
</tr>
<tr>
<td>UMSCC1-D2sh</td>
<td>Human squamous cell carcinoma cell line of the head and neck, floor of mouth; FANCD2 short hairpin sequence (D2sh), HPV (-)</td>
<td>DMEM with Hydrocortisone</td>
</tr>
<tr>
<td>UMSCC47-NTsh</td>
<td>Human squamous cell carcinoma cell line of the head and neck, lateral tongue; non-targeting short hairpin sequence (NTsh), HPV (+) integrated</td>
<td>DMEM</td>
</tr>
<tr>
<td>UMSCC47-D2sh</td>
<td>Human squamous cell carcinoma cell line of the head and neck, lateral tongue; FANCD2 short hairpin sequence (D2sh), HPV (+) integrated</td>
<td>DMEM</td>
</tr>
<tr>
<td>UMSCC47-Ash</td>
<td>Human squamous cell carcinoma cell line of the head and neck, lateral tongue; FANCA short hairpin sequence (Ash), HPV (+) integrated</td>
<td>DMEM</td>
</tr>
</tbody>
</table>

The cell lines originated from the University of Michigan Repository (gift from Dr. Thomas Carey, University of Michigan). The UMSCC1 and UMSCC47 lines originated from two different donors and are not isogenic (Brenner et al. 2010). The FANCD2 and FANCA knockdowns as well as non-targeting vector cell lines were donated from the University of Cincinnati (gift from Dr. Susanne Wells, Cincinnati Children’s Hospital Medical Center). These were achieved by transducing the cells with lentiviral supernatant containing either the FANCD2 short hairpin or non-targeting NTsh short hairpin sequences. The lentiviral constructs used to make the supernatant were purchased from the Sigma-Aldrich Mission Program (Lin et al. 2007). All of the UMSCC cell lines were established from head and neck cancer patients who gave written informed consent in studies reviewed and approved by the University of Michigan Medical School Institutional Review Board (Brenner et al. 2010).

Table 2. Bacterial Strain for Plasmid DNA Isolation

<table>
<thead>
<tr>
<th>Bacterial strain</th>
<th>Description</th>
<th>Origin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasmid</td>
<td>Description</td>
<td>Origin</td>
</tr>
<tr>
<td>---------------------------------</td>
<td>-----------------------------------------------------------------------------------------------</td>
<td>---------------------------------------------</td>
</tr>
<tr>
<td>pBlueScriptII KS(+/−) (pBS)</td>
<td>Plasmid derivate from pBR322 with multiple cloning sites</td>
<td>Stratagene, Heidelberg</td>
</tr>
<tr>
<td>pCMV-I-SceI</td>
<td>Expression vector for meganuclease I-SceI under the control of the CMV promoter</td>
<td>(Rouet et al. 1994)</td>
</tr>
<tr>
<td>P5bPuroCMV-wtEGFP (wtEGFP)</td>
<td>Wild-type EGFP expression under control of the CMV promoter serving as the positive control</td>
<td>(Akyüz et al. 2002)</td>
</tr>
<tr>
<td></td>
<td>in recombination analysis to determine transfection efficiency</td>
<td></td>
</tr>
<tr>
<td>EJ5SceGFP</td>
<td>Plasmid substrate used for assessment of total non-homologous end joining (NHEJ)</td>
<td>(Bennardo et al. 2009)</td>
</tr>
<tr>
<td>p5xEsCMVHygb PuroCMV-HR (HR-EGFP/5’EGFP)</td>
<td>Plasmid substrate used for assessment of conservative homologous recombination with long homology (HR)</td>
<td>(Akyüz et al. 2002)</td>
</tr>
<tr>
<td>p5bPuroCMV-EJ (EJ-EGFP)</td>
<td>Plasmid substrate used for assessment of micro-homology mediated non-homologous end joining</td>
<td>(Akyüz et al. 2002)</td>
</tr>
<tr>
<td></td>
<td>(MMEJ)</td>
<td></td>
</tr>
<tr>
<td>p5bPuroCMV-N’-CMV-Red-HR (5’EGFP/HR-EGFP)</td>
<td>Plasmid substrate used for assessment of nonconservative homologous recombination (SSA)</td>
<td>(Hinsch 2000)</td>
</tr>
</tbody>
</table>

Constructs NHEJ (EJ5SceGFP), HR (HR-EGFP/5’EGFP), MMEJ (EJ-EGFP) and SSA (5’EGFP/HR-EGFP) were used for assessment of different DSB repair pathways. Dark gray box with arrow, promoter sequence; white triangle, I-SceI recognition site (note 2 for NHEJ); black box, spacer sequence; light gray box, mutated EGFP genes; cross, deleted EGFP sequence; gray triangles, microhomologies.
Table 4. Primary Antibodies

<table>
<thead>
<tr>
<th>Name</th>
<th>Dilution Western Blot</th>
<th>Description</th>
<th>Origin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-DNA-PKcs</td>
<td>1:1000</td>
<td>Mouse monoclonal antibody against DNA-PKcs, (18-2) Ab 1832-500/146443</td>
<td>Abcam, Cambridge, UK</td>
</tr>
<tr>
<td>Anti-GAPDH</td>
<td>1:2500</td>
<td>Mouse monoclonal antibody against Glyceraldehyd-3-Phosphat-Dehydrogenase (GAPDH), Ab 94/84</td>
<td>Abcam, Cambridge, UK</td>
</tr>
<tr>
<td>Anti-FANCD2</td>
<td>1:5000 (1:500)</td>
<td>Rabbit IgG polyclonal antibody against Fanconi Anemia Group D2 Protein (FANCD2), clone NB100-182/ N-4</td>
<td>Novus Biologicals, Littleton, CO, USA</td>
</tr>
<tr>
<td>Anti-Phospho-RPA (P-RPA)</td>
<td>1:1000</td>
<td>Rabbit polyclonal antibody against P-RPA, S33, A300-246A-2</td>
<td>Bethyl Laboratories, Montgomery, AL, USA</td>
</tr>
<tr>
<td>Anti-RPA</td>
<td>1:500</td>
<td>Mouse monoclonal antibody against RPA, (Ab-2) Cat #NA18 (RPA34-19)</td>
<td>Calbiochem, Darmstadt</td>
</tr>
<tr>
<td>Anti-Phospho-CHK1 (P-CHK1)</td>
<td>1:1000</td>
<td>Rabbit polyclonal antibody against P-CHK1, Ser345 #2341S/ 4, #2348S/11</td>
<td>Cell Signaling, Danvers, MA, USA</td>
</tr>
<tr>
<td>Anti-CHK1</td>
<td>1:1000</td>
<td>Mouse monoclonal antibody against CHK1, 2G1D5/ Lot 3 Ref 06/2010</td>
<td>Cell Signaling, Danvers, MA, USA</td>
</tr>
<tr>
<td>Anti-53BP1</td>
<td>(1:1000)</td>
<td>Rabbit polyclonal antibody against 53BP1</td>
<td>Novus Biologicals, Littleton, CO, USA</td>
</tr>
<tr>
<td>Anti-RAD51</td>
<td>1:1000 (1:1000)</td>
<td>Rabbit polyclonal antibody against RAD51, (H-92) SC-8359/ G3012</td>
<td>Santa Cruz Biotechnology, Heidelberg</td>
</tr>
</tbody>
</table>

(Italics) indicates dilution used for Immunofluorescence

Table 5. Secondary Antibodies

<table>
<thead>
<tr>
<th>Name</th>
<th>Dilution</th>
<th>Origin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Goat anti-mouse, horseradish peroxidase (HRP)- labeled</td>
<td>1:1000</td>
<td>Pierce/Perbio, Rockford, IL, USA</td>
</tr>
<tr>
<td>Goat anti-rabbit, horseradish peroxidase (HRP)-labeled</td>
<td>1:1000</td>
<td>Pierce/Perbio, Rockford, IL, USA</td>
</tr>
<tr>
<td>Alexa Fluor® 555 anti-mouse</td>
<td>1:1000</td>
<td>Invitrogen, Darmstadt, Germany</td>
</tr>
<tr>
<td>Alexa Fluor® 555 anti-rabbit</td>
<td>1:1000</td>
<td>Invitrogen, Darmstadt, Germany</td>
</tr>
</tbody>
</table>
2.2 Methods

2.2.1 Cell Culture Techniques

2.2.1.1 Cultivation of adherent cells

The UMSCC adherent cell cultures were grown on tissue culture plates (Sarstedt, Nümbrecht) in Dulbecco’s Modified Eagles Medium (DMEM) (Invitrogen, Karlsruhe), supplemented with 10% fetal bovine serum (FBS) (Biochrom, Berlin), 1% penicillin/streptomycin (PEES) (Invitrogen), 1.25 µg/ml puromycin (Sigma-Aldrich, Steinheim) and either with or without 0.4 µg/ml hydrocortisone (+CO or – CO) (Sigma-Aldrich, Steinheim). UMSCC1 cell lines were HPV (-) and grown in hydrocortisone DMEM media whereas UMSCC47 cell lines were HPV (+) and grown in DMEM media free of hydrocortisone. Culture plates with cells were maintained at 5 % CO₂ and 37°C. Cell growth was monitored under the light microscope (Axiovert 25 Zeiss, Jena) and based on confluence or experimental need the cells were passaged and distributed to new tissue culture plates. DMEM (+CO or – CO), 1x PBS and Trypsin-EDTA (PAA, Pasching) were placed in a water bath at 37.5°C. After removing the media the adherently crowing cells were washed with 1x PBS and incubated with Trypsin-EDTA for 5-10 min at 37.5°C. Once cells had detached adequately from the tissue culture plate, about 10 ml of fresh DMEM (+CO or – CO) media was added for deactivation of Trypsin-EDTA. The cells were resuspended and distributed based on growth pattern and crowding tendencies onto new tissue culture plates labelled with the subsequent passage. Another 15 ml of DMEM (+CO or – CO) media was added prior to returning the cells to the normal incubated maintenance conditions. The experiments were performed using antibiotic free DMEM (+CO or – CO) media. All cell lines used had previously been tested negative for *Mycoplasma* contamination.

2.2.1.2 Storage of adherent cells

Cells stored at -80°C were defrozen by rapid placement in a 37°C water bath, followed by immediate seeding in tissue culture plates (Sarstedt, Nümbrecht) with 15 ml of DMEM (+CO or – CO) media. Adherent cell cultures were maintained at 5 % CO₂ and 37°C. To prepare adherent cell cultures for storage, cells were centrifuged (5 min at 300 x g) and the
pellet dissolved in 4.5 ml cryomax solution to prepare 3 vials of cells for storage. The vials were placed in isopropanol and placed in the -80°C freezer.

2.2.1.3 Trypsinizing of adherent cells

Trypsin-EDTA was normally used to trypsinize the cells. Certain cell lines, through their particular adhesive growth patterns, often required at higher passages the use of DetachKIT (Promocell, Heidelberg) in order to detach. The procedure was followed using manufacturer instructions and exposure time at room temperature Trypsin-EDTA was set to 2 min for the UMSCC1-NT cell line and 10 min for the other adherent cell lines. The same time intervals were used for cell lines detached using the DetachKIT.

2.2.2 Molecular Biology Methods

2.2.2.1 Isolation of plasmid DNA from E.coli using Maxi-prep

Competent bacteria were transformed with the desired plasmid according to Inoue et al. (Inoue et al. 1990). For plasmid isolation the transformed bacteria with the plasmid of interest were taken from -80°C storage. Media derived from 10xLB media was diluted to 1xLB media and combined 1:1000 with ampicillin to a final concentration of 100 µg/mL. These bacteria were pre-cultured in 5 mL of the 1xLB medium with ampicillin and placed in incubator spinning for 5-7 h. To isolate the plasmid a 2500 mL overnight bacterial culture was prepared and incubated in 1-2 L Erlenmeyer flasks. The bacteria were then centrifuged (3 min at 4500 x g), the supernatant discarded and either placed in -20°C storage for later use or plasmid isolated by use of the PowerPrep™ HP Plasmid Maxiprep Kits (Marligen Biosciences Inc., Ijamsville, MD, USA) following the instructions of the manufacturer.

2.2.2.2 Determination of plasmid DNA concentration using NanoDrop®

The DNA concentration as well as the purity of the plasmid investigated was determined using NanoDrop® 2000 Spectrophotometer (Thermo Scientific, Wilmington, DE, USA) at an absorbance of 260 nm. The plasmid DNA was applied in 1-2 µL quantities for measurement and concentrations were afterwards set to 1 µg/ml. The ratio of absorbance at 260 nm and 280 nm was used to determine the purity of DNA. A value of ~ 1.8 was
considered as pure DNA. A differing ratio would imply impurity from proteins or other contaminants. The purified plasmid DNA was stored at -20°C for further experiments.

### 2.2.3 DSB Repair

#### 2.2.3.1 Fluorescence-based DSB repair test system

The fluorescence-based DSB repair test system was established in the lab of Professor Wiesmüller (Akyüz et al. 2002) and is based on the restoration of a functional wild-type *EGFP* (wt-*EGFP*) gene through genetic exchange. Several plasmid substrates, including EJ5SceGFP, HR-EGFP/5'EGFP, EJ-EGFP, and 5'EGFP/HR-EGFP, have been designed and used to measure NHEJ, HR, MMEJ and SSA respectively (Bennardo et al. 2008). The constructs designed for SSA or HR contain an *EGFP*- mutated acceptor gene receiving genetic information with an integrated I-SceI recognition site while the donor *EGFP* gene is C-terminally truncated. The plasmid mixture, containing the meganuclease expression plasmid (pCMV-I-SceI), one of the plasmid DSB repair substrates listed above for NHEJ, HR, MMEJ or SSA and either filler plasmid pBS or wt-*EGFP* expression plasmid was introduced into the UMSCC cells by transfection using Amaxa nucleofection described in Section 2.2.3.3. The DSB repair is initiated via I-SceI meganuclease expression causing targeted DSB formation. Through homologous sequence exchange with the uncleaved donor *EGFP* (HR, SSA; Figure 6), rejoining of the cleaved ends (MMEJ; Figure 6), or non-homologous end joining between two I-SceI sites (NHEJ), functional EGFP is regenerated which could be analyzed by flow cytometry described in Section 2.2.3.2 (Böhringer and Wiesmüller 2010).
Figure 6 Basic principle of EGFP reconstitution in fluorescence-based DSB repair test system. The figure is based on the principle described by Akyüz et al. (Akyüz et al. 2002). The DSBs are introduced by expression of meganuclease I-SceI (scissor) in the acceptor EGFP that leads to targeted DSB. Functional EGFP reconstitution takes place and resulting appearance of green fluorescence is measured by flow cytometry. The representation shows a construct measuring HR that contains a mutated EGFP-acceptor sequence (upper left green box) harboring an I-SceI recognition site (white triangle). The donor sequence (upper right green box) contains truncated EGFP (black cross). The promoter sequence is shown as a dark blue box and the spacer sequences as light blue boxes. In the case of the displayed HR construct, homologous sequence exchange takes place with use of the uncleaved donor EGFP to restore functional EGFP.

2.2.3.2 Flow cytometry

Flow cytometry (FCM) method was used to determine the DSB repair frequency, cell cycle distribution and apoptosis induction with the flow cytometer (FACSCalibur™) from Becton-Dickinson. The software BD Cell QuestTM Pro 5.2.1 (BD Biosciences, Heidelberg, Germany) was used to analyze the data. Flow cytometry is a laser based technology that uses the principles of light scattering, excitation of fluorochrome molecules in the cells and emission of fluorescent light. Cells in solution are injected into the flow cytometer and assorted into a stream of single particles which are then exposed to a laser beam of a specific wavelength. The resulting scattering of light is monitored in two ways. Light that is scattered in the forward direction is collected by a detector known as
the forward scatter channel (FSC). The intensity of the FSC corresponds to the particle size and can therefore be used to distinguish between larger living cells and smaller cellular debris. Light measured rectangular to the excitation line is called side scatter. The side scatter channel (SSC) gives information about the granularity of a cell. Both FSC and SSC are specific for every particle and a combination of both may be used to distinguish different cell types in a heterogeneous sample. Light emitted by fluorescent molecules such as EGFP can be detected by a number of detectors, where specificity of detection is controlled by optical filters, which block certain wavelengths while transmitting others. Acquired data was processed and analyzed by computer.

2.2.3.3 Transfection using Amaza® nucleofection

To determine the DSB repair frequencies, DNA mixtures containing one of the DSB repair plasmids (NHEJ, HR, MMEJ or SSA) described in Table 3 were introduced by Amaza nucleofection (Lonza, Cologne, Germany) into each UMSCC cell line. In order to see how well these DNA repair plasmids supported repair in transfected cells, a comparison of wt-EGFP-positive cells (green glow) transfected with a mixture containing pBS filler plasmid versus wt-EGFP-positive cells after transfection with a mixture containing wt-EGFP plasmid was made, which demonstrated how well the repair mechanism worked in transfected cells. UMSCC cells were harvested and 1 x10^6 cells were centrifuged for 5 min at 300 x g for each transfection sample. Plasmid mixes containing a total of 10 μg of DNA per transfection, consisting of 5 μg of one of the DSB repair substrates, 2.5 μg of the meganuclease expression plasmid pCMV-I-ISceI and 2.5 μg of either the filler plasmid pBS or wt-EGFP were prepared. The UMSCC cells were resuspended in 100 μL Amaza transfection solution Kit V and the prepared 10 μg DNA plasmid mixture. Resuspended cells were quickly transferred into the electroporating cuvettes and transfected with Amaza program S.28. Afterwards they were seeded on 6-well plates and incubated for 24 h. The transfected cells were collected after this time, centrifuged for 5 min at 300 x g and resuspended in 200 μL of 0.2% PBS-EDTA and transferred into FACS tubes.

2.2.3.4 Recombination analysis (Akyüz et al. 2002)

Flow cytometric analysis was performed using the FACSCalibur™ (Becton Dickinson, Germany) equipped with a 488nm laser to determine the DNA repair frequency as a
fraction of green fluorescent cells within a population of non-fluorescent cells with the use of the diagonal gating method in the FL1/FL2 dot plot (FACSCalibur™) where FL-1 (530nm) detects green fluorescence and FL-2 (585nm) detects orange auto-fluorescence of the cells. Cells introduced into the FACS were first separated into living and dead cells using a FSC/SSC dot plot. Live cells from the FSC versus SSC gate were plotted with FL-1 (530nm, green fluorescence) versus FL-2 (585nm, orange fluorescence) without compensation between FL1 and FL2 to detect the green fluorescent EGFP-expressing cells above the FL1-FL2 diagonal of non-EGFP expressing cells (Böhringer and Wiesmüller 2010). To correct for potential differences in transfection, transcription, translation, proliferation and lethality, transfection efficiencies were determined for each assay. To this end each cell culture was split for co-transfection with one of the DSB repair plasmid (NHEJ, HR, MMEJ or SSA) and I-SceI expression plasmid plus pBS filler plasmid (sample for DSB repair measurement) or co-transfection with DSB repair plasmid, I-SceI expression plasmid plus wt-EGFP expression plasmid (sample for determination of transfection efficiencies) (Keimling and Wiesmüller 2009). For each wt-EGFP sample 50,000 cells and for each specific DSB repair sample 100,000 living cells were analyzed for EGFP-positivity. Each DSB repair measurement was normalized by use of the individually determined transfection efficiency to calculate the absolute frequency using the following formula:

\[
\text{DSB repair frequency (\%) = \frac{\text{DSB repair events per live cell count (\%)}}{\text{Transfection efficiency per live cell count (\%)}}}
\]

The statistical significance of differences was determined using nonparametric Mann-Whitney-U test using the GraphPad Prism software version 5.01. Transfection efficiencies ranged from 20 to 54% in all the UMSCC cell lines.

2.2.4 Immunodetection/Immunofluorescence and Biochemistry

2.2.4.1 Poly-L-Lysine covered slide preparation

Poly-L-Lysine was diluted 1:10 in dH₂O. The glass slides were incubated in this solution for 5 min at RT. After incubation they were removed to dry and stored at RT.
2.2.4.2 Treatment and Staining of UMSCC Cells for Immunofluorescence Analysis

The UMSCC1 and UMSCC47 cell lines were seeded on the poly-L-lysine coated glass slides and given 24 h to attach. The cells were then exposed to MMC (2 µM) for 1 h and allowed to recover in media for 6, 16 or 24 h. Depending on the specific antibody to be used, cells were pre-extracted for 2 min with pre-extraction buffer at 0°C (RAD51 and FANCD2) or immediately fixed for 10 min with 3.7% formaldehyde (53BP1). The untreated cells (no MMC exposure) were pre-extracted and/or fixed after media change and parallel incubation for 6 h. After washing 3 times for 5 min with 1x PBS, cells were permeabilized with 0.5 % triton/PBS for 15 min (53BP1) or 30 min (RAD51 and FANCD2). Fixed and permeabilized cells were washed 3 times for 5 min with 1x PBS, blocked with 5 % goat serum 1x PBS for an hour at RT to block unspecific binding sites and immunostained with primary antibody in 5 % goat serum 1x PBS solution for 1h at 37°C. Subsequently, the cells were washed 3 times for 5 min with 1x PBS, incubated in the dark with an AlexaFluor555 secondary antibody for 1h at 37°C, washed a final 3 times for 5 min with 1x PBS. Stained cells were mounted with Vectashield containing DAPI (Vector Laboratories, Burlingame, CA, USA). The slides were stored at RT overnight to dry, then stored at 4°C and evaluated within 1 week.

2.2.4.3 Analysis of focal accumulations (foci)

The focal accumulations of the damage marker 53BP1, the recombinase RAD51 and the Fanconi Anemia complementation group protein FANCD2 in the UMSCC nuclei were analyzed in time course experiments following MMC damage using an Olympus BX51 epifluorescence microscope and a 100x oil immersion objective fitted with an Olympus XC10 camera and CellF 2.5 Software (Olympus Soft Imaging Solutions, Münster, Germany). Focal accumulations per nucleus in 50 foci positive cells (n≥2) were counted from two independent slides. The intensity threshold was maintained throughout one experimental set.

2.2.5 MTT assay

In order to assess cell survival upon exposure to drugs known to target the HR pathway, MTT assay was performed. PARP-inhibitor sensitivities and MMC sensitivities for all cell lines differing in their FANCD2 proficiency and HPV infection status were determined by
this assay which is a colorimetric assay used to assess cell viability. Live cells that are metabolically active are able to reduce the yellow 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium bromide (MTT) by mitochondrial dehydrogenases to its insoluble form formazan. The resulting purple crystals are quantified using ELISA by measuring the absorbance at a wavelength of 630 nm (Mosmann 1983). For survival analysis UMSCC cells were seeded in a 96-well plate at a density of 20,000 cells per well and allowed to attach for 24 h in antibiotic free media. Post seeding, the cells were treated with increasing concentrations of the PARP-inhibitor 1,5-Isoquinolinediol (IQD) ranging from 3.9 µM to 1000 µM or MMC ranging from 16.625 nM to 16 µM. For each IQD or MMC concentration a DMSO solvent control in media was also prepared, so that the amount of DMSO in the control corresponded to the amount of DMSO in the IQD or MMC sample. The cells were cultivated for 8 d during which fresh IQD was applied every second day. For MMC the cells were cultivated uninterrupted with MMC. After 2 d for MMC or 7 d for IQD treatment, cells were centrifuged (300 x g for 5 min), media removed and 100 µL MTT solution was added to each well for a final concentration of 0.5 μg/mL. Plates were incubated at 37°C for additional 3 h, centrifuged again, MTT removed and cells with formazan crystals were dissolved in 200 µL of HCl-Isopropanol fixing solution. Following a 20 min incubation time, light absorption was measured at the wave length of 570 nm with the reference filter of 630 nm by use of microtiterplate photometer MRX (Dynatech Laboratories, Chantilly, VA, USA). Quantitative analysis was performed with GraphPad Prism version 5.04 (LaJolla, USA) to obtain IC$_{50}$ values and the statistical significance between them. Values were obtained from a log(inhibitor) versus response-variable slope fit for IQD, or log(inhibitor) versus response-variable slope fit with bottom constraint equal to zero for MMC. The mean values for the DMSO controls were defined as 100% each.

2.2.6 Cell Cycle and Apoptosis Measurements

Propidium iodide (PI) staining and flow cytometric analysis of cells were used to determine cell cycle distribution and apoptosis levels. The assay is based on the principle of different DNA contents in individual cell cycle phases and that apoptotic cells are characterized by DNA fragmentation and thus loss of DNA. PI is a fluorescent molecule which intercalates into DNA and thus it is used as a measure of DNA content. In order to obtain these measurements 30,000 cells were seeded in culture dishes and given 24 h to
attach. The cells were exposed to 2 µM of MMC for 1 h and compared to the mock group of the same cells which were not treated with MMC. Both groups were given 24 h to recover and then fixed in ice-cold 40 % (v/v) ethanol/50 % acetone solution for at least 1 h at -20°C. After fixation, the cells were rehydrated and resuspended in 50 µg/ml propidium iodide solution with freshly added 50 µg/ml RNase A. Following incubation in the dark for 30 min, cells were analyzed by flow cytometry on a FACS Calibur®.

2.2.7 Western Blot Analysis

2.2.7.1 Seeding, treatment and preparation of cell lysates

Cells (1 x 10⁶ to 1 x 10⁷) were seeded onto tissue culture plates in media without antibiotics and incubated for 24 h. On the following day the cells were treated with 2 µM of MMC for 1 h, followed by incubation in fresh media and harvesting at 16 h and 24 h post treatment by adding 2 mL of iced PBS and carefully scraping cells from the culture plates. Another 1 mL of PBS was added to wash remaining cells from the culture plates. The treated and untreated cells were centrifuged for 5 min with 300 x g at 4°C and the supernatant removed. Cell pellets were resuspended in 250 µL of cell lysis buffer for protein extraction, filled in 1.5 mL Eppendorf tubes, vortexed briefly and incubated on ice for 20 min. Cell lysates were centrifuged (16 000 x g, 20 min, 4°C) to remove the cellular debris and the supernatant was transferred into fresh, cooled Eppendorf tubes and frozen at -20°C.

2.2.7.2 Determination of protein concentration by Bicinchoninic acid (BCA) assay

Protein concentrations of cell lysates were determined by use of the BCA Protein Assay Kit (Thermo Scientific, Rockford, IL, USA) according to the instructions of the manufacturer. This included a serial dilution of BSA standard from 2 mg/mL to 0.125 mg/mL and pipetting of 10 µL of each concentration of BSA/lysate in the 96-well plate. The reagents A and B provided by the kit were prepared in a 1:20 ratio and 200 µL were pipetted into each well. The 96-well plate was then incubated for 30 min at 37°C before the optical density was measured spectrophotometrically at 570 nm by use of the microtiterplate photometer MRX (Dynatech Laboratories, Chantilly, VA, USA). Protein concentrations in lysates were calculated by use of the BSA calibration curve and finally protein amounts in samples were equilibrated by adding appropriate volumes of 6 x SDS-buffer freshly
supplemented with 10% β-Mercaptoethanol at a final concentration of 3 µg/µL. Samples were heated to 99°C for 5 min (shaken at 250 rpm) and stored at -80°C.

2.2.7.3 SDS-polyacrylamide gel electrophoresis (Laemmli 1970)

Separation of the proteins was performed by discontinuous SDS-polyacrylamide gel electrophoresis (SDS-PAGE). To that end combs, glass and ceramic plates (size: 8 x 10 x 0.075 cm; Amersham Pharmacia Biotech, Freiburg) were cleaned with isoseptol and assembled with 0.1 cm spacers between. Solutions for the gels were prepared as presented in Table 6 for five gels each. Ammonium persulfate (APS) and N, N, N', N'-tetramethylethlenediamine (TEMED) were added at the end to start polymerization. The separating gel solution was casted in the gel caster comb SE200 (Hoefer Pharmacia Biotech, San Francisco, CA, USA) with approximately 2 cm space remaining on top, which was filled with 200 – 400 µL of isopropanol. Gradient gels ranging from 5% at the bottom to 20% at the top were created by adding sucrose and a drop of blue stain and using a funnel system to create a separating gel solution better able to allow for separation of small and larger proteins simultaneously. After polymerization of approximately 15 min isopropanol was poured away, loading gel solution was added and combs were placed between the glass and ceramic plates to create the wells. Once the loading gel was solidified, the gel was clamped in the gel electrophoresis chamber, which was then filled two-thirds of the height with 1 x Rotiphorese SDS-Page buffer (Roth, Karlsruhe). Combs were carefully removed and well pockets were loaded with 10 µL of protein molecular mass marker (Page Ruler™ Pre-Stained and Pre-Stained Prest Plus Protein Ladder) and previously prepared samples of 45 µg protein each, which were pre-heated to 99°C for 5 min (shaken at 250 rpm). Separation of the proteins was accomplished by electrophoresis of the gel with 20 mA per gel, 200 V and 5-10 Watts.
Table 6. Solutions for the preparation of SDS-PAGE gels

<table>
<thead>
<tr>
<th>Protein Size</th>
<th>≥ 200 kDa Separating gel (6%)</th>
<th>60-200 kDa Separating gel (8%)</th>
<th>16-70 kDa Separating gel (10%)</th>
<th>16-60 kDa Separating gel (12%)</th>
<th>10-30 kDa Separating gel (15%)</th>
<th>Loading gel</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acrylamide 30 %, 0.8 Bisacrylamide</td>
<td>9 mL</td>
<td>12 mL</td>
<td>15 mL</td>
<td>18 mL</td>
<td>22.5 mL</td>
<td>3.9 mL</td>
</tr>
<tr>
<td>4 x SDS/Tris pH 8.8 (Separating buffer)</td>
<td>11.25 mL</td>
<td>11.25 mL</td>
<td>11.25 mL</td>
<td>11.25 mL</td>
<td>11.25 mL</td>
<td>-</td>
</tr>
<tr>
<td>4 x SDS/Tris pH 6.8 (Loading buffer)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>7.5 mL</td>
</tr>
<tr>
<td>dH₂O</td>
<td>24.75 mL</td>
<td>21.75 mL</td>
<td>18.75 mL</td>
<td>15.75 mL</td>
<td>11.25 mL</td>
<td>18.3 mL</td>
</tr>
<tr>
<td>10 % APS</td>
<td>150 μL</td>
<td>150 μL</td>
<td>150 μL</td>
<td>150 μL</td>
<td>150 μL</td>
<td>150 μL</td>
</tr>
<tr>
<td>TEMED</td>
<td>30 μL</td>
<td>30 μL</td>
<td>30 μL</td>
<td>30 μL</td>
<td>30 μL</td>
<td>30 μL</td>
</tr>
</tbody>
</table>

2.2.7.4 Transfer of proteins on the membrane (Towbin et al. 1979)

After electrophoresis was performed, separated proteins were transferred on nitrocellulose membrane (Hybond-C™ Extra, Amersham Pharmacia Biotech, Freiburg). The SDS-PAGE gel, nitrocellulose membrane and Whatman filter paper were first soaked and equilibrated for 15 min in 1 x HMV-buffer. These were arranged beginning at the anode with two buffer-equilibrated Whatman papers, the nitrocellulose membrane, the SDS-PAGE gel and two more Whatman papers. Transfer was performed in 1 x HMV buffer, on ice and in the cold room (4°C) at either 50V for 16 h, for proteins exceeding the size of 200 kDa, or 100V for 2.5 h, for smaller proteins. The membrane surface was stained with Ponceau solution to visualize the proteins transferred then washed 1x with dH₂O and 3 x with TBS-T 0.1% until excess Ponceau was removed.
2.2.7.5 **Immunodetection of transferred proteins** (Blake et al. 1984)

The membrane with transferred proteins was first incubated in 5 % milk-powder solution prepared in TBS-T 0.1 % for 60 min at room temperature (RT) to block unspecific binding of antibodies. Then the membrane was incubated with primary antibody diluted in TBS-T 0.1 % for 1-2h at RT or overnight at 4°C while shaking. After that the membrane was washed 3 times for 5 min with TBS-T 0.1 %, incubated for another hour with HRP labeled secondary antibody diluted in TBS-T 0.1% and washed again 3 times for 5 min with TBS-T 0.1 %. The membrane was incubated with Clarity Western ECL substrate (Bio-Rad Laboratories, München, Germany) for 5 min and then with SuperSignal West Pico or SuperSignal West Dura Extended Substrate (Thermo Scientific, Rockford, IL, USA). The bands were visualized following the recommendations of the manufacturer using the ChemiDoc™ MP Imaging System (Bio-Rad Laboratories, München, Germany). Quantification of the band intensities was performed using the Image Lab 4.1 software (Bio-Rad Laboratories, München, Germany).

2.2.8 **Statistical Analysis**

The statistics and graphs were calculated and created using GraphPad Prism version 5.04 (La Jolla, USA). Statistical significance of differences for DSB repair measurements and immunofluorescence microscopy results were calculated using the nonparametric Mann-Whitney U-test. For MTT survival assays statistical calculations are described in section 2.2.5 where IC\(_{50}\)-values and Hill Slopes (variable slope) were compared. Protein level analysis by western blotting was evaluated calculating mean values and SD from ≥ 2 independent analyses. Cell cycle analysis was performed calculating mean values and SEM from ≥ 3 replicates obtained in as least 3 experiments and statistical significances between G2 cell cycle phase was calculated using nonparametric Mann-Whitney U-test.
3 RESULTS

3.1 DSB repair pathway investigation

Investigation of the DSB repair pathway usage upon FANCD2 deficiency and HPV infection was accomplished by introducing different EGFP-based reporter constructs for HR, NHEJ, MMEJ or SSA repair mechanisms into the cells based on the system developed in the lab of Professor Wiesmüller (Akyüz et al. 2002).

3.1.1 FANCD2 deficiency and DSB repair pathway choice

In this investigation the DNA damage response pathways to DSB and ICL stress were investigated in head and neck cancer cell lines with and without the knockdown of Fanconi Anemia complementation group proteins FANCD2 and FANCA in both HPV (-) UMSCC1 and HPV (+) UMSCC47 cell lines. An EGFP based DSB repair model system was used to determine each cell lines’ preferred repair mode. Using Amaxa® technology, the cells were nucleofected with 2.5µg each of pCMV-I-SecI and pBS control plasmids and 5 µg of a DNA construct enabling detection of HR, NHEJ, MMEJ or SSA. The cell lines were given 24 h to exponentially grow, after which repair was examined for each mechanism. A DSB introduced via I-SecI meganuclease-mediated cleavage in the EGFP reporter and subsequent repair was measured by flow cytometry by which the number of green fluorescent cells, reflecting repair of the DSB in the EGFP reporter was determined. Transfection efficiency was determined in split samples each. The percentage of green fluorescence among non-fluorescent cells obtained was compared to the overall transfection efficiency to calculate repair frequencies normalized individually for each construct. The results of this investigation revealed a significant 8.5 fold downregulation of error-free HR repair in FANCD2 deficient cells (UMSCC1-D2) compared to the control UMSCC1-NT cells carrying the expression plasmid for the non-targeting NT short hairpin RNA (Figure 7a; p value ≤ 0.01). At the same time FANCD2 deficient cells showed a 3.7 fold upregulation of error-prone NHEJ repair compared to the control cells (Figure 7b; p value ≤ 0.0001). DSB repair occurring via MMEJ or SSA did not show any significant differences for FANCD2 deficient cells compared to the corresponding control (Figure 7c-d).
Figure 7  Determination of double strand break repair (DSBR) mechanisms in head and neck cancer cell lines with and without the knockdown for Fanconi Anemia complementation group proteins FANCD2 and FANCA in both HPV (-) and HPV (+) cell lines. DSB was analyzed in the UMSCC1 and UMSCC47 cell lines 24 h after nucleofection using Amaxa® with 2.5 µg each of pCMV-I-SceI and pBS control plasmid and 5 µg of one of four DNA constructs for testing either (a) HR, (b) NHEJ, (c) MMEJ or (d) SSA. Using flow cytometry the fraction of green fluorescent cells in each cell line was determined to establish the repair frequency (%) based on the overall transfection efficiency. The mean values (n=3-15) and standard errors (SEM) are indicated in the graph with white bars indicating non-HPV cell lines and red bars indicating HPV positive cell lines. Control cell lines carry expression plasmid for the non-targeting NT short hairpin RNA (UMSCC1-NT and UMSCC47-NT) while FANCD2 or FANCA silenced cell lines carry either shRNA targeting FANCD2 (UMSCC1-D2 and UMSCC47-D2) or FANCA (UMSCC47-FA). Significant differences were established using the nonparametric Mann-Whitney-U test for all cell lines except UMSCC47FA (*p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001, ****p≤0.0001), as no corresponding non-HPV cell line was available.
3.1.2 HPV infection and DSB repair pathway choice

The effect of HPV infection alone on DSB repair frequency was analyzed in FANCD2 proficient cells carrying the expression plasmid for the non-targeting NT short hairpin RNA. The respective UMSCC47-NT cells being HPV (+) showed a 3.0 fold downregulation of the HR repair frequency compared to HPV (-) UMSSCC1-NT cells (Figure 7a; \( p \leq 0.01 \)). The NHEJ repair frequency in these HPV (+) cells was concomitantly significantly upregulated 2.0 fold compared to their HPV (-) counterparts (Figure 7b; \( p \leq 0.01 \)). In these results it can be seen that HPV infection mimics the FANCD2 deficient phenotype regarding the DSB repair phenotype. The MMEJ and SSA repair frequencies (Figure 7c-d) do not significantly differ between FANCD2 proficient cells with or without HPV infection suggesting that HPV infection does not appear to play a role in repair via the MMEJ and SSA pathway.

3.1.3 Effect of FANCD2 deficiency and HPV infection on DSB repair pathway choice

While cells not infected with HPV showed a difference in HR versus NHEJ repair frequency between FANCD2 proficient and FANCD2 deficient cells, this difference was no longer seen in HPV (+) cells. HR repair was almost equally downregulated (3.0 fold and 2.7 fold) in HPV (+) cells (UMSCC47-NT and UMSCC47-D2, respectively) compared to the non-infected control, UMSCC1-NT (Figure 7a; both \( p \) values \( \leq 0.01 \)). Further, HR frequencies in these two HPV (+) cells were not significantly different. At the same time an upregulation of NHEJ was seen for FANCD2 proficient (2.0 fold) and deficient (2.3 fold) UMSGCC47-cells with \( p \leq 0.01 \) and \( p \leq 0.001 \) respectively. The extent of upregulation of DSB repair by NHEJ was less than for the HPV (-) FANCD2 deficient compared to the control UMSCC1-NT cells (2.0 fold and 2.3 fold vs. 3.7 fold; \( p \leq 0.0001 \)) (Figure 7b). Similar to the downregulation of HR, NHEJ frequencies in the two HPV (+) cells were not significantly different. While HPV infection had an influence on DSB repair via the HR and NHEJ pathway, the influence was not as pronounced regarding MMEJ and SSA repair. The only significant difference in these pathways was seen for the HPV (+) FANCD2 deficient cell, that displayed an 1.8 fold upregulation of MMEJ repair compared to the non-infected control expressing FANCD2 (Figure 7c; \( p \leq 0.05 \)). Regarding SSA repair, all cell lines showed similar frequencies.
The UMSSCC47FA cell line with stable knockdown of Fanconi Anemia complementation group protein FANCA was not included in the statistical calculations for its lack of an HPV (-) counterpart. Moreover, this cell line particularly showed a growth defect and prompted caution to be taken in its interpretation. Any statistical differences seen in this cell line for HR, NHEJ, MMEJ or SSA repair would be subjected to the uncertainty regarding the causal effect of FANCA protein deficiency versus HPV infection status. Nonetheless, the results have been included for completeness and potential interest for further investigations.

### 3.2 Immunofluorescence Microscopy Analysis

In this investigation immunofluorescence microscopy was employed as a technique to dissect the DNA repair pathway and evaluate the effect of FANCD2 deficiency and HPV infection on foci formation. The DNA damage was introduced by MMC resulting in DNA intra- and interstrand crosslinks, replication fork stalling and finally DSB. The FA pathway coordinates several distinct repair activities including HR to remove crosslinks (Moldovan and D’Andrea 2009, Thompson and Hinz 2009). The focal accumulations of damage markers 53BP1, recombinase RAD51 and FANCD2 were analyzed at set time points post treatment. All images were acquired using the Olympus BX51 microscope and quantitative analysis was performed with *CellF 2.5 Software* with focal accumulations (foci) per nucleus in 50 foci positive cells (n≥2) counted.

#### 3.2.1 53BP1 foci analysis

The 53BP1 nuclear focal formation (foci) in head and neck cancer cell lines with and without the knockdown of Fanconi Anemia complementation group protein FANCD2 in both HPV (-) UMSCC1 and HPV (+) UMSCC47 cell lines was analyzed. The 53BP1 nuclear foci formation at 6, 16 and 24 h after 1 h of 2 µM MMC exposure, in UMSCC1 and UMSCC47 cell lines, was investigated by quantitative immunofluorescence microscopy. The UMSCC1 and UMSCC47 cell lines were first seeded on glass slides and incubated 24 h to attach. Following seeding the cells were then exposed to 2 µM of MMC for 1 h and allowed to recover for 6, 16 or 24 h in media prior to being fixed for 10 min with 3.7% formaldehyde. The untreated cells, those not exposed to the genotoxic substance MMC, were fixed after a media change corresponding to treatment and
subsequent cultivation at the 6 h time point of the treated cells. To visualize the foci formation microscopically immunostaining was done by using a specific anti-53BP1 primary antibody and an Alexa Fluor 555 labeled secondary antibody. The slides were counterstained with DAPI for nuclear identification and nuclear foci counted. The results are represented graphically in Figure 8a and demonstrate the foci formation at set time intervals after release from MMC as well as foci formation in untreated cells. The increase in foci represents DSB formation and reduction implicates removal via DSB repair. For FANCD2 proficient HPV (-) cells (UMSCC1-NT) peak foci formation was seen at 16 h with less foci present at 24 h. The other cells showed a continuous increase in foci. The results showed that at 24 h post-MMC exposure a significant increase in 53BP1 foci formation for FANCD2 deficient cells (UMSCC1-D2, p value ≤ 0.001) and HPV (+) cells (UMSCC47-NT, p value ≤ 0.01 and UMSCC47-D2, p value ≤ 0.001) was seen in comparison to the FANCD2 proficient HPV (-) cells (UMSCC1-NT) which were the only cells displaying a reduction in foci formation at 24 h (Figure 8b). In addition, no significant differences in foci formation were found at the other time points post-MMC exposure among the cell lines.
53BP1 nuclear foci formation

Figure 8  53BP1 nuclear foci formation 6, 16 and 24 h after 1 h of 2 µM MMC exposure in UMSCC1 and UMSCC47 cell lines.  (a-c) The UMSCC1 and UMSCC47 cell lines were seeded on glass slides (24 h attachment). The cells were then exposed to 2 µM of MMC for 1 h and allowed to recover for 6, 16 or 24 h in fresh media prior to fixation for 10 min with 3.7% formaldehyde. The untreated cells were fixed after corresponding media change and cultivation up to 6 h time point. Immunostaining was accomplished using a specific anti-53BP1 primary antibody and an Alexa Fluor 555 labelled secondary antibody. The slides were counterstained with DAPI (blue) and nuclear 53BP1 foci (red) counted using the Olympus BX51 microscope. Quantitative analysis was performed with CellF 2.5 Software and foci per nucleus in 50 foci positive cells per slide counted. Mean and SEM are based on 7-8 measurements from 4 repeat experiments. (a) Graphical presentation of recovery post-MMC treatment. (b) Foci formation 24 h after MMC exposure. White bars indicate HPV (-) cells and red bars indicate HPV (+) cells. Significant differences in foci formation between the cell lines were calculated using the nonparametric Mann-Whitney-U test and indicated (**p ≤ 0.01, ***p ≤ 0.001). No significant differences in foci formation were found at the other time points post-MMC exposure. (c) Representative images of 53BP1 nuclear foci formation in untreated cells and at 6, 16 and 24 h after MMC exposure. The blue (DAPI) staining shows the DNA and the red foci the secondary antibody labeling 53BP1.
The changes in 53BP1 foci numbers following MMC treatment did not vary significantly between HPV (+) cells regardless of FANCD2 status at 24 h whereas in HPV (-) cells the FANCD2 deficiency resulted in the same increase in foci accumulations at 24 h. Representative images acquired show 53BP1 nuclear foci formation in untreated cells and at 6, 16 and 24 h after MMC exposure (Figure 8c).

3.2.2 RAD51 foci analysis

The foci of the recombinase RAD51, known to play a major role in HR, were quantified at different time points in the same way as 53BP1. The immunostaining was done by using a specific anti-RAD51 primary antibody and an Alexa Fluor 555 labeled secondary antibody. The results are represented graphically in Figure 9a and demonstrate the foci formation at set time intervals after release from MMC as well as foci formation in untreated cells. An increase in RAD51 foci after 6 and 16 h was seen as well as a slight decrease in foci formation at 24 h in most cell lines. Only the HPV (+) FANCD2 proficient cell line UMSCC47-NT did not demonstrate this slight decrease at 24 h. The results showed that at 16 and 24 h post-MMC exposure a significant increase in RAD51 foci formation was only seen for FANCD2 deficient HPV (-) cells (UMSCC1-D2, p value ≤ 0.05 at 16 h and p value ≤ 0.01 at 24 h) in comparison to the FANCD2 proficient HPV (-) cells (UMSCC1-NT) (Figure 9b and c). Further, no significant differences between foci formation were found at the other time points post-MMC exposure. The images acquired (Figure 9d), showed RAD51 nuclear foci numbers in untreated cells and at 6, 16 and 24 h after MMC exposure. The increase in RAD51 foci at 6 and 16 h with a slight decrease at 24 h can be seen graphically but the changes were not statistically significant. A significant increase in RAD51 foci was seen in the FANCD2 deficient UMSCC1-D2 cells compared to the FANCD2 proficient UMSCC1-NT cells at 16 and 24 h. HPV status, however, did not significantly increase the number of RAD51 foci seen at these time points compared to the FANCD2 proficient control cell.
a) RAD51 Foci after MMC Treatment

![Graph showing RAD51 foci over time after MMC treatment.](image)

b) RAD51 Foci at 16 hours

![Bar graph showing RAD51 foci at 16 hours.](image)

- * Significance

Rad51 MMC (16h)

- ** Significance

Rad51 MMC (24h)
Figure 9  RAD51 nuclear foci formation 6, 16 and 24 h after 1 h of 2 µM MMC exposure in UMSCC1 and UMSCC47 cell lines. (a-c) The UMSCC1 and UMSCC47 cell lines were seeded on glass slides (24 h attachment). The cells were then exposed to 2 µM of MMC for 1 h and allowed to recover for 6, 16 or 24 h in fresh media prior to fixation for 10 min with 3.7% formaldehyde. The untreated cells were fixed after corresponding media change and cultivation up to the 6 h time point. Immunostaining was accomplished using a specific anti-RAD51 primary antibody and an Alexa Fluor 555 labelled secondary antibody. The slides were counterstained with DAPI (blue) and nuclear RAD51 foci (red) counted using the Olympus BX51 microscope. Quantitative analysis was performed with CellF 2.5 Software and foci per nucleus in 50 foci positive cells per slide counted. Mean and SEM are based on 7-8 measurements from 4 repeat experiments. (a) Graphical presentation of recovery post-MMC treatment. (b) Foci formation 16 h and (c) 24 h after MMC exposure. White bars indicate HPV (-) cells and red bars indicate HPV (+) cells. Significant differences in foci formation between the cell lines were calculated using the nonparametric Mann-Whitney-U test and indicated (*p ≤ 0.05, **p ≤ 0.01). No significant differences in foci formation were found at the other time points post-MMC exposure. (d) Rad51 nuclear foci formation in untreated cells and at 6, 16 and 24 h after MMC exposure. The blue (DAPI) staining shows the DNA and the red foci the secondary antibody labeling RAD51.
3.2.3 FANCD2 foci analysis

The foci of the Fanconi Anemia complementation group protein, FANCD2, monoubiquitinated in response to DNA damage and involved in homology directed repair, were quantified at different time points in the same way as 53BP and RAD51. The immunostaining was done by using a specific anti-FANCD2 primary antibody and an Alexa Fluor 555 labeled secondary antibody. The results are represented graphically in Figure 10a and demonstrate the foci formation at set time intervals after release from MMC as well as foci formation in untreated cells. For the HPV (+) cell line a steady increase in FANCD2 foci after 6, 16 and 24 h was seen. For the HPV (-) cell line there was also a steady increase in foci formation at 6 and 16 h but a decrease in foci formation at 24 h was observed. The foci formation for the HPV (+) cell line was significantly elevated compared to the HPV (-) cell line at 16 and 24 h post-MMC exposure (UMSCC47-NT, p value ≤ 0.01 at 16 h and p value ≤ 0.001 at 24 h) (Figure 10b and c).

3.3 Survival analysis after MMC and IQD exposure

To investigate the effect of HPV infection on the UMSCC cell line-specific MMC sensitivities, survival of all cell lines differing in FANCD2 proficiency and HPV infection status was determined by MTT assay-based analysis after 48 h of MMC treatment. Optimal MMC concentration ranges were previously established. Further, to investigate the response of the UMSCC cell lines to the PARP inhibitor, IQD, known to target deficiencies in the HR pathway, MTT assay was performed after 7 days of IQD treatment. Again, optimal IQD concentrations ranges were previously established. Quantitative analysis was performed with GraphPad Prism version 5.04 to obtain IC_{50} values and the statistical significance of differences calculated between them.
a) FANCD2 Foci after MMC Treatment

![Graph showing FANCD2 Foci after MMC Treatment]

- Blue line: UMSCC1NT
- Red line: UMSCC47NT

b) FANCD2 Foci at 16 hours

c) FANCD2 Foci at 24 hours
FANCD2 nuclear foci formation

Figure 10  FANCD2 nuclear foci formation 6, 16 and 24 h after 1 h of 2 µM MMC exposure in UMSCC1 and UMSCC47 cell lines. (a-c) The UMSCC1 and UMSCC47 cell lines were seeded on glass slides (24 h attachment). The cells were then exposed to 2 µM of MMC for 1 h and allowed to recover for 6, 16 or 24 h in fresh media prior to fixation for 10 min with 3.7% formaldehyde. The untreated cells were fixed after corresponding media change and cultivation up to the 6 h time point. Immunostaining was accomplished using a specific anti-FANCD2 primary antibody and an Alexa Fluor 555 labelled secondary antibody. The slides were counterstained with DAPI (blue) and nuclear FANCD2 foci (red) counted using the Olympus BX51 microscope. Quantitative analysis was performed with CellF 2.5 Software and foci per nucleus in 50 foci positive cells per slide counted. Mean and SEM are based on 4-10 measurements from 3 to 4 repeat experiments. (a) Graphical presentation of recovery post-MMC treatment. (b) Foci formation 16 h and (c) 24 h after MMC exposure. White bars indicate HPV (-) cells and red bars indicate HPV (+) cells. Significant differences in foci formation between the HPV (+) and HPV (-) control cell lines were calculated using the nonparametric Mann-Whitney-U test and indicated (**p ≤ 0.01, ***p ≤ 0.001). (d) FANCD2 nuclear foci formation in untreated cells and at 6, 16 and 24 h after MMC exposure. The blue (DAPI) staining shows the DNA and the red foci the secondary antibody labeling FANCD2.

3.3.1  FANCD2 deficiency and sensitivity to MMC

The MTT assay was used first to verify the increased sensitivity to ICL inducing agents such as MMC in cells with a defective FA pathway. The IC_{50} values were obtained from each cell survival curve and the significances in survival were calculated by use of GraphPad Prism version 5.04. The survival curves of all cell lines are shown in Figure 11a, and individual comparisons in Figure 11b – e. Significant 92% reduction in survival (IC_{50}) or increased sensitivity to MMC was seen in the FANCD2 deficient UMSCC1-D2 line compared to its proficient UMSCC1-NT counterpart (Figure 11b; p ≤ 0.0001). Although the reduction in survival could still be seen for the FANCD2 deficient HPV (+)
UMSCC47-D2 line compared to its proficient UMSCC47-NT counterpart, the significant reduction was only 33% (Figure 11c; $p = 0.0184$).

To investigate the effect of HPV infection, the FANCD2 proficient cell lines UMSCC1-NT and UMSCC47-NT were compared as were the FANCD2 deficient cell lines UMSCC1-D2 and UMSCC47-D2. It was seen that HPV infection alone did not significantly reduce survival in cells exposed to MMC with a working FA pathway (Figure 11d; $p = 0.2074$). In the case of the FANCD2 deficient cell lines, HPV infection also did not reduce survival; it had even significantly improved survival (Figure 11e; $p \leq 0.0001$).
Figure 11  MMC sensitivities of UMSCC1 and UMSCC47 cell lines. (a-e) For all cell lines 20,000 cells were seeded in a 96-well plate and given 24 h to attach. MMC sensitivities for all cell lines differing in their FANCD2 proficiency and HPV infection status were determined by MTT assays after 48 h of treatment with MMC, concentrations ranging from 16.625 nM – 16 µM. Quantitative analysis was performed with GraphPad Prism version 5.04 to obtain IC_{50} values and the statistical significance between them. The survival values were obtained from a log(inhibitor) versus response-variable slope fit with bottom constraint equal to 0. Mean and SEM are based on 12-16 measurements in 4 repeat experiments. Mean values for media controls were defined as 100% each. (a) Graphical representation of all cell survival curves after 48 h of exposure to MMC. Survival of (b) HPV (-) and (c) HPV (+) cells with and without FANCD2 proficiency. Significantly decreased survival was seen in FANCD2 deficient versus FANCD2 proficient cells, but this difference was less with HPV infection. Survival of (d) FANCD2 proficient and (e) FANCD2 deficient cells with and without HPV infection. Significantly increased survival was seen in HPV (-) versus HPV (+) FANCD2 deficient cells, with greater IC_{50} values for HPV infection. No significant difference in survival was seen between HPV (-) and HPV (+) FANCD2 proficient cells.

3.3.2 HPV infection and sensitivity to IQD in FANCD2 proficient cells

The response of the UMSCC cell lines to the PARP inhibitor, IQD, was investigated by MTT assay to see how HPV infection and FANCD2 deficiency influence cell survival by inhibiting alternative repair mechanisms and promoting HR. The IC_{50} values were obtained for each cell survival curve and the significances in survival were calculated by use of GraphPad Prism version 5.04. The IQD sensitivities of all cell lines are shown in Table 7. No significant reduction in survival or increased sensitivity to IQD was seen in the HPV (-) FANCD2 deficient UMSCC1-D2 line compared to its proficient HPV (-) UMSCC1-NT counterpart (Table 7 first data row; p = 0.8650). This did not change with the HPV infected FANCD2 deficient UMSCC47-D2 line, compared to the UMSCC47-NT line (Table 7 second data row; p = 0.3946).
To investigate the effect of HPV infection, the FANCD2 proficient cell lines UMSCC1-NT and UMSCC47-NT were compared as were the FANCD2 deficient cell lines UMSCC1-D2 and UMSCC47-D2. When comparing the FANCD2 proficient cell lines, it was seen in two repeat experiments that HPV infection significantly reduced survival in cells exposed to IQD (Table 7 fourth data row; \( p = 0.0209 \)). However, an extension of the data by a colleague indicated that survival or IQD sensitivity of HPV (+) and HPV (-) FANCD2 proficient cells was not statistically different. In the case of the FANCD2 deficient cell lines, HPV infection did not significantly reduce survival (Table 7 third data row; \( p = 0.6961 \)). In FA pathway functioning cells, the HPV infection significantly reduced survival with an increased sensitivity to the PARP inhibitor IQD in this investigation. Though HPV altered survival in FA pathway functioning cells, it did not significantly decrease survival or increase sensitivity to IQD in FANCD2 deficient cell lines.

Table 7. PARP inhibitor (IQD) sensitivities of UMSCC1 and UMSCC47 cell lines

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>IC(_{50}) (µM)</th>
<th>Cell Line</th>
<th>IC(_{50}) (µM)</th>
<th>p</th>
<th>Cell Type</th>
</tr>
</thead>
<tbody>
<tr>
<td>UMSCC1-NT</td>
<td>338</td>
<td>UMSCC1-D2</td>
<td>315</td>
<td>0.8650 (ns)</td>
<td>Survival of HPV (-) Cells</td>
</tr>
<tr>
<td>UMSCC47-NT</td>
<td>220</td>
<td>UMSCC47-D2</td>
<td>257</td>
<td>0.3946 (ns)</td>
<td>Survival of HPV (+) Cells</td>
</tr>
<tr>
<td>UMSCC1-D2</td>
<td>315</td>
<td>UMSCC47-D2</td>
<td>257</td>
<td>0.6961 (ns)</td>
<td>Survival of FANCD2 Deficient Cells</td>
</tr>
<tr>
<td>UMSCC1-NT</td>
<td>338</td>
<td>UMSCC47-NT</td>
<td>220</td>
<td>0.0209 (*)</td>
<td>Survival of FANCD2 Proficient Cells</td>
</tr>
</tbody>
</table>

For all cell lines 20,000 cells were seeded in a 96-well plate and given 24 h to attach. IQD sensitivities for all cell lines differing in their FANCD2 proficiency and HPV infection status were determined by MTT assays after 7 days of treatment with IQD, concentrations ranging from 3.9 µM – 1000 µM. Quantitative analysis was performed with GraphPad Prism version 5.04 to obtain IC\(_{50}\) values and the statistical significance between them. The survival values were obtained from a log(inhibitor) versus response using variable slope fit. Mean and SEM are based on 6-8 measurements in 2 repeat experiments. Mean values for DMSO controls were defined as 100% each. Red text denotes the HPV (+) cell lines and black text denotes the HPV (-) cell lines. A significant difference in survival was only seen between the FANCD2 proficient cells with and without HPV infection. Significances are defined as *\( p \leq 0.05 \), **\( p \leq 0.01 \), ***\( p \leq 0.001 \), ****\( p \leq 0.0001 \).
3.4 Protein level analysis by Western Blot

The effect of MMC treatment on proteins involved in DSB repair and cell cycle checkpoints was investigated by Western Blot analysis in order to investigate the influence of FANCD2 deficiency and HPV infection on protein level expression. Proteins investigated included CHK1 (56kDa) as well as the phosphorylated form of CHK1 (p-CHK1; 56kDa), the phosphorylated form of RPA (p-RPA; 32kDa), RAD51 (37-43 kDa) and FANCD2 (166 kDa). Mean values were obtained from 3 independent experiments whereby the healthy FANCD2 proficient cell line, UMSCC1-NT, after 24 h of MMC treatment, was used as a reference and set to 100% each. All cell lines were investigated with no MMC and at 16h and 24h post 1h MMC treatment. The values were quantified from signal intensities of bands measured with ChemiImagerTM5500 in Western Blot. The loading control GAPDH (36-40 kDa) was used for each protein band except FANCD2, which required DNA-PK (460 kDa) detection due to FANCD2’s larger size. Though these calculations revealed no significant differences, trends in protein level changes could be observed (Figure 12a – d). The representative Western Blots are shown in Figure 12e. Phosphorylated proteins p-CHK1 and p-RPA indicated checkpoint activation and DNA damage and ssDNA repair intermediates respectively, post-MMC treatment. The CHK1 protein was used to quantify phosphorylated versus overall protein levels. Protein levels of RAD51 and FANCD2 post DNA damage revealed insight into pathway activation whereby RAD51 is the principal recombinase in HR and FANCD2 a central protein that connects the FA pathway to HR mediated repair (Pilonetto et al. 2009, De Vos et al. 2012). The trend regarding p-CHK1 levels was a steady increase from non-treatment to 16h and 24h post exposure to MMC. Thus, the HPV (-) FANCD2 proficient cell line UMSCC1-NT, showed a gradual increase in p-CHK1 levels. This observed increase was much greater for the cell lines deficient in FANCD2 and those HPV (+), with or without FANCD2 proficiency. To exclude changes in total CHK1 levels rather than its phosphorylation, p-CHK1 levels were normalized by the total CHK1 concentration each (Figure 12a). The overall protein level of CHK1 revealed no major changes for the different cell lines with or without MMC treatment. Very high CHK1 protein levels observed for the UMSCC1-NT cell line, as seen in Figure 12a, were only observed in one repeat experiment and therefore not representative but rather due to low protein concentrations in these samples. With regard to the p-RPA levels, UMSCC1-NT cells showed low levels with and without MMC treatment (Figure 12b). Cell lines with
Figure 12  DNA repair protein expression of UMSCC1 and UMSCC47 cell lines after treatment with MMC. (a-d) All cell lines were grown maximally to subconfluence and treated for 1 h with 2 µM of MMC or left untreated. At 16 h and 24 h post treatment, cells were lysed. The protein extracts were analyzed by western blot using antibodies specific for the indicated protein and quantification of protein signal relative to loading control each, (GAPDH or DNA-PK), was carried out using ChemiImagerTM5500. The signals for (a) p-CHK1/CHK1, (b) FANCD2/DNA-PK, (c) p-RPA/GAPDH, and (d) RAD51/GAPDH were determined. Values represent the mean and SD of 3 independent experiments. (e) Western blots showing indicated protein expression levels in MMC treated and non-treated cell lines. GAPDH or DNA-PK immunolabeling to control for loading is displayed below the corresponding specific immunoblots.

FANCD2 deficiency and those HPV (+) either with or without FANCD2 proficiency showed similar low levels of p-RPA when not exposed to MMC. However, upon MMC exposure the levels of phosphorylated RPA drastically increased, mostly in FANCD2-deficient and HPV (+) cells (Figure 12b). Compared to levels of p-RPA, the RAD51 protein level trends did increase for all cell lines upon exposure to MMC but not nearly as greatly (Figure 12d). Finally, the FANCD2 protein concentration increased post MMC
exposure in the UMSCC1-NT cell. The HPV infected cell line counterpart showed a similar concentration increase, albeit lower overall protein concentrations. The FANCD2 protein concentrations according to Western Blot were as expected very low for the cell lines UMSCC1-D2 and UMSCC47-D2, silenced for FANCD2 (Figure 12c). Due to high protein expression levels in the NT cell line, no quantification of the bands could be determined. Note the increase of the high molecular weight (MW) band likely indicating ubiquitination (Ub) of FANCD2 after MMC exposure. Garcia-Higuera et al. were able to show that the FANCD2-L isoform, that differs by 7 kDA from the FANCD2-S isoform, was the Ub form of FANCD2-S that was required for nuclear FANCD2 foci formation (Garcia-Higuera et al. 2001).

3.5 Cell Cycle Analysis

The DNA content analysis was performed by propidium iodide staining of cells in order to determine cell cycle phases as well as apoptosis/sub G1 phase. The UMSSC cell lines were investigated for cell cycle distribution before and after exposure to MMC in order to investigate the effect of this DNA cross-linking agent on the cell cycle distribution.

Alterations in cell cycle influence DNA repair through differences in repair protein expression and differential protein modifications (Rothkamm et al. 2003, Wohlbold and Fisher 2009). Cell cycle distribution in HPV (-) cell lines UMSSC1NT and UMSSC1D2 was very similar during mock treatment with predominant (> 60%) G1 phase cells. Upon MMC treatment a G2 phase increase from just below 20% to > 40% was seen (Figure 13a; green *). The HPV (+) cell lines UMSSC47NT and UMSSC47D2 during mock treatment showed a higher proportion of G2 phase cells (>40%) than their HPV (-) counterparts (Figure 13a; red *). Upon MMC treatment little change in cell cycle distribution was seen for the FANCD2 proficient UMSSC47NT cell line compared to mock treatment, however, the FANCD2 deficient cell line showed an even greater percentage of cells arrested in G2 phase (>60%) with resulting decrease in G1 phase (Figure 13a; black *). No significant changes in S phase distribution were seen for all HPV (+) and HPV (-) cell lines (Figure 14a). Table 8 lists further significances in cell cycle distribution differences not included in the graphical presentation for clarity.
Table 8. MMC treatment and HPV Effect on UMSCC1 G2 Cell Phase Distribution

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>Treatment</th>
<th>Cell Line</th>
<th>Treatment</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>UMSCC1-NT</td>
<td>Mock</td>
<td>UMSCC47-D2</td>
<td>Mock</td>
<td>****</td>
</tr>
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<td>MMC</td>
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<td>Mock</td>
<td>UMSCC47-NT</td>
<td>MMC</td>
<td>***</td>
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<td>Mock</td>
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<td>***</td>
</tr>
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<td>MMC</td>
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<td>UMSCC47-D2</td>
<td>MMC</td>
<td>***</td>
</tr>
</tbody>
</table>

Red text denotes the HPV (+) cell lines and black text denotes the HPV (-) cell lines. Significances are defined as

*p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001, ****p ≤ 0.0001

For HPV (-) cell lines the percentage of cells in sub G1 (indicating apoptosis) did not significantly change upon exposure to MMC with the percentage of cells in this phase maintaining 1.0% to 1.5% for mock treated and MMC treated UMSCC1NT and UMSCC1D2 cell lines. Further, FANCD2 deficiency did not significantly alter the percentage of cells in sub G1 phase and demonstrated behavior similar to FANCD2 proficient cells in both mock and MMC treated states. The HPV (+) cell lines UMSCC47NT and UMSCC47D2 showed a sub G1 fraction of ~0.5% with mock treatment. Exposure to MMC resulted in a slight increase to ~1.0% in the sub G1 phase, however, not of statistical significance. Also in the HPV (+) cell lines FANCD2 deficiency did not significantly alter the percentage of sub G1 fraction compared to its FANCD2 proficient counterpart possibly attributing this trend in increase of sub G1 function to HPV infection and not FANCD2 status (Figure 13b).
Figure 13 Effect of MMC treatment on the cell cycle distribution of UMSCC1 and UMSCC47 cells. (a-b) For all cell lines 300,000 cells were seeded in a 6-well culture dish and given 24 h to attach. Following attachment, control cell lines (left side of graphs) were subjected media change (Mock) or treated for 1 h with 2 µM with MMC (right side of graphs, MMC). Both groups were then given 24 h to recover. (a) Cellular distribution of cell cycle phases with viable cells divided into G1, S and G2 (represented in %) and (b) apoptosis were measured for all cell lines by flow cytometry following staining with propidium iodide. Apoptosis was defined as Sub G1 fraction. The mean values are based on 3 measurement and standard errors (SEM) are indicated in the graphs. For clarity, statistical significances are indicated only for ****p values (****p≤0.0001).
4 DISCUSSION

The discovery of human papillomaviruses and their causal relationship to cancers in the 1970s was awarded the 2008 Nobel-prize in Physiology or Medicine to Dr. Harald zur Hausen for his discovery that HPV caused cervical cancer (zur Hausen 2009). Since this discovery a rapid expansion of knowledge in this field took place with many more links of HPV to cancer such as laryngeal, esophageal (Syrjänen et al. 1982) and of the oral cavity (Syrjänen et al. 1983). With advent of modern virology in the 1970s many more types of HPV could be identified and classified as high risk, with oncogenic potential, or low risk. Further characterization of viral genes such as the E6 and E7 oncogenes eventually led to the development of the HPV vaccine (Malisic 2013). Of the many types of HPV known, it is specifically the HPV-16 type that is involved in head and neck cancers and often present in squamous cell cancers of the oropharynx (Gillison 2000, Kreimer et al. 2005).

FA patients have an increased risk for SCC, especially at sites subject to HPV-16 infection such as the oral cavity (Spardy et al. 2007). This clinical connection illustrates the relevance of gaining knowledge on the FA pathway in these patients, as it can offer insights into how HPV influences such a DNA repair pathway. Though much is known about how HPV interferes with cellular processes, this study could show that HPV resembles a FA-like phenotype in various aspects of DNA repair.

4.1 FANCD2 deficiency and HPV infection alter DSB repair pathway usage

DSB repair analysis of HPV infected and FANCD2 deficient HNSCC cells in this investigation was initiated with analysis of pathway-specific DSB repair activities using the DSB repair test system established in the lab of Professor Wiesmüller (Akyüz et al. 2002). There are several pathways that compete for DSB repair including HR, NHEJ, MMEJ and SSA each with a different spectrum and degree of mutagenic consequences including deletions, loss of heterozygosity, translocations, and chromosome loss that can contribute to cancer development (Wyman and Kanaar 2006, Shrivastav et al. 2008). Entire fragments of chromosomes can be lost if such breaks are not repaired prior to cell division (Bennardo et al. 2009). Identifying factors regulating DSB repair pathway choice
can provide mechanistic insight into how cellular responses to DSB damage are affected and potentially provide targets for future gene therapies.

HR repair normally uses the identical sister chromatid as a template for RAD51-mediated strand invasion, and repair DNA synthesis making is a relatively precise form of repair. HR repair can be mutagenic, such as in the case of exchanges of imperfectly homologous sequences or loss of heterozygosity, also HR’s intrinsic properties of genetic exchange through gene conversion or crossing over can generate genetic instability (Guirouilh-Barbat et al. 2014). End-joining pathways show various degrees of mutagenesis depending on the degree of end-processing and fidelity of end-pairing. In the case of NHEJ, also called classical end joining, DSB repair relies on the Ku70–Ku80 and DNA ligase IV–XRCC4 heterodimers. Repair is often error-prone but can be fairly accurate regarding the ends such as during end-joining via V(D)J recombination when DSB ends are ligated without significant processing (Ma et al. 2005). However, V(D)J recombination deletes DNA segments, thus creating mutations with respect to the overall chromosomal DNA sequence. Another end-joining pathway in the absence of the classical NHEJ repair factors is MMEJ or micro-SSA. MMEJ is Ku-independent, mediated by base pairing between microhomologous sequences of up to 25 nucleotides and always leads to deletion mutations. The SSA pathway is a mutagenic mechanism that operates between long direct repeats flanking a DSB and results in loss of one of the repeats and intervening sequence. Sharing features with MMEJ, it differs in the amount of homology used for end alignments requiring long homologous sequences (> 25 nucleotides) but always causes genomic deletions (Bennardo et al. 2008, McVey and Lee 2008, Sfeir and Symington 2015). The balance between the pathway choices is affected by a variety of factors including cell type, cell cycles phases and as could be shown in this study also by HPV infection and downregulation or inactivation of Fanconi Anemia repair proteins like FANCD2.

4.1.1 FANCD2 deficiency shifts HR towards a more error-prone NHEJ DSB repair pathway

In this investigation it was found that FANCD2 deficient cells showed a shift from HR repair to NHEJ repair with a 3.7 fold increase in error-prone NHEJ repair compared to the isogenic FANCD2 proficient cell line. This finding is consistent with those of Adama et al. who showed that FANCD2 deficiency caused an upregulation of the error-prone DSB
repair pathway NHEJ (Adamo et al. 2010). Such increases in NHEJ repair inevitably lead to genomic instability resulting from partially inappropriate joining of chromosome ends. FANCD2 is necessary for a variety of HR-mediated DNA repair processes such as gene conversion or SDSA. Yamamoto et al. could show that FANCD2 is required for efficient gene targeting and that FANCD2 knockout was defective in HR-mediated DSB repair because FANCD2 was necessary for an HR-mediated DNA repair processes involving a sub-pathway of HR. This sub-pathway normally mediates gene conversion without increased frequency of nontemplated point mutations (Yamamoto et al. 2005). These findings were demonstrated in this investigation as seen in the 8.5 fold downregulation of error-free HR repair in the FANCD2 deficient UMSCC1-D2 cell line compared to the healthy UMSCC1-NT cell line. Another group showed that FANCD2 may have a role in preventing NHEJ factors such as Ku70/80 from binding to DSB ends by direct inhibition or facilitating HR initiating events such as DNA end resection (Pace et al. 2010). These findings could in part explain the upregulated NHEJ in FANCD2 deficient cells of this investigation. The consistencies in these findings served as establishment of the DSB repair system used in the investigation of the effects of HPV infection in the HNSCC cell lines.

4.1.2 HPV infection downregulates error-free HR and upregulates error-prone NHEJ

The FA-like phenotype of HPV-16 infected HNSCC cells is supported by studies demonstrating interplay between HPV and the FA pathways. Previous reports showed that the HPV-16 E7 oncogene stimulated transcription of FANCD2, thereby activating the FA pathway (Spardy et al. 2007; Hoskins et al. 2008). In this investigation, HPV infection could not activate the FA pathway by stimulation of FANCD2 transcription in the FANCD2 deficient cells, UMSCC47-D2. Rather, DNA repair behavior of HPV infected proficient and deficient cells in their reduced ability to activate HR repair was similar suggesting a mechanistic overlap between the purely HPV attributed HR repair defect and the combined HPV and FA attributed HR defect. A recently published study showed that HNSCC cells deficient in HR, upon exposure to PARP1 inhibitor olaparib made them radiosensitive by disabling the DNA replication fork elongation response (Wurster et al. 2016). This shows the principle of ‘synthetic lethality’ of PARP1 inhibitors in cancers impaired in HR repair, as is already known for BRCA1/2 mutations. These HNSCCs did
not carry a corresponding mutation, suggesting that alternative determinants of PARP1 sensitivity exist. Understanding these alternative determinants could lead to better therapies in the future.

It is still not clear, however, how the connection between HPV and the FA pathway leads to carcinogenesis but a balance between DNA repair interference and viral replication possibly plays a role in analogy to differences in HR and DNA replication in HNSCC in general (Wurster et al. 2016). HPV infection causes a mutagenic environment by distorting correct DNA repair pathway usage by mechanisms including altered DNA repair, as shown in this investigation for upregulated NHEJ and downregulation of HR, most likely resulting from altered expression of DNA repair proteins, discussed in detail in section 4.2. An intact FA pathway would normally oppose these HPV deregulations, but it is possible that the HPV E6 and E7 oncogenes directly target or overcome this deregulation and interfere with the functions of critical DNA repair proteins, namely FANCD2 and the HR repair factor BRCA1, normally activated by FA signaling (Smith et al. 2015). This interference with BRCA1 could also be seen by further data from the Wiesmüller lab, that demonstrated the expected reduced BRCA1 foci formation as a result of FANCD2 deficiency but also a reduction of BRCA1 foci in HPV infected FANCD2 proficient cells (Salles, unpublished data). This observation of reduced BRCA1 function may offer an explanation for the downregulation of HR seen in this HPV infected FANCD2 proficient cell line. Previous work had also shown that mutations in various genes such as *BRCA1*, *BRCA2*, and *PALB2*, led to a shift from error-free to error-prone DSB repair pathway usage (Keimling et al. 2011; Obermeier et al. 2016). However, different from these previous findings, here HPV (+) cells showed a shift from HR to NHEJ predominantly, rather than to MMEJ or SSA. HPV may intervene with additional proteins in the HR pathway and could in part explain the shift towards error-prone NHEJ. Further study of the FA pathway and HR targets could provide more insights for better mechanistic understanding of HPV in carcinogenesis especially in terms of viral replication as was suggested in two other studies that found that HPV oncogenes activate the ATM pathway for use by viral genomes for replication (Moody and Laimins 2009, Gillespie et al. 2012).
4.1.3 HPV infected FANCD2 proficient and deficient cells show no further difference in DSB repair usage

No significant differences in HR or NHEJ between HPV infected FANCD2 proficient and deficient cells were observed. HR was almost equally downregulated and NHEJ was almost equally upregulated in the HPV infected cell lines regardless of the FANCD2 status. This could suggest that HPV infection may act in a FA-like manner interfering in HR repair much like FANCD2 deficiency.

Though generally an upregulation of NHEJ and downregulation of HR is seen in all HPV (+) cell lines compared to the control, differences towards FANCD2 deficient cells were seen. Thus, HPV infected cells still showed significantly more HR compared to FANCD2 deficient cells (p ≤ 0.01). Though reflecting generally the FA-like phenotype of downregulated HR, the difference seen could be perhaps explained by viral intervention to promote viral replication or possibly influence cell longevity. Some groups have demonstrated that the HPV-16 virus E2 protein can bind to TopBP1, an interaction partner required for optimal virus replication (Boner et al. 2002, Gauson et al. 2015), and more recent evidence has shown that TopBP1 is required for full activation of ATR (Kumagai et al. 2006). Recent findings have shown that TopBP1 is necessary for HR (Brown et al. 2014, Moudry et al. 2016). The beneficial role of HR lies in bypassing replication obstacles and failure to do so leads to replication fork collapse and eventual cell death. TopBP1 or ATR activation by HPV could explain slightly elevated HR in FANCD2 deficient HPV (+) cells versus FANCD2 deficient HPV (-) cells. Exploitation of DNA repair proteins by viruses to aid in their replication is a quite common strategy, with several of these described in a review by Lilley et al. (Lilley et al. 2007).

At the same time, the difference in upregulation of NHEJ in HPV infected cells compared to FANCD2 deficient cells was significant with significantly more pronounced error-prone upregulation of NHEJ seen in FANCD2 deficient cells than HPV infected cells (p ≤ 0.001). The FA-like phenotype of NHEJ upregulation was again revealed but the relative differences were possibly due to the virus sustaining HR processes of the cell in favor of its own life cycle resulting indirectly in upregulation of NHEJ as a result of less available HR. This idea is supported by evidence from the behavior of other viruses. Viruses can not only activate DNA damage proteins and inhibit DNA damage proteins, some even have
dual roles in first exploiting and then inactivating specific DNA repair pathways, while others are either inhibited or activated by certain DNA repair pathways (Lilley et al. 2007). Darbinyan et al. showed that the NHEJ protein KU70 inhibits HSV-1 replication (Darbinyan et al. 2004). The same KU70 protein, however, is bound and mislocalized by a late protein encoded by the polyomavirus JCV, underlying the ability of JCV to inhibit NHEJ (Taylor and Knipe 2004). Another virus, the human cytomegalovirus (HCMV), first induces activation of ATM and other downstream targets recruiting certain repair components such as the MRN or MRE11 complex into viral replication centers. Then, HCMVs late viral proteins limit the function of repair proteins by excluding them from viral replication centers and mislocalizing them into cytoplasmic aggregates or by causing downregulation of key response elements such as 53BP1 (Gaspar and Shenk 2006, Luo et al. 2007). These examples of viral influence on DNA repair pathway manipulation should encourage further investigation of corresponding HPV activities. Already it is known that HPV-16 upregulates the DEK proto-oncogene promoting growth of HNSCC tumors and further resulting in upregulated NHEJ. However, DEK knockdown inhibited the proliferation of not only HPV (+) HNSCCs but also of HPV (-) HNSCC cells, establishing a functional role for DEK in HNSCC growth in general (Adams et al. 2015). Given the similarity in phenotype of HPV infected and FANCD2 deficient cell responses in downregulating HR and upregulating NHEJ, it may be interesting to look further into the effects of HPV infection on the FA-pathway to gain better understanding of where exactly these differences in up- and downregulation of HR and NHEJ arise.

4.1.4 Cell cycle analysis supported the FA-like phenotype in HPV (+) cells

In response to DNA damage, cells respond by arresting the cell cycle at checkpoints G1/S, intra-S and G2/M. A cell cycle signaling cascade through phosphorylation of specific Chks, is triggered by ATM and ATR and leads to cell cycle arrest. ATM can directly activate Chk2 and ATR requires claspin protein to phosphorylate and activate Chk1 (Kumagai et al. 2004). At the first G1/S cell cycle checkpoint, employed to prevent replication in cells with damaged DNA, Chk1 and Chk2 inactivate a critical phosphatase, cell division cycle (Cdc) 25A protein, and thereby keep cyclin-dependent kinase (CDK) 2/Cyclin E kinase in an inactivated state. Similarly, intra-S phase checkpoint mechanisms are reliant on Cdc25A inactivation. The G2/M checkpoint functions to prevent segregation of damaged DNA into daughter cells and depends on the inhibition of Cdc2/CDK1 kinase.
Phosphorylation of Cdc25C by Chk1 or Chk2 creates a binding site for 14-3-3 proteins, leading to nuclear export of the phosphatase Cdc25C and retention in the cytoplasm. As a result, the nuclear Cdc2 stays phosphorylated and prompts G2 phase arrest (Lopez-Girona et al. 1999).

Cell cycle analysis has long been used as a diagnostic tool in FA patients and typically shows a G2 arrest in response to treatment with interstrand DNA cross-linking agents (Kaiser et al. 1982, Kubbies et al. 1985, Moreira et al. 2008). The inability for FANCD2 deficient cells to pass the G2 checkpoint upon ICL damage can be partially attributed to two key points. The first is the inability of the FANCD2 complementation group to be either ubiquitinated, resulting in the larger FANCD2-L variant, or deubiquitinated, resulting in the smaller FANCD2-S variant. Taniguchi et al. showed that if FANCD2 deficient cells were exposed to cross-linkers during S phase, cells failed to progress from G2 to M and showed that monoubiquitination of FANCD2 during S phase was required for normal completion of subsequent phases of the cell cycle (Taniguchi et al. 2002). Similarly, Oestergaard et al. could show that the absence of FANCD2 deubiquitination made cells more sensitive to crosslinking agents such as MMC (Oestergaard et al. 2007) Further, Garcia-Higuera et al. went on to show that after DNA repair is completed, the FANCD2-L protein appears to be deubiquitinated and recycled back to the FANCD2-S isoform, allowing the cell cycle to resume (Garcia-Higuera et al. 2001). Knowing that this response cannot be seen in the UMSCC1-D2 cells of this investigation, there are several factors possibly contributing to this. Kaiser et al. observed the best discrimination between normal cells and FA cells to occur 48 h after 2 h exposure to approximately 0.3 µM of MMC. Further they observed that fibroblasts from patients with Fanconi Anemia were, on average, more prone to blockage in late S or G2 phase after MMC exposure than fibroblasts from normal individuals (Kaiser et al. 1982). The UMSCC cells investigated were not primary cells but cancerous epithelial cells. These were exposed for only 1 h but at a concentration of 2 µM to MMC and evaluated 24 h after this exposure. These experimental differences, might in part explain the lacking G2 arrest in this work. However, given that the concentration of MMC used was around the IC₅₀ for these UMSCC1-D2 cells, it is conceivable that cell death is responsible for the inability to see the G2 arrest. This would not explain, however, that the G2 arrest is seen in the HPV infected cells. Thus, further investigation would be needed to clarify. Another limitation to the numerical accuracy of the data concerned the biological variability within the
individual cell lines. Kaiser et al. also showed the growth rate of a given cell line depended on the passage number and the particular batch of culture medium employed (Kaiser et al. 1982). It is noteworthy that a particularly long generation time of the UMSCC1-D2 cell line was observed in these experiments and that the media used for cell culture may also have been of a different batch as the non-HPV cells were cultivated in media with hydrocortisone and therefore source media would always have been from a different bottle. Further, the UMSCC1-NT cell line had a particularly short generation time and consequently a high passage number accounting in part for the unexpected high G2 arrest seen in this cell line. Another group tested differences in intercalating agents on 17 lymphoblast cell lines (LCLs) derived from patients registered with the International Fanconi Anemia Registry (IFAR) and found a significant induction of cell cycle arrest 48 h after treatment with either 0.5 µg/ml melphalan or 100 nM MMC (Chandra et al. 2005). Melphalan was found to produce a significantly higher percentage of cells in G2/M than MMC, an interesting point as melphalan is commonly used for such cell cycle studies in FA diagnosis and the investigations in this work were carried out with MMC. However, the most likely explanation for the decreased G2 arrest in this UMSCC1-D2 cell line is the appearance of compensatory mutations in the other genes of the FANCD2/BRCA pathway which e.g. are frequent in the downstream BRCA1 or BRCA2 genes (Swisher et al. 2008, Gottipati et al. 2010).

The second reason for the characteristic FA G2 arrest is thought to be the inability of FANCD2 deficient cells to limit p-CHK1 inactivation of G2/M checkpoint cell cycle progression factors, causing cells to arrest in G2. Guervilly et al. demonstrated that the ICL-induced accumulation of FA cells in late S/G2 phase was dependent on ATR and CHK1 and consistently found that CHK1 phosphorylation was enhanced in FA cells (Guervilly et al. 2008). Enhanced CHK1 phosphorylation was also demonstrated in the Western Blot studies of this investigation with an increase in p-CHK1 protein concentration found in the FANCD2 deficient UMSCC1-D2 cell line. Guervilly et al. went on to describe that in normal cells p-CHK1 activated the FANC/BRCA pathway through FANCD2 monoubiquitination whereas FANCD2 deficiency led to a p-CHK1-dependent G2 accumulation and concluded that the FANC/BRCA pathway activity downregulated CHK1 activation (Guervilly et al. 2008). Downregulation of p-CHK1 is also seen in the Western Blot results of this investigation in which the FA proficient cell line UMSSC1-NT demonstrated a lower level of p-CHK1 concentration compared to the
FA deficient cell line UMSCC1-D2 after MMC treatment. Though the findings of this study demonstrate this difference in p-CHK1 concentration of FA proficient and deficient cells, the expected effect on the cell cycle could not be demonstrated but could very well be due to experimental factors and compensatory mutations as previously explained.

Though this G2 arrest was not seen in the FANCD2 line UMSCC1-D2 in this study, the FA-like phenotype was recovered in the HPV infected cells. The HPV cells demonstrated FA behavior with enhanced CHK1 phosphorylation also being observed. The intact FA pathway as suggested by Guervilly would downregulate this CHK1 activation suggesting perhaps that HPV may interfere in an FA-like manner to prohibit this downregulation. Both FANCD2 proficient and deficient HPV-16 infected cells not only demonstrated the increased p-CHK1 protein levels according to Western Blotting, but they also displayed the FA-like phenotype of G2 arrest in the cell cycle studies. These findings are in line with studies on the HPV-16 E7 oncogene that was found to induce elevated expression of phosphorylated ATM(Ser1981) and downstream phosphorylated Chk1, Chk2 and JNKs, known to inactivate Cdc25c (Banerjee et al. 2011). Cdc25c is needed for cell cycle progression and inhibitory Cdc25c phosphorylation at tyrosine 15 causes G2 arrest (Gould and Nurse 1989, Enserink and Kolodner 2010). Duensing et al. found that HPV-16 E7 cells showed a 3.5 fold increase in phosphorylated Cdc25 and showed that expression of HPV-16 E7 triggers the activation of cell cycle checkpoints similar to defective FA pathway (Duensing and Münger 2002). Knowing this increase in G2 arrest, it is even more surprising that HR was downregulated, as HR takes place in S/G2 phase (Saleh-Gohari and Helleday 2004, Shibata et al. 2011).

This G2 arrest is favorable to HPV as the viral genome amplification persists as differentiating epithelial cells progress from an S-like phase to a G2-like phase, whereby the viral genome amplification occurs primarily in G2 after completion of cellular replication (Wang et al. 2009; Banerjee et al. 2011; Doorbar et al. 2012). The study by Banerjee et al. revealed that prolonged G2 phase in these differentiating cells following S phase was attributed to E7 (Banerjee et al. 2011). Altogether the G2 arrest in DNA-damaged FA cells was suggested to be dependent on activation of CHK1 by phosphorylation (Guervilly et al. 2008). Here, the G2 arrest attributed to HPV through viral activation of CHK1 phosphorylation resembles this FA-like mechanism of cell cycle arrest.

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4.2 FANCD2 deficiency and HPV infection lead to differences in the focal accumulation of DNA damage and DNA repair markers according to Immunofluorescence Microscopy and Western Blot Data

Immunofluorescence microscopic analysis was carried out to dissect the effect of FANCD2 deficiency and HPV-16 infection on foci formation and disassembly of three proteins involved in DSB repair post ICL damage. The proteins investigated were 53BP1, RAD51 and FANCD2. Additionally the protein levels of relevant proteins and phosphorylated forms of these proteins involved in DSB repair namely RAD51, FANCD2 and p-RPA as well as the cell cycle checkpoint control protein p-CHK1, were investigated at basal levels and after MMC treatment by Western Blot analysis.

4.2.1 53BP1 foci accumulate in cross-linker treated FANCD2 deficient cells and HPV (+) cells

The p53 binding protein 1 (53BP1) is a protein whose function was originally thought to primarily lie in the transcriptional coactivation of the tumor suppressor, p53. It does this by binding p53 and as such, its function is part of the early cellular response to DNA double strand breaks (DSB). By localizing to discrete foci in the nucleus, i.e. regions flanking the chromatin at a DSB to which DNA repair factors are recruited, the 53BP1 foci represent sites of DNA damage and subsequent DNA processing as part of the repair process (Schultz 2000). Further Schultz could show that the number of the DSB labeled by 53BP1 peaked early after damage exposure and decreased over time in a manner reflecting the rate of DSB repair. Knowing this about 53BP1, this investigation compared the effects of HPV and FANCD2 deficiency on 53BP1 foci formation after MMC treatment in order to gain a better understanding of the repair defects and kinetics involved under unperturbed growth conditions, as well as following treatment causing replication blockage. DNA damage that is caused by stalled replication forks is repaired by HR or crosslink repair including HR (Hoeijmakers 2001; Thompson and Hinz 2009). If this is not possible, an increase in 53BP1 foci numbers would be expected to indicate DNA damage accumulation and genomic instability would accumulate due to the inability of the cells to repair the lesions effectively. As such 53BP1 promotes DNA repair via the NHEJ pathway and also antagonizes RAD51-mediated HR. Further, 53BP1 is an antagonist to the BRCA1/2
pathway, especially in BRCA1/2 mutated cells (Difilippantonio et al. 2008; Bouwman et al. 2010; Bunting et al. 2010). Kee and D’Andrea could show that the NHEJ promoting event was inhibition of C-terminal binding protein-interacting protein (CtIP)-mediated DNA end resection by 53BP1 and that 53BP1 deletion restored RAD51 foci formation and homologous repair in BRCA1-deficient cells via alternative routes (Kee and D’Andrea 2010).

The results showed a cross-linker-induced increase in 53BP1 foci in all cell lines, though disassembly of foci was only seen in the control UMSCC1-NT cell line at 24 h. These findings of increased 53BP1 foci formation upon genotoxic stress are consistent with proficient DNA repair via NHEJ, as seen in the DNA repair assays for FANCD2 deficient and HPV infected cell lines. However, since cross-linker induced damage cannot be repaired by NHEJ, this 53BP1 foci assembly can only be considered an attempt to resolve the damage.

As for DNA repair kinetics, results also showed that the control UMSCC1-NT cells reached peak foci formation at 16 h with a decline at 24 h. This data reflects known DNA repair kinetics. The FANCD2 deficient cell line, UMSCC1-D2 did not show this decline. Instead foci formation further increased without peaking, suggesting that the absence of the FANCD2 protein impaired DSB repair. The effect of HPV infection on 53BP1 foci formation was strikingly similar to FANCD2 deficiency, with no peak in foci formation and significantly more 53BP1 foci still present at 24 h compared to the control UMSCC1-NT cell line. Moreover, the results of the HPV infected cell lines UMSCC47-NT and UMSCC47-D2 were similar in the 53BP1 foci response indicating that HPV infection alone and FANCD2 deficiency are epistatic, targeting the same pathway. These findings show that HPV may have a similar effect as the absence of the DSB repair protein FANCD2 with non-resolution of foci suggesting an accumulated, unrepaired DNA damage leading to increased genomic instability. An important role of the FANCD2 protein in preventing chromosome instability and aneuploidy was previously suggested by many reports, suggesting a corresponding genome destabilizing role of HPV infection (Bogliolo et al. 2007, Naim and Rosselli 2009, Lachaud et al. 2016).
4.2.2 RAD51 foci formation is increased by FANCD2 silencing but not by HPV positivity in HNSCC cell lines

RAD51, a well conserved recombinase, is central to DSB repair through HR (Balacescu et al. 2014, Velic et al. 2015). It is recruited with help of mediator proteins to sites of DSB where it polymerizes on resection-generated ssDNA ends and forms a nucleoprotein filament that initiates strand invasion for exchange of homologous DNA sequences (Davies et al. 2001, Takata et al. 2001). This recombinase activity can be visualized through formation of discrete RAD51 nuclear foci at sites of DNA damage (Haaf et al. 1995).

Though RAD51 is a marker for HR, which was found in DNA repair assays of this study to be reduced in the FANCD2 deficient and HPV-16 infected cells, RAD51 foci formation was not reduced in any of the corresponding cell lines investigated. This may be due to the fact that the HNSCC cell lines are epithelial cancer cells and an overexpression or upregulation of RAD51 has been linked to a variety of cancers including leukemia (Raderschall et al. 2002), breast (Maacke et al. 2000) and prostate cancers (Mitra et al. 2009). These findings have suggested that RAD51 regulation may be important for cell homeostasis as an increase in RAD51 protein in tumor cells has been shown to protect them from undergoing apoptosis in response to DNA damage and that these increased levels were associated with enhanced recombination and genomic instability (Raderschall et al. 2002, Henning and Stürzbecher 2003). Its expression is tightly controlled in normal healthy cells to maintain genomic stability (Richardson 2005). However, HR was decreased in FANCD2 deficient and HPV-16 infected cells and not increased. Therefore, RAD51 foci formation may simply reflect the attempt to repair the cross-linker damage by HR, and the lacking decline would indicate the failure to accomplish repair. This implies that other mechanisms than RAD51 foci assembly are affected in FANCD2 deficient UMSCC1-D2 and HPV (+) UMSCC47-NT and UMSCC47-D2 cell lines. Possible candidate mechanisms could affect correct end processing when BRCA1 foci are decreased such as alternative excessive end processing mechanisms via BLM/DNase 2 or EXO1. These may destroy the HR substrate as p-RPA levels were normal indicating functionality of end processing (Nimonkar et al. 2011).
DNA damage persistence thus may explain the findings of this investigation that the FANCD2 deficient cell line UMSCC1-D2 showed a significant increase in RAD51 foci formation at 16 hr (p \leq 0.05) and at 24 hr (p \leq 0.01) post MMC treatment compared to the FANCD2 proficient UMSCC1-NT cell line. As an alternative explanation for the missing RAD51 foci resolution at 24 h in the UMSCC1-D2 cell line, dissociation of RAD51 from DNA may have been disturbed. Hilario et al. suggest a separate mechanism responsible for this, a mechanism that could be inactive in FANCD2 deficient cells and only partially in the HPV-infected cell lines (Hilario et al. 2009). Supporting this non-dissociation are findings by Park et al. that demonstrated RAD51 levels to remain elevated in HPV infected HNSCC cell lines compared to non-HPV infected HNSCC cell lines (Park et al. 2014). In this work, however, RAD51 foci formation in HPV infected cells did not show significant differences neither to control UMSCC1-NT cells nor to FANCD2 deficient UMSCC1-D2 cells, suggesting an intermediate phenotype. It could be speculated from these findings that HPV, though inducing DNA damage, does so to a lesser extent perhaps to prevent or postpone apoptosis not profitable to viral propagation. The significance of elevated RAD51 levels as related to tumor development and prognosis has made it an interesting target for small molecule or gene therapies as a possible anticancer treatment. Studies have shown inhibition of RAD51 to have been successful in reducing treatment resistance of tumor cells (Russell et al. 2003, Qiao et al. 2005).

### 4.2.3 HPV infection seems to reduce the ability to bypass replication obstacles as compared to FANCD2 deficient cells according to Western Blotting

The pattern of RAD51 foci formation is mirrored also in the RAD51 protein expression profile of the UMSCC cell lines according to Western Blotting. The persistence and even increase of RAD51 foci and protein expression but concomitantly reduced HR in FANCD2 deficient UMSCC1-D2 cells could be attributed to its status as a cancer cell with elevated S/G2 phase cell numbers or might simply reflect a halt during HR. A trend towards less RAD51 foci formation and protein accumulation was seen in HPV infected FANCD2 deficient cells compared to non-HPV infected FANCD2 deficient cells suggesting a role of HPV in destabilization of the RAD51 nucleofilament or reduced expression of RAD51. The reduced RAD51 filament formation in HPV infected cells cannot be explained through cell cycle results as HPV infected cells showed an increase in the G2 phase that normally promotes RAD51 filament formation. These findings suggest an additional component of
HPV’s possible interference with RAD51 foci formation independently of FANCD2. The findings of the FANCD2-deficient cells of this investigation are in line with studies by Yamamoto et al. that showed RAD51 focus formation was not impaired in the absence of FANCD2 (Yamamoto et al. 2005). The exact mechanism by which HPV interferes in DSB repair has not been elucidated but evidence from this study shows that the repair defect lies closely connected to FANCD2.

4.2.4 FANCD2 foci and protein analysis in HPV infected cells suggest the repair defect is not upstream of FANCD2 ubiquitylation

The results of this study revealed that the HPV-16 infected cell line UMSCC47-NT formed FANCD2 foci normally upon exposure to MMC. At 16 h post MMC treatment the number of FANCD2 foci was significantly elevated ($p \leq 0.01$) showing 30% more foci than the HPV (-) cell line UMSSC1-NT. At 24 h post MMC treatment a reduction in FANCD2 foci was seen in the UMSSC1-NT cell line, not however in the HPV (+) UMSCC47-NT cell line that accumulated more FANCD2 foci resulting in a significant 3 fold elevation of FANCD2 foci ($p \leq 0.001$). This increased accumulation of FANCD2 at sites of DNA damage in HPV infected cells is suggestive of HPV’s ability to influence the FA pathway. It further suggests that the repair defect present in HPV infected cells lies downstream of FANCD2 monoubiquitination. Intact FANCD2 monoubiquitination in HPV-infected cells was further supported by the appearance of an additional, more slowly migrating FANCD2 form in Western Blots upon MMC treatment. Increased FANCD2 foci formation following replication stress has been reported also by Spardy et al. who could show that the HPV-16 E7 oncoprotein was responsible for the increase in FANCD2 nuclear foci (Spardy et al. 2006). Another group went on to report that increased FANCD2 activation in HPV (+) HNSCC could be a response to hyperproliferation and DNA damage accumulation driven by E7 (Hoskins et al. 2012). FANCD2 colocalization with other proteins is crucial for stabilization and processing of stalled replication forks. Hence, it is understandable that dysfunction of this leads to more DSB and genomic instability through radial chromosome formation and explains also the susceptibility to cancer especially for FA patients (D’Andrea and Grompe 2003; Spardy et al. 2006; Hoskins et al. 2012). Spardy et al. were also able to show that FANCD2 foci may increase in HPV cells due to the increase in replication fork stalling that resulted from the E7 replication stress at telomeric DNA (Spardy et al. 2008). They argued that this in turn would activate the recombination based
telomere maintenance pathway underlying alternative lengthening of telomeres (ALT) which is involved in telomere homeostasis in the absence of telomerase, and thus provide an advantage to HPV infected cells to extend host cell life span and in case of this investigation offer another explanation to increased FANCD2 foci numbers.

From this investigation and others, the epistatic relationship of FANCD2 deficiency and HPV positivity but normal formation of FANCD2 foci in HPV-16 infected cells suggest that the effect of HPV on the repair process occurs downstream of FANCD2 ubiquitination. The persistence of FANCD2 foci 24 h post MMC treatment might suggest that HPV prevents foci resolution or deubiquitination of FANCD2. This in turn might be advantageous for viral propagation as failure to deubiquitinate would halt the cell cycle in the virus amplification favoring phase (Reinson et al. 2015). FA-like phenotype caused by the virus may thus reflect its FA-like interference with the activation/deactivation step necessary for cell cycle progression. It is possible, though, that the resolution of FANCD2 foci may simply occur at a later time point due to a repair defect and that this repair defect of HPV-16 infected cells is due to downstream or independent factors. Such factors that could be investigated as candidates cover a wide spectrum of other repair cascades including but not limited to ubiquitin binding proteins such as RAP80 or CCDC98 (Abraxas), a localizer of BRCA1 to sites of DNA damage (Yuan et al. 2010). Interestingly Wang et al. found that cells depleted of CCDC98 or RAP80 exhibited mild HR defects, a finding compatible with the results of this investigation pointing to the need to investigate HPV interference with these BRCA1 recruiting factors (Wang et al. 2007). Further hints into this direction came from the Wiesmüller lab, showing reduced BRCA1 foci formation in HPV (+) UMSCC47 cell lines and perhaps implicating HPV to interfere via factors localizing BRCA1 to chromatin (Yuan et al. 2010). Alternatively, and in analogy to a recent report on β-HPV5 and 8 E6 effects on HR, BRCA1 and BRCA2 expression could be downregulated in HPV (+) cells (Wallace et al. 2015). These and a number of other factors should be considered in further investigations of the effect of HPV infection on DNA damage repair pathways especially in relation to the FA pathway. Not only HPV-16 but also other viruses including simian virus 40 (SV40) and adenovirus 5 (Ad5) were shown to induce FANCD2 monoubiquitination (Cherubini et al. 2011), adding FANCD2 to a class of common viral targets that include p53 and pRb.
4.2.5 HPV infection reduces FANCD2 protein in FANCD2 proficient cells and alters the DNA damage response according to Western Blot

The findings of this study revealed that HPV-16 infection in the UMSCC47-NT cell lines resulted in a trend of lower total expression of FANCD2 protein with and without exposure to MMC compared with the HPV negative UMSCC1-NT cell lines. HPV’s ability to influence total FANCD2 levels without reducing the nuclear FANCD2 foci suggests that the cells are still able to respond to DNA damage. Evidence of this response to DNA damage is seen in the FANCD2-Ub band present in the Western Blot with band formation being consistent with the FANCD2 foci accumulation in the HPV (+) cells. HPV’s effect on DNA repair was also evident, as previously mentioned, in the decreased BRCA1 and BRCA2 levels seen in HPV infected FANCD2 proficient cells (Wallace et al. 2015). However, the specific influence of HPV on the FANCD2 levels by still unknown mechanisms remains unknown. Further investigation could be carried out in analogy to Wallace et al. in which FANCD2 transcription would be investigated. If no change is found, then protein stability or translation by inhibitors could be investigated (Volcic et al. 2012).

4.2.6 HPV infection causes an FA-like increase in replication stress markers that are not further amplified by FANCD2 deficiency according to Western Blotting

Replication protein A, (RPA), is considered a guardian of the genome due to its functions in DNA replication, during which it binds ssDNA to prevent it from re-association and degradation. In repair processes RPA in its phosphorylated form (p-RPA), acts as a sensor to initiate DDR upon exposure to genotoxic stresses (Fanning et al. 2006, Zou et al. 2006, Oakley and Patrick 2010, Maréchal and Zou 2015). Genotoxic stresses or DNA replication problems are signaled by detection of persistent RPA coated ssDNA by ATR and ATRIP, in which ATR activation subsequently leads to cell cycle checkpoint activation and stabilization of stalled forks and repair (Liu et al. 2000a, Zou and Elledge 2003, Masai et al. 2010). Vassin et al. showed that p-RPA prevented RPA from promoting DNA replication and showed that p-RPA could serve as marker for DNA damage (Vassin et al. 2004).
Investigations by Western Blotting showed an increase in p-RPA levels upon exposure to MMC whereby the response of HPV infected cells mimicked that of FANCD2 deficient cells. The HPV (+) cell line UMSCC47-NT, showed approximately twice the p-RPA level compared to its HPV (-) counterpart, UMSCC1-NT. This increase very much resembled that of the HPV (-) FANCD2 deficient UMSCC1-D2 cell line. The effect of HPV infection on the amount of p-RPA protein could not be further increased by loss of FANCD2 function, suggesting an epistatic relationship. Though the normalization of p-RPA to RPA would have been a better comparison than the p-RPA to GAPDH normalization, RPA detection failed despite repeated attempts. The difficulty most likely can be attributed to problems with the commercial antibody. Nonetheless, the results strangely suggest increasing replication stress in the FANCD2 deficient and HPV infected cells.

The same type of response is mirrored by elevated p-CHK1 levels in HPV infected cells which were again quite similar to the p-CHK1 levels of FANCD2 deficient cells. Phosphorylation of CHK1 in comparison to CHK1 levels was used as the basis for this comparison with p-CHK1 levels being normalized to total CHK1 levels. Since p-CHK1 amplifies the damage signal by phosphorylation and activation of further damage control proteins (Deakyne and Mazin 2011), as discussed already in detail in section 4.1.4, the p-CHK1 protein expression levels served as a marker of DNA replication stress (Gole et al. 2014). From equally elevated p-RPA and p-CHK1 levels, it became clear that FANCD2 deficiency in HPV infected cells had no additional effect, meaning that it did not further increase these markers of increased replication stress. Altogether the similar increase in replication stress proteins, p-RPA and p-CHK1, of HPV infected cell lines compared to the FANCD2 deficient non-HPV cells confirmed the FA-like phenotype. This increase in replication stress of cells by mechanisms via the FA pathway might improve viral replication, which replicate adjacent to replication stress susceptible fragile sites, thereby increasing the chances of viral integration (Jang et al. 2014).

4.3 FANCD2 deficiency and HPV infection lead to an increase in sensitivities to MMC

Mitomycin C (MMC) has been used in the clinical treatment of certain cancers since 1956, after it was found to possess significant anti-cancer properties that at the time were still
unknown. It is now known that MMC causes significant cytotoxicity to cells based on the various types of DNA damage that it causes, most notably its ability to induce interstrand crosslinks (ICL) that prevent the cells from performing critical cellular process of replication and transcription by prohibiting strand separation (Lee et al. 2006, Deans and West 2011). Interstrand crosslinking by MMC targets two guanines at the 2-amino group in the minor groove of DNA, and resulting damage of both strands is repaired by multiple pathways including nucleotide excision repair (NER), homologous recombination (HR) and translesion bypass repair (TLS) (Li et al. 1999, Silva et al. 2000). As DNA crosslinks represent serious DNA damage, it is evident that the cellular ability to repair this damage is not only crucial to survival but also a determinant for drug sensitivity.

Poly(ADP-ribose) polymerase (PARP) comprises a family of 17 proteins involved in repair of single and double-strand breaks through post-translational modification involving transfer of ADP-ribose moieties, using NAD\(^+\) as a substrate, onto itself and proteins forming covalently linked polymers of ADP-ribose (Krishnakumar and Kraus 2010). The long-chain polymers in turn recruit other repair proteins. PARP is involved in base excision repair (BER) of alkylating agent-induced damage or IR induced oxidative damage or breaks, where it binds to resulting single-strand breaks (SSB) to form SSB intermediate (Satoh et al. 1993). PARP is further involved in HR mediated stalled replication fork restart (Bryant et al. 2009).

PARP inhibitors have long been of interest for use in targeted tumor therapy of cancers defective in HR due to the ability of PARP inhibitors to block alternative repair mechanisms, leading to eventual cell death. Ström et al. found that PARP inhibitors trap PARP on the SSB intermediate formed during BER thus preventing completion of repair (Ström et al. 2011). This is the underlying principle of synthetic lethality in which persistence of unrepaired SSB leads to DSB after DNA polymerases encounter SSB at the replication fork (Bryant et al. 2005, Farmer et al. 2005). These DSB breaks would then normally be repaired by HR, but to cells deficient in this repair, it leads to cell death (De Vos et al. 2012). In this study potential synthetic lethality was tested by investigating the effect of PARP inhibition by 1,5-Isoquinolinediol (IQD) in combination with FANCD2 deficiency and HPV infection.
4.3.1 FANCD2 deficiency results in elevated sensitivity to MMC

The investigation by MTT survival assays showed that the FANCD2 deficient cells, UMSSC1-D2, were significantly more sensitive to MMC than the FANCD2 proficient UMSSC1-NT cells. Concentrations of MMC were gradually increased in the survival assays in order to see at which concentration cells reached 50 percent survival (IC$_{50}$). The IC$_{50}$ value for the FANCD2 deficient cells was reached already after continual 48 h exposure to an MMC concentration of 782 nM compared to a concentration of 10255 nM for FANCD2 proficient cells resulting in a significant difference (Figure 11b; p < 0.0001). These results confirm well established knowledge that FANCD2 deficient cells are more sensitive to DNA crosslinking agents such as MMC and present with decreased cell survival (Fujiwara and Tatsumi 1977, Weksberg et al. 1979, Kaiser et al. 1982). In addition, FANCD2 cells have an abnormal response to replicative DNA synthesis already at low MMC concentrations (150 nM) as was found by Claasen et al. in that FA cells were inhibited to perform DNA synthesis whereas normal cells were insensitive at this low dose (Claasen et al. 1986). This finding was reflected in the results of this investigation in which the concentration of 150 nM did not affect survival of FANCD2 proficient cells but already led to decreased survival of FANCD2 deficient cells consistent with DNA synthesis inhibition. A recent study by Lombardi et al. has shown, however, that MMC sensitivities for a FANCC deficient cell line did not differ significantly from the counterpart FANCC proficient HNSCC cell line. Experimental differences in MMC concentration used may explain these results in addition to the different FANC protein status investigated. The experimental design used in the study by Lombardi included a consecutive 5 day exposure to an MMC concentration range of 0.1 nM to 100 nM (Lombardi et al. 2015). This differed from this investigation in which a consecutive 48 h of MMC exposure at a concentration range from 16.625 nm to 16 µM was applied. Hence, resulting differences in IC$_{50}$ values obtained may be explained from these two different experimental approaches.

4.3.2 Sensitivity to MMC in HPV (+) FANCD2 proficient and deficient cells is similar and HPV partially desensitizes FANCD2 deficient cells

No significant reduction of survival of FANCD2 proficient cells infected with HPV (Figure 11d) compared to their non-infected counterpart was seen. Interestingly, however,
a difference in MMC sensitivity of HPV infected FANCD2 proficient and deficient cells, was noticeable (Figure 11c). The correspondingly improved survival attributed to HPV may indicate a partial recovery of FANCD2 deficient cells survival. Moreover, comparing the survival of non-HPV infected and HPV infected FANCD2 deficient cell lines, again reduced survival for the non-HPV infected FANCD2 deficient cells was observed compared to HPV infected FANCD2 deficient cells (Figure 11e; p ≤ 0.0001). The resulting conclusion that HPV infection would increase cell survival versus FANCD2 deficient cells, appears to be contradictory to published results as studies have shown that HPV (+) cells, like normal human mammary epithelial cells (HMEC) and human keratinocytes (HKC), were highly sensitive to apoptosis induced by MMC due to ubiquitination and subsequent destruction of p53 by E6 (Xu et al. 1995, Liu et al. 2000b). Here, the difference between HPV (-) and HPV (+) cells did not reach statistical significance. One critical difference for this could be attributed to their status as squamous cell carcinomas. In the case of this present investigation, HPV infected cells showing better survival were also FA pathway deficient, a factor that alone would have rendered the cell very susceptible to the intercalating action of MMC. This poses the possibility that HPV may reduce sensitivity to MMC and provide a survival benefit that would prolong cell life and possibly promote viral replication. This was suggested in findings by Hoskins et al. in which downregulation of FANCD2 and HPV infection in keratinocytes synergistically increased hyperplasia (Hoskins et al. 2012). Clinically hyperplasia is a precursor to SCCs and this study showed that HPV through hyperplasia contributed to cancer development. As HPV depends on the cellular machinery for genome replication, normally limited to differentiated keratinocytes that are growth arrested, its ability to uncouple cell differentiation from proliferation is part of the viral strategies to control cell growth patterns for its benefit (Syrjänen and Syrjänen 1999). This could in part explain better survival rates found in this investigation for FANCD2 deficient cells. It is known that FA patients are more susceptible to HPV infection (Spardy et al. 2008; Park et al. 2010). Also FA patients in particular have a 14% risk for developing HNSCCs by the age of 40 (Alter 2003; Kutler 2003; Rosenberg, Greene, and Alter 2003). Taken this knowledge and the results of the survival assays carried out in this investigation showing increased survival for HPV infected FANCD2 deficient cells, it would be interesting to further investigate whether HPV specifically exploits such deficiencies or itself leading to local FA-like phenotypes at infection sites that then lead to SCC development in the general population.
4.3.3 HPV infection increases sensitivity to IQD in FANCD2 proficient cells

When MTT survival assays with IQD were carried out, statistically significant differences of the IC$_{50}$ values of the UMSCC cell lines were compared to investigate if the principle of synthetic lethality applies to PARP inhibition in combination with HPV infection or FANCD2 deficiency. Only FANCD2 proficient UMSCC47-NT cells showed a significant reduction (Table 7 fourth data row; p = 0.0209) in survival upon HPV infection compared to the non-infected counterpart UMSCC1-NT. FANCD2 status did not appear to play a role in the sensitivity to IQD. This suggests that HPV infection may be PARP inhibitor responsive. However, this is somewhat contradictory to a report by Lombardi et al. which described PARP inhibitor sensitivity in FANCA and FANCC deficient cells (Lombardi et al. 2015b). It might indicate critical differences between FANC proteins upstream of FANCD2. Though the mechanism by which HPV is able to do this would require further investigation, it is an interesting result because it is known that HPV degrades p53, a regulator of HR, and leads to impaired RAD51 repression known to contribute to malignant transformation (Gatz and Wiesmüller 2006). If HPV infected cells are truly susceptible to PARP inhibition, this could present a new powerful therapeutic option for HPV linked cancers such as HNSCCs. Use of PARP inhibitors had previously been suggested by Polyak et al., stating that PARP inhibitors may be used to treat tumors with similar defects in DNA damage repair responses as BRCA1 or BRCA2 mutated tumors (Polyak and Garber 2011). Clinically, the use of PARP inhibitors is still very new with the 2015 approval and licensing of the first PARP inhibitor, olaparib (Lynparza) for BRCA-mutated ovarian cancer (Drew 2015). These agents targeting DNA repair are well tolerated and show significant efficiency in treating tumors that are HR deficient (Wielgos and Yang 2013). Though currently only in use for BRCA1 or BRCA2 mutated tumors, it may be interesting to expand the use to HPV linked cancers in the future. In agreement with the findings of this study, Weaver et al. were also able to demonstrate the sensitivity of HPV (+) HNSCC to the PARP inhibitor veliparib and found that the sensitivity was magnified by low dose IR (Weaver et al. 2015). Additionally Güster et al. showed HPV (+) HNSCCs to be responsive to olaparib in that the cells had decreased proliferation and increased radiosensitivity, resulting from the combination of PARP inhibition with Chk1 inhibition leading to abolished G2-checkpoint response (Güster et al. 2014). These findings suggest the application of PARP inhibitors as a potential therapy option for HPV linked cancers, even though the evidence seen in the HPV (+) UMSCC47-NT cells was not
very strong and based only on 6-8 measurements in two repeat experiments. The necessity for investigating other types of PARP inhibitors is evident from these results but also suggested by findings by Jelinic and Levine, who could show that another PARP inhibitor, NU1025, caused a dramatic decrease in DNA damage repair irrespective of the inhibitory potency (Jelinic and Levine 2014). Altogether these reports show that not only could PARP be an effective single agent for targeted therapy but that it may be used in combination with radio- and chemotherapy to provide better treatment outcome for cancers such as HNSCCs.

4.4 Conclusions

DNA repair phenotypes such as the Fanconi-Anemia like phenotype demonstrated in the HPV-16 infected HNSCCs of this investigation can potentially be used as a marker of response to therapy as well as serve as a therapy target. The analysis of the molecular components of the Fanconi Anemia pathway and those of HPV infected HNSCCs provided better knowledge of the DNA damage response that could be used as markers of therapy response and also revealed a possible therapy target, as seen in the sensitivity of HPV infected cells to PARP inhibition.

Knowing that FA patients are at high risk for developing SCCs, it was interesting to investigate DNA damage response after HPV infection in the context of FA pathway deficiency. FA pathway deficient FANCD2 cells demonstrated a shift from error-free HR to error-prone NHEJ, a DNA repair response demonstrated in this study and to a lesser extent also mimicked by HPV infection. Further, immunofluorescent analysis of HPV infected cells showed an increase in DNA damage response proteins 53BP1, RAD51 and FANCD2 upon exposure to ICL inducing agent MMC, demonstrating impaired DNA repair progression and cross-link resolution, in a manner often reflective of FA pathway deficiency. S-phase checkpoint activation was found also to be FA-like in HPV infected cells according to Western Blotting showing similarly increased p-CHK1 and p-RPA level in response to MMC. G2 arrest was detectable in DNA content analysis in HPV infected cells after MMC exposure, a diagnostic response characteristic of FA cells. The analysis of these DNA repair components provide a basis of how potential targeted therapies such as PARP inhibitors could be monitored; for example, through the evaluation of DSB repair by DNA repair assay or monitoring of DNA repair proteins as carried out in this
investigation. Though PARP inhibitor sensitivity was not seen for the FANCD2 deficient cells and seems contradictory to findings by Lombardi et al., it must be emphasized that their investigation studied FANCA and FANCC deficient cell lines (Lombardi et al. 2015b). It highlights the importance of FANCD2 in crosslink repair. However, HPV alone increased PARP inhibitor sensitivity of the cells and suggests that HPV may have a crosslink independent effect on the PARP inhibitor sensitivity, i.e. replication stress bypass mechanism. It is suggested by a study by Bunting et al. that indicated BRCA1 to have an additional role in DNA crosslink repair distinct from HR (Bunting et al. 2012). This was also compatible with lab internal findings of decreased BRCA1 foci and is suggestive of a potential HPV target. A second key factor to consider is HPV E6 driven p53 inactivation, that could potentially explain the slight HR increase seen in HPV (+) FANCD2 deficient cells as well as the increased survival to MMC. It is a possibility, therefore, that a combined effect due to BRCA1 and p53 impairment is observed here. These results are presented in the supplement under S1 for clarity and overview.

Limitations to this investigation arise in the small spectrum of cell lines investigated. Although a paired analysis of isogenic cell lines was carried out, it is possible that differences between the UMSCC1 and UMSCC47 cells lines beyond HPV infection status are possible. To mitigate these effects, many other HPV (-) and HPV (+) cell lines should be investigated in order to pursue the conclusions presented in this work and reduce the possibility of clonal interference.

The importance of this investigation is demonstrated in the clinical need for effective treatment of HPV (+) HNSCCs that have lower toxicity and hence, provide better quality of life. Targeted molecular therapies are the future of such treatments. The need for the development in this direction is apparent in patients suffering from HPV (+) HNSCC which continue to be difficult to treat. The patients are diagnosed at a relatively young age and often have a prolonged cancer survivorship with conventional therapies of surgery, radiation or chemotherapy resulting in several debilitating side effects including speech and swallowing dysfunction, xerostomia, visual impairment, cognitive decline and PEG tube dependence, all affecting normal physiological function (Wise-Draper et al. 2012). These current therapies alter normal physiological function, physical appearance and do not always prevent disease recurrence. Therefore, a molecular understanding of how HPV infection plays a role in various aspects of DNA repair could provide insight for
development and monitoring of new targeted therapies or in the adaptation of existing individual therapies. The analysis of molecular components causing FA-like phenotype in HPV-16 infected HNSCCs carried out in this investigation in part provided a closer look at how HPV infection affects DNA repair and may potentially be used as a starting point for further investigations of targeted molecular therapy possibilities.
5 SUMMARY

DNA repair especially of DNA double strand breaks is necessary not only for cell survival but also to prevent malignant progression arising from genome instability attributed to insufficient repair. Cells are able to accomplish DNA double strand break repair and repair of other DNA lesions through various pathways with pathway choice depending on lesion type and cell cycle phase at the time point of damage. Non-homologous end joining (NHEJ), available at all cell cycle stages, single-strand annealing (SSA) and microhomology-mediated end joining (MMEJ) represent the error-prone pathways. Homologous recombination (HR) is the most error-free type of repair, and inability for cells to employ this type of repair results in an increased risk of accumulated damage, genome instability and subsequent increased risk of cancer. The Fanconi Anemia (FA) pathway in particular executes repair specific to DNA damage resulting from interstrand crosslinks. Insufficiencies in this repair pathway, like FANCD2 deficiency, increase the risk of malignancy especially if DNA is exposed to agents causing interstrand crosslinks, such as the widely used chemotherapy drug mitomycin C.

Head and neck squamous cell carcinomas are a type of cancer for which detection at an early stage is not common and treatment at a late diagnosis time point is complex often with grave side effects. An etiological shift of risk factors from alcohol and tobacco use to human papillomavirus (HPV) infection has taken place in the last decades. Observation of better treatment outcomes in this new group is promising but insufficient research has been conducted to change the clinical course of therapy of these etiologically different tumors. New targeted therapies are necessary to improve cancer survivorship and increase quality of life by shifting the focus to therapies devoid of the significant side effects of current therapies. Development of such therapies requires an understanding of the underlying mechanisms of DNA repair. Given that links between the FA pathway and HPV infection were suggested, it was the aim of this investigation to analyze various molecular components of HPV-16 infected head and neck squamous cell carcinoma cell lines targeting DNA double strand break repair. To accomplish this, a set of University of Michigan squamous cell carcinoma cell lines (UMSCC), FA pathway proficient and deficient by silencing of FANCD2, were compared in several ways to another set of these cell lines with HPV positive status.
The initial method employed was identification of DNA double strand break (DSB) repair activities using the EGFP-based reporter system in which constructs for relevant DSB repair mechanisms (HR, NHEJ, SSA, MMEJ) were introduced by transient infection. The analysis showed a significant increase in error-prone NHEJ and a significant decrease in error-free HR for FA deficient cells as well as all for HPV infected cell lines compared to the reference cell line. This pattern of repair shift is known for FA deficient cells and was thus mirrored by HPV infected FA proficient cell lines and corroborated a FA-like phenotype exerted by the HPV-16 virus. To further investigate the effect of HPV on DSB repair, immunofluorescent microscopic analysis was performed to dissect the molecular defect attributed to HPV. The cells were stained with antibodies targeting proteins the 53BP1, RAD51 and FANCD2 after treatment with DNA damaging mitomycin C (MMC). The HPV infected cells demonstrated an increase in these protein markers for DNA damage similar to FA deficient cells but varied slightly in the extent of damage accumulation. Western Blot analysis was then used to investigate the levels of RAD51, FANCD2 and p-RPA involved in DSB repair in addition to p-CHK1, known as a marker for replication stress in S-phase. The analysis showed for HPV infected cells a similar pattern of p-RPA and p-CHK1 upregulation compared to the FANCD2 deficient cell line. These data together suggested FA-like interference with DNA repair and cell cycle control by HPV. This information could be used to further study how HPV interferes with the DNA damage response by providing a basis for further investigation and development of potential therapeutic options. This idea was tested with MTT assay to assess survival responses to PARP inhibitor IQD, a drug targeting HR defective cells, and DNA crosslinker MMC, extensively damaging FA deficient cells. Increased IQD sensitivity was observed for HPV infected cells compared to the reference cell and implicated the effective use of PARP inhibitors in the treatment of HPV positive cancers. In summary, this study revealed a phenotype with increased frequency in error-prone NHEJ repair and decreased error-free HR repair for HPV-16 infected UMSCC47 cell lines. This and further insight from this study into the DNA repair behavior of these cells could contribute to a better understanding of HPV-exerted carcinogenic activity and E6 and E7 oncogenes. Thus data may also support potential development of new therapies better suited to treat head and neck cancers of HPV etiology.
6 BIBLIOGRAPHY


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Salles, D.: (unpublished data)


SUPPLEMENT

S1. Overview of Findings

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These values are based on comparison to the control UMSCC1-NT. The data for foci derives from the 24 h time point. Black text denotes the HPV (-) cell line UMSCC1-D2 (FANCD2). Red text denotes the HPV (+) cell lines UMSCC47-NT (HPV (+)) and UMSCC47-D2 (HPV (+)/FANCD2). Arrows indicate increase or decrease, +/- indicates not significant, n.a. is not applicable, and n.d. is not determined. * Notes that even though RAD51 foci formation in HPV infected cell lines did not show significant differences neither to control UMSCC1-NT cell line nor to FANCD2 deficient UMSCC1-D2 cell line, it is suggestive of an intermediate phenotype as discussed in section 4.2.2.
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The last step of a very long road - the final page and yes, it is 3:20 am. To all my family and friends who know this is my usual time, I want to say thank you because without your moral support throughout this long process I would not be at this point!

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