CHAPTER 1

General introduction
Many thousands of toxic DNA lesions, such as mutations or breaks arise in each human cell every day\textsuperscript{1–3}. While the majority of these lesions are induced by mutagenic by-products of normal cell metabolism or during DNA replication, they also occur due to exogenous sources (\textit{e.g.} sunlight, radiation or cigarette smoke). Since DNA damage can have deleterious effects as it interferes with DNA replication and gene transcription, and can lead to permanent changes which might drive carcinogenesis, the cell is equipped with a variety of lesion-specific DNA repair mechanisms (\textit{e.g.} base excision repair, nucleotide excision repair, mismatch repair, non-homologous end joining, homologous recombination and crosslink repair)\textsuperscript{3}. The importance of DNA damage repair is further demonstrated by the finding that inherited mutations in DNA repair genes often predispose to cancer\textsuperscript{2,3}. For example, individuals with mutations in any of the seventeen known genes involved in the rare genomic instability syndrome Fanconi anemia (FA) have a defect in DNA crosslink repair and are prone to develop acute myeloid leukemia (AML) and malignancies of the head and neck region\textsuperscript{4–7}. Interestingly, patients with other diseases, such as the telomere maintenance disease Dyskeratosis congenita, have also an extremely high risk to specifically develop AML and head and neck tumors\textsuperscript{4,8}.

The study of cancer predisposition diseases has helped to reveal and understand biological mechanisms that secure genomic stability and prevent carcinogenesis. This is highlighted by studies of FA, which have elucidated important insights into cancer pathogenesis and chemotherapy resistance. In this chapter, general introductions on FA as well as the defective DNA repair mechanism in this syndrome are described. The aim of the research described in this thesis is to better understand the role of an FA-defect in the etiology of sporadic head and neck cancer and to which extent this defect can be exploited in anti-cancer therapy. Insights in the molecular pathogenesis of head and neck squamous cell carcinoma (HNSCC) are therefore also discussed.

\textbf{1. Hallmarks and diagnosis of Fanconi anemia}

Fanconi anemia was first described by and named after the Swiss pediatrician Guido Fanconi in the early 1900s\textsuperscript{9}. In a case report published in 1927, he described a family in which three brothers had a blood picture which was typical of pernicious anemia and died\textsuperscript{10}. They also suffered from several physical abnormalities, such as microcephaly, intensive brown skin pigmentation and hypoplasia of the testes. Later on it became clear that the disease is heterogeneous and characterized by a broad variety of congenital malformations, progressing bone marrow failure and increased cancer risk\textsuperscript{11}. 
1 General introduction

1.1 The chromosomal breakage assay as the gold standard in diagnosing Fanconi anemia

The clinical manifestations of FA are highly variable. Patients may present with an overall short stature, low birth weight, congenital abnormalities of the head (e.g. microcephaly), eyes (e.g. microptalmia), skin (e.g. hyperpigmentation or café-au-lait spots) or abnormalities involving central nervous, gastrointestinal and skeletal system (e.g. absent or extra thumbs). The majority (75%-90%) of FA patients develops bone marrow failure during the first decade of life. However, since physical abnormalities can be subtle or absent, hematological problems are often the first indication for FA and were the main cause of mortality. Several patients lack both the clinical manifestations of bone marrow failure and congenital abnormalities, and in these cases AML or head and neck squamous cell carcinoma (HNSCC) at a young age may be the first sign of FA. The variability of clinical symptoms and the low prevalence of the disease can make accurate diagnosis of FA difficult. However, at the cellular level FA patients have a single unifying feature; FA-deficient cells are hypersensitive to DNA interstrand cross-linking (ICL) agents (e.g. mitomycin C (MMC), diepoxybutane (DEB) and cisplatin (CDDP)) and to endogenous...
aldehydes. Upon treatment with these compounds, FA-deficient cells arrest in the G2 phase of the cell cycle and cells that managed to enter M phase exhibit many chromosomal aberrations, such as chromosome breaks, radials and gaps (Fig. 1). Therefore, this hallmark is used in the diagnosis of FA by performing a chromosomal breakage assay on T lymphocytes of suspected FA individuals. Those individuals with a significant increase in the number of ICL-induced breaks are further screened for mutations in any of the 17 FA genes (further discussed in section 2). Conventional Sanger sequencing of all FA genes without first performing a chromosomal breakage test can also immediately be used in diagnosing FA. The high number of FA and FA-associated genes, some of which have many exons, makes Sanger sequencing time-consuming and costly, but it is beneficial if the specific mutation is known within an FA family. Although sequencing of all FA genes is not yet routinely used to test for FA, it is likely that next generation sequencing may become a valuable tool for FA genotyping in the future. Nevertheless, to determine the pathogenicity of unclassified sequence variants in FA genes, the chromosomal breakage assay will still be relevant.

1.2. Complications of the chromosomal breakage test: diagnostic overlap and somatic mosaicism

Although the chromosomal breakage assay has been considered as the gold standard to test for FA, there are two complications. First, the chromosomal breakage assay is not entirely specific for FA and therefore, misdiagnosis may occur. MMC-induced chromosomal breakage has been observed in lymphocytes of individuals suffering from Nijmegen breakage syndrome, Roberts syndrome or Warsaw breakage syndrome. Nijmegen breakage syndrome is caused by mutations in a DNA repair gene (NBS1), while the last two syndromes are caused by defective sister chromatid cohesion (see Box 1).

Second, the presence of somatic mosaicism (reversion of a pathogenic allele to wild type) in hematopoietic progenitor cells from FA patients may hamper the interpretation of the chromosomal breakage data. Somatic mosaicism results in two subpopulations of lymphocytes, one of which is hypersensitive to DNA interstrand cross-linking agents, while the reverted cells are not sensitive anymore. Somatic mosaicism is relatively common in FA patients (estimated at 10-30%) and can be caused by new compensatory DNA mutations in the affected genes (e.g. additional insertions/deletions that restore the reading frame), spontaneous genetic reversion of FA mutations (back mutations) or in case of compound heterozygous FA patients by intragenic mitotic recombination. Occasionally, the number of reverted cells can reach such a high level in mosaic FA patients that a false negative result may occur. To avoid this complication, skin fibroblasts of FA patients can be
tested for ICL sensitivity, since mosaicism has not been observed in tissues other than blood. 

2. Genetic basis of Fanconi anemia: 17 FA genes

Fanconi anemia is not only a heterogeneous disease with respect to the clinical features but also at the genetic level; mutations in several genes can cause FA. This genetic heterogeneity among FA patients was revealed by cell fusion experiments between different FA cell lines (also known as complementation analysis). These cell lines were able to complement each other’s ICL sensitivity, indicating the involvement of multiple genes. Currently, seventeen complementation groups (FA-A, -B, -C, -D1, -D2, -E, -F, -G, -I, -J, -L, -M, -N, -O, -P, -Q and -S) representing seventeen separate FA genes are known (Table 1). Fanconi anemia is primarily inherited in an autosomal recessive pattern, except for complementation group B, in which FANCB mutations are inherited in an X-linked recessive manner. Similar to other X-linked recessive syndromes in which males are much more frequently affected, no female FANCB patients have yet been reported.
2.1. Identification of presently known FA genes and their contribution to FA

Several approaches have been used to identify the presently known FA genes: complementation cloning (FANCA, FANCC, FANCE, FANCF and FANCG)\(^{39,40,42-44}\), protein interaction studies by using co-immunoprecipitation followed by mass spectrometry (FANCB, FAACL and FANCM)\(^{47,48,51}\), positional cloning (FANCA, FANCD2, FANCI, FANCI and FANCO)\(^{41,45,49,52,56}\), candidate-gene approaches (FANCD1, FANCI, FANCN and FANCP (see Chapter 2 of this thesis))\(^{46,53-55,57,58}\) and next generation sequencing (FANCO (see Chapter 4 of this thesis) and FANCS)\(^{59,62}\). FANCC was the first FA gene identified in 1992 (ref. 39), followed by FANCA in 1996 (refs. 40 and 41). Pathogenic mutations in these two genes together with mutations in FANCG are found in the majority of FA individuals (Table 1). It has been suggested that the FANCA gene might be hypermutable, whereas founder effects may explain the high numbers of FA-C and FA-G patients\(^{41}\). In contrast, for some complementation groups only a few patients have been described in literature. Remarkably, the first patient described belonging to complementation group FA-M has in addition to bi-allelic FANCM mutations, also bi-allelic mutations in FANCA\(^{64}\). Although this raised the question whether FANCM is an FA gene, correction of the FANCA-defect in lymphoblasts of this patient did not restore ICL sensitivity, indicating that the ICL-sensitivity did not depend on the FANCA mutation. Moreover, FANCM can interact with other FA proteins and is part of the FA core complex, consisting of eight FA proteins which are involved in mono-ubiquitination of FANCD2 and FANCI (further discussed in section 5.5). Taken together, this indicates that FANCM is important for ICL repair and plays a role in the FA pathway.

Besides FA patients with FANCM mutations, the occurrence of individuals with bi-allelic mutations in FANCO, FANCO or FANCS is also rare (Table 1). Only one consanguineous family (2 affected individuals) with mutations in FANCO have been reported\(^{56}\). Of note, FANCO is a provisional term as individuals with mutations in FANCO (RAD51C) present with an FA-like syndrome: they have a milder ICL-induced chromosomal breakage phenotype and thus far they do not display bone marrow failure or cancer. Likewise, hematological problems were also absent in two patients with bi-allelic mutations in FANCS/BRCA1 and, therefore, are also associated with an FA-like disorder\(^{61,62}\). Finally, in a small percentage of patients with FA-like symptoms no mutations were found in any of the 17 known FA genes, suggesting the involvement of more, yet to be identified, FA genes.

2.2. Different mutations in one gene can cause distinct clinical outcomes

One of the newest members of the FA protein family is FANCQ (also known as ERCC4/XPF)\(^{59}\) (see Chapter 4 of this thesis). Remarkably, mutations in the FANCQ/ERCC4/XPF gene have been associated with four different syndromes: Xeroderma pigmentosum (XP), XFE progeroid syndrome, Fanconi anemia and a syndrome with
combined features of XP and Cockayne syndrome (CS), termed XPCS\textsuperscript{65}.

The product of the \textit{FANCQ/ERCC4/XPF} gene forms a complex with the DNA binding protein ERCC1 to function as a structure-specific DNA endonuclease\textsuperscript{65}. This nuclease is involved in both ICL-repair as well as in the removal of sunlight-induced UV photolesions and bulky DNA adducts by nucleotide excision repair (NER)\textsuperscript{65–67}. NER can be divided into two subpathways (global genome NER and transcription-coupled NER), and although these subpathways differ in their initial DNA damage recognition process, XPF acts in both pathways in the incision step near the adduct\textsuperscript{68}. XPF is also responsible for the incisions that unhook the cross-linked nucleotide during ICL repair\textsuperscript{69}. Since XPF is involved in both NER as well as ICL repair, the type of mutation in \textit{XPF} and the balance between NER and ICL-repair activities dictates outcome\textsuperscript{59}. \textit{XPF} mutations that primarily affect NER will cause XP, while an FA manifestation is caused by mutations in \textit{XPF} that disturb ICL-repair. Moreover, when both repair pathways are compromised, individuals will present with XFE or XPCS.

\textbf{Table 1 Fanconi anemia genes}

<table>
<thead>
<tr>
<th>Gene</th>
<th>also known as</th>
<th>Chromosome location</th>
<th>First reported in</th>
<th>Percentage of patients</th>
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<tbody>
<tr>
<td>\textit{FANCA}</td>
<td></td>
<td>16q24.3</td>
<td>1996</td>
<td>64.5</td>
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<tr>
<td>\textit{FANCB}</td>
<td>FAAP95</td>
<td>Xp22.2</td>
<td>2004</td>
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<td>\textit{FANCC}</td>
<td></td>
<td>9q22.32</td>
<td>1992</td>
<td>10.6</td>
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<tr>
<td>\textit{FANCD1}</td>
<td>BRCA2</td>
<td>13q13.1</td>
<td>2002</td>
<td>2.5</td>
</tr>
<tr>
<td>\textit{FANCD2}</td>
<td></td>
<td>3p25.3</td>
<td>2001</td>
<td>2.9</td>
</tr>
<tr>
<td>\textit{FANCE}</td>
<td></td>
<td>6p21.31</td>
<td>2000</td>
<td>1.3</td>
</tr>
<tr>
<td>\textit{FANCF}</td>
<td></td>
<td>11p14.3</td>
<td>2000</td>
<td>1.7</td>
</tr>
<tr>
<td>\textit{FANCG}</td>
<td></td>
<td>9p13.3</td>
<td>1998</td>
<td>9.0</td>
</tr>
<tr>
<td>\textit{FANCI}</td>
<td>KIAA1794</td>
<td>15q26.1</td>
<td>2007</td>
<td>1.5</td>
</tr>
<tr>
<td>\textit{FANJ}</td>
<td>BRIP1/ BACH1</td>
<td>17q23.2</td>
<td>2005</td>
<td>2.0</td>
</tr>
<tr>
<td>\textit{FANCL}</td>
<td>PHF9/ FAAP43</td>
<td>2p16.1</td>
<td>2003</td>
<td>0.4</td>
</tr>
<tr>
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<td>14q21.2</td>
<td>2005</td>
<td>0.1</td>
</tr>
<tr>
<td>\textit{FANCN}</td>
<td>PALB2</td>
<td>16p12.2</td>
<td>2007</td>
<td>0.6</td>
</tr>
<tr>
<td>\textit{FANCO}</td>
<td>RAD51C/ RAD51L2</td>
<td>17q22</td>
<td>2010</td>
<td>0.1</td>
</tr>
<tr>
<td>\textit{FANCP}</td>
<td>SLX4/ BTBD12</td>
<td>16p13.3</td>
<td>2011</td>
<td>0.5</td>
</tr>
<tr>
<td>\textit{FANCQ}</td>
<td>XPF/ ERCC4</td>
<td>16p13.12</td>
<td>2013</td>
<td>0.1</td>
</tr>
<tr>
<td>\textit{FANCS}</td>
<td>BRCA1</td>
<td>17q21.31</td>
<td>2013/ 2014</td>
<td>0.1</td>
</tr>
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\* X-linked recessive inheritance, \textsuperscript{§} Individuals with mutations in RAD51C are associated with an FA-like syndrome as these patients did not yet develop bone marrow failure or cancer and their chromosomal breakage test showed less chromosomal aberrations compared to other FA patients. Therefore, \textit{FANCO} is a provisional term. \* Likewise, the reported two reported FA-S patients did not develop bone marrow failure yet and therefore bi-allelic mutations in \textit{BRCA1} are also associated with an FA-like disorder. Data obtained from the Rockefeller University Fanconi Anemia database (www.rockefeller.edu/fanconi/).
3. Fanconi anemia and increased cancer risk

Approximately 75%-90% of FA individuals develop bone marrow failure during their first decades of life. In addition, the incidence of other life-threatening hematological abnormalities, such as myelodysplastic syndrome and acute myeloid leukemia (AML), is also high in FA patients. Specifically, the risk to develop AML is 800-fold higher than in the general population, with a median onset age of 14 years. Unless treated, the progressive depletion of normal bone marrow or the development of leukemia represents the primary cause of morbidity in FA patients. The treatment of choice for FA patients with severe bone marrow failure is hematopoietic stem cell transplantation. Since outcomes of bone marrow transplantations have improved tremendously, the next challenge that FA patients face is the high predisposition to develop solid tumors, in particular squamous cell carcinomas of the head and neck region, esophagus and anogenital area. The risk for all solid tumors combined is approximately 50-fold higher than in the general population, whereas the susceptibility to develop HNSCC is even 500- to 700-fold higher. Remarkably, the occurrence and the type of cancer can differ between the various FA complementation groups. Patients with bi-allelic mutations in FANCD1 (BRCA2) or FANCN (PALB2) have the most severe phenotype as they have a higher probability, different spectrum and earlier onset of malignancy. Unlike individuals of other FA complementation groups, these patients have a severe predisposition to develop childhood solid cancers, such as Wilms tumor (kidney cancer), neuroblastoma and medulloblastoma.

Inactivating mutations in some FA genes confer already susceptibility to cancer in monoallelic carriers. Individuals with heterozygous germline mutations in FANCD1 (BRCA2), FANCJ, FANCN (PALB2), FANCO (RAD51C) or FANCS (BRCA1) are prone to develop breast and ovarian cancer. FANCD1 (BRCA2) mutation carriers have also an increased risk of developing melanoma, prostate or pancreas cancer. Pancreas cancer susceptibility is also seen in FANCN (PALB2) mutation carriers, and two recent articles showed a potential increased risk of breast and colorectal cancer for FANCM germline mutation carriers. In conclusion, FA patients as well as some FA mutation carriers are prone to develop a tumor, demonstrating the important role of FA proteins in suppressing tumorigenesis.

4. DNA interstrand cross-linking agents in the treatment of cancer

ICL-inducing drugs, such as mitomycin C and cisplatin have remarkable anti-cancer properties and are widely used in the treatment of cancer. The ICL-inducing agent nitrogen mustard, which is also a well-known chemical warfare agent, was actually the first chemotherapeutic drug used in the treatment of cancer. The idea to use this ICL agent for cancer treatment arose more than 60 years ago and originated from observations during the First World War. Autopsy findings from soldiers dying
of mustard gas showed severe suppression of hematopoiesis\(^{88,89}\). During World War II in 1943, these observations were extended after a German air raid bombed the US Merchant Ship S.S. *John Harvey*, which was anchored in the Italian harbor Bari and contained a secret cargo of sulphur mustard bombs. The attack caused the detonation of the bombs and no one aboard survived. Consequently, the inhabitants of Bari were not warned or aware that they were under attack by mustard gas. The survivors of the disaster became leukopenic, confirming the remarkable sensitivity of normal lymphoid tissue to the cytotoxic action of mustard gas\(^{88}\). One year before this disaster, Louis Goodman and Alfred Gilman were already assigned to investigate the potential therapeutic value of nitrogen mustard. They reasoned that nitrogen mustard might also have therapeutic possibilities for treating lymphomas, which they first validated

<table>
<thead>
<tr>
<th>Table 2 Cancer types in FA patients and mutation carriers</th>
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<tbody>
<tr>
<td><strong>Gene</strong></td>
</tr>
<tr>
<td><strong>FANCA</strong></td>
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<tr>
<td><strong>FANCB</strong></td>
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<tr>
<td><strong>FANCC</strong></td>
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<tr>
<td><strong>FANCD1 (BRCA2)</strong></td>
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<tr>
<td><strong>FANCD2</strong></td>
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<tr>
<td><strong>FANCE</strong></td>
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<tr>
<td><strong>FANCJ</strong></td>
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<tr>
<td><strong>FANCl</strong></td>
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<tr>
<td><strong>FANCJ</strong></td>
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<tr>
<td><strong>FANCM</strong></td>
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<tr>
<td><strong>FANCN (PALB2)</strong></td>
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<tr>
<td><strong>FANCO (RAD51C)</strong></td>
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<tr>
<td><strong>FANCP (SLX4)</strong></td>
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<tr>
<td><strong>FANQ (XPF)</strong></td>
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<tr>
<td><strong>FANCS (BRCA1)</strong></td>
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</tbody>
</table>

*Depending on the type of mutation, individuals present with one of 4 syndromes: XP, FA, XFE progeroid syndrome or XPCS. Table adjusted from Kottemann and Smogorzewska\(^9\).*
with animal studies and later by treating a patient with lymphoma. Their findings were published in 1946 and opened new avenues in the treatment of cancer.\textsuperscript{87,90,91}

4.1. Different DNA interstrand cross-linking agents and sources

Nitrogen mustards (\textit{e.g.} chlorambucil or melphalan) are still used in the treatment of cancer, in particular lymphoid tumors. In addition to nitrogen mustards, other classes of cross-linking agents, such as platinum compounds (\textit{e.g.} cisplatin), mitomycin C and psoralens, have been identified.\textsuperscript{92,93} Mitomycin C is an anti-cancer antibiotic isolated from \textit{Streptomyces} and needs to be metabolized by the cell to become an active ICL-inducing agent, whereas psoralens form ICLs upon activation by UV irradiation. Cisplatin, discovered by Dr. Rosenberg in 1965 and the most widely used cross-linking drug today, does not require activation. Rosenberg found that cisplatin inhibits bacterial cell division, which led to the hypothesis that cisplatin may also inhibit the proliferation of rapidly dividing cancer cells. The anti-cancer activity of cisplatin was indeed confirmed in a mouse model and it is now used either alone or in combination with radiotherapy or other anti-cancer agents in the treatment of a wide spectrum of solid tumors, including testicular, ovarian, head and neck as well as small-cell lung cancers.\textsuperscript{95,96} Although cisplatin is one of the most effective anti-cancer drugs, major problems are the toxicity profile and the intrinsic resistance or the development of resistance in patients who initially responded to this therapy. The clinically acquired resistance can be caused by metabolic inactivation, increased DNA damage repair or decreased drug accumulation due to reduced uptake or increased efflux of cisplatin.\textsuperscript{97} Of note, the various classes of cross-linking agents induce other damaging DNA lesions besides interstrand cross-links, such as mono-adducts and intrastrand cross-links. Moreover, these chemotherapeutic drugs cause different numbers of ICLs: psoralens form the highest fraction of ICLs (up to 40\%), whereas mitomycin C causes 5-10\% ICLs and CDDP even less than 5\%\textsuperscript{92,98–100}. Although a small fraction of the DNA lesions induced by mitomycin C and cisplatin are ICLs, these lesions are believed to be the main determinant of toxicity as ICLs interfere with important cellular processes, such as DNA replication and transcription.

Undoubtedly, we did not evolve mechanisms of ICL repair to defend ourselves only against the toxic effects of chemotherapeutic agents. Moreover, FA patients have not necessarily been exposed to these drugs but nonetheless frequently develop congenital abnormalities and cancer, raising the question about the identity of the endogenous agent that generates FA-associated DNA damage. A potential source of endogenous cross-linking agents are by-products of normal cellular metabolism. For example, lipid peroxidation (oxidative degradation of lipids) causes the production of aldehydes, which are able to damage DNA. Aldehyde concentrations are also influenced by a high fat diet and alcohol use.\textsuperscript{101–104} As expected, FA cells are indeed
sensitive to aldehydes (e.g. acetaldehyde and formaldehyde), suggesting that the endogenous DNA damage caused by aldehydes is counteracted by the FA repair pathway. Another endogenous source that could be responsible for the generation of ICLs include abasic sites (site on DNA without a purine/ pyrimidine base), which can arise spontaneously (~10,000 sites per cell per day) or as an intermediate of base excision repair. Spontaneous hydrolysis of the glycosidic bond in DNA causes abasic sites, which exist in equilibrium between a ring-open aldehyde and ring closed hemiacetal. The ring-open aldehyde can form ICLs by reacting with the exocyclic amino group of adenine or guanine residues on the opposite strand.

5. The FA pathway

The FA pathway (Fig. 2 and Table 3) is involved in the repair of replication blocking lesions (in particular ICLs) and can be divided into two components: an upstream part, which is required for mono-ubiquitination of FANCD2 and FANCI, and a downstream part that is not necessary for this posttranslational modification. Eight upstream FA proteins (FANCA, -B, -C, -E, -F, -G, -L and -M) together with several FA-associated proteins form the FA core complex, which functions as an E3 ubiquitin ligase to mono-ubiquitinate FANCD2 and FANCI. Mono-ubiquitination of these two FA proteins is believed to be a key event in the pathway and results in their localization to damaged chromatin, where they coordinate downstream repair events. Although repair of ICLs can occur in a replication independent way, the FA pathway is coupled to DNA replication. Subsequent sections discuss the FA pathway and the different steps in ICL repair in more detail.

5.1. Step 1: Sensing of DNA interstrand cross-link damage by Fanconi anemia proteins

ICLs that covalently join both strands of a DNA double helix have always intuitively been regarded as absolute blocks for the replicative machinery, necessitating repair prior to resumption of DNA replication. Longstanding models of ICL repair indicated that the encounter of an ICL by a single replication fork initiates repair. However, work based on replication of a crosslinked plasmid in *Xenopus laevis* egg extracts resulted in a new model in which collisions on both sides of an ICL are envisioned as the trigger for repair. The stalled replication forks, either from one or both sides of an ICL, are recognized by the DNA translocase FANCM and its binding partners FAAP24, MHF1 (FAAP16) and MHF2 (FAAP10). Deletion of these proteins results in a cellular FA phenotype with reduced FANCD2 mono-ubiquitination, increased chromosomal breakage and ICL sensitivity. They can form three distinct complexes: one containing all 4 proteins (FANCM-FAAP24-MHF1/2), a heterodimer of FANCM-FAAP24 and a complex consisting of FANCM-MHF1/2, in
which multiple MHF1 and MHF2 proteins form a heterotetrameric structure\textsuperscript{115,117,118}. The conserved HhH\textsubscript{2} domain at the C-terminus of FANCM enables FANCM to form a heterodimer with FAAP24, whereas the interaction with the MHF1/2 protein complex is mediated via the region following the helicase domain of FANCM\textsuperscript{114–116}. Many studies have been performed to elucidate the binding preference of FANCM, FAAP24 and MHF1/2 to various DNA substrates. FANCM binds specifically to Holliday junctions and replication forks\textsuperscript{110,120}, whereas FAAP24 and MHF prefer to bind to single-stranded and double-stranded DNA, respectively\textsuperscript{114–116,121}. Moreover, FAAP24 also displays affinity to splayed arm DNA and 3’-flap DNA, while the MHF complex, like FANCM, can bind to branched DNA structures (e.g. Holliday junctions and replication forks). This resulted in an initial model in which FANCM, FAAP24 and MHF simultaneously bind different parts of a stalled replication fork: FANCM at the branch point and FAAP24 and MHF at the surrounding ssDNA and dsDNA, respectively\textsuperscript{115}. Two alternative models were suggested when, in addition to the DNA binding preferences of FANCM and its partners alone, the impact of the interaction between FANCM, FAAP24 and MHF1/2 on DNA binding activity was investigated. The DNA binding preference of FANCM was not affected by the presence of FAAP24 or vice versa\textsuperscript{119}, while the binding affinity of the MHF-FANCM complex changed. Although there is some discrepancy regarding the preference of MHF alone to bind to dsDNA over branched DNA (most likely depending on salt concentrations), the FANCM-MHF complex prefers and has an increased affinity to bind to branched DNA\textsuperscript{122,123}. This resulted in two models: 1) both FANCM and MHF bind to the branch point of a stalled replication fork due to the remodeling activity of FANCM on MHF, thereby changing the DNA binding preference of MHF from dsDNA by MHF alone to branched DNA by the FANCM-MHF complex\textsuperscript{122}. 2) MHF functions as a heterotetramer\textsuperscript{117,118} and binds concurrently to the two DNA arms of a replication fork, followed by the recruitment and increased binding affinity of FANCM to the DNA junction\textsuperscript{123}. Regardless of which model is true, both FAAP24 as well as the MHF heterotetramer are required for the localization of FANCM to the stalled replication fork to trigger repair or stimulate replication fork restart\textsuperscript{116,124}.

5.2. Step 2: Remodeling of the stalled replication fork and replication traverse by FANCM

Besides a role in sensing ICL-induced stalled replication forks, FANCM exerts multiple roles downstream this step in maintaining genome stability. First, biochemical studies have shown that FANCM contains an ATP-dependent branch point translocase activity, which promotes migration of Holliday junctions, replication fork reversal and D-loop dissociation\textsuperscript{51,119,120,125}. The branch migration activity of FANCM is independent of FAAP24, while MHF exerts a stimulatory effect on FANCM’s ability to process branched DNA structures\textsuperscript{115,116,119}. As the result of the
combined branch migration and fork reversal activities of FANCM, replication fork regression occurs, providing an important mechanism to stabilize stalled replication forks or to allow repair factors to gain access to the replication-blocking lesion. Thereby, FANCM is essential for constant progression of replication forks as well as for resumption of DNA replication after DNA damage. Since accumulating evidence showed that replication fork collisions on both sides of an ICL are the trigger for repair, the remodeling activity of FANCM-MHF might aid in stabilizing the first stalled replication fork or promoting dormant origin firing until a second replication fork approaching from the opposite direction encounters the ICL.

As an alternative to an approaching replication fork from the opposite direction, replication traverse has been suggested. This is a translocase-based mechanism in which DNA replication forks bypass ICLs without unhooking them (leaving the unrepaired ICL behind and allowing fork progression). Similar to the dual fork collision model, replication traverse will also result in an X-shaped DNA molecule that triggers repair. Although it requires the translocase activity of FANCM-MHF1/2 complex and only takes a few minutes, it is unclear how DNA replication forks bypass or “traverse” ICLs. Possible scenarios could be the translocation of the MCM replicative helicase complex by FANCM (MCM complex jumps over the ICL) or a new MCM or a different replicative DNA helicase is recruited on the other side of the ICL. However, the recruitment of a new MCM or other replicative DNA helicase is unlikely given the absence of known MCM loading mechanisms during S-phase. Of note, it has been demonstrated by using Xenopus egg extract that BRCA1 promotes unloading of the replicative helicase complex, which is an essential and early event in the repair of cross-links. Finally, it is remarkable that FANCM and the MHF proteins are the only FA core complex proteins with orthologs in yeast, implying that the traverse activity as well as the branch migration activity of these proteins occurred before the emergence of the complete FA pathway.

5.3. Step 3: Checkpoint activation mediated by FAAP24

The third important step in repairing DNA ICLs is to allow enough time to repair the lesion before going to the next phase of the cell cycle. Several studies demonstrated a role of FANCM and FAAP24 in promoting intra-S-phase and G2-phase cell cycle arrest by checkpoint activation when a replication fork encounters an ICL. However, comprehensive research of one research group showed that FAAP24 plays the primary role in checkpoint activation thereby stopping the cell cycle. Activation of the checkpoint, which is mediated by the serine/threonine-specific protein kinases ATR and CHEK1, begins when a stretch of single-stranded DNA coated by RPA is generated as consequence of an ICL-induced stalled replication fork. ATRIP, a binding partner of ATR, binds to the RPA-coated ssDNA and recruits ATR. Activation of these two proteins (ATR-ATRIP) requires
**Chapter 1**

**Step 1: Sensing of DNA interstrand crosslink damage**

Step 3: Checkpoint activation

Step 5: Mono-ubiquitination of FANCD2 and FANCI

**Step 2a: Replication fork regression - remodeling**

Step 4: Recruitment FA core complex and BLM complex

**Step 2b: Replication traverse - bypass of ICL**

**Step 6: Unhooking**
General introduction

Figure 2 The Fanconi anemia (FA) pathway.

A stalled replication fork due to encountering of an interstrand crosslinks (ICL) will be detected by FANCM, FAAP24 and MHF1 and 2 (step 1). Given that an X shaped DNA molecule is the trigger for repair, FANCM-MHF1/2 complex may remodel the replication fork to stabilize the fork until a second replication fork approaching from the opposite direction encounters the ICL (alternative step 2a) or FANCM-MHF promotes replication traverse, in which the ICL is bypassed or “traversed” by the replicative MCM DNA helicase leading to replication fork progression. The precise mechanism is unclear and it could also be that a different DNA helicase is loaded onto the distal site of the ICL (alternative step 2b). FAAP24 activates ATR, which leads to activation of the intra-S-phase and G2/M checkpoints, allowing enough time to repair the DNA damage. In addition, ATR phosphorylates several FA proteins, such as FANCD2 and FANCI (step 3 and 5). FANCM recruits two protein complexes to the site of DNA damage: the FA core complex and the BLM complex (step 4). The FA core complex mono-ubiquitinates FANCD2 and FANCI, which is a central event in the FA pathway and is required for downstream repair events (step 5). Mono-ubiquitinated FANCD2 promotes incisions near the ICL. The different structure-specific endonucleases can interact with the scaffold protein SLX4 (FANCP). XPF (FANCQ) is the primary enzyme that makes the 3’ incision to the ICL. The 5’ side of the ICL may be incised by SLX1, FAN1 or even also XPF (step 6). As a consequence of unhooking, an intact but crosslinker damaged and a broken sister chromatid are generated. The intact sister chromatid is repaired by translesion synthesis, in which REV1 and Polymerase ζ (zeta) play an important role (step 7) combined with nucleotide excision repair, whereas the broken sister chromatid is repaired by homologous recombination (step 8). Homologous recombination is initiated by DNA end resection (facilitated by the MRN (MRE11, RAD50, NBS1) complex, CtIP, BLM EXO1 and DNA2), followed by RAD51 nucleofilament formation (mediated by BRCA1, BRCA2 and PALB2) and strand invasion. Finally, the Holliday junction that is generated during homologous recombination is dissolved by the BLM complex or resolved by SLX4-SLX1, MUS81-EME1 or GEN1.
additional regulators, such as the RAD17-RFC complex, the Rad9-Hus1-Rad1 (9-1-1) complex and TOPBP1 (refs. 135 and 136). The 9-1-1 complex is loaded onto DNA by the RAD17-RFC complex at the junction of ssDNA and dsDNA. This is eventually followed by the recruitment of TOPBP1, which can interact with RAD17, the 9-1-1 complex and autophosphorylated ATR to increase the kinase activity of ATR and facilitate ATR to recognize its substrates. Finally, the phosphorylation of ATR substrates (e.g. CHEK1) leads to intra-S-phase and G2/M checkpoint activation. The process of ATR activation by FANCM-FAAP24 is still not fully understood, but may occur: 1) via interaction with the checkpoint protein HCLK2, which facilitates ATR activation and CHEK1 stabilization, 2) through regulating RPA recruitment at ICL-induced stalled replication forks, 3) or by FANCM mediated TOPBP1 retention.

Interestingly, ATR or its downstream phosphorylation target CHEK1 appears to directly regulate the FA pathway by the phosphorylation of multiple FA proteins (FANCA, FANCD2, FANCE, FANCG, FANCI and FANCM). ATR-mediated phosphorylation of FANCM (S1045) is required to localize FANCM to ICLs and to mediate FANCM-FAAP24-dependent ATR/CHEK1 signaling. Phosphorylation of FANCA (S1449 by ATR), FANCD2 (T691, S717 by ATR/ATM and S331 by CHEK1), FANCE (T346 and S374 by CHEK1), FANCG (S7 by CHEK1) and FANCM (S1045 by ATR) are required for resistance to ICL-inducing agents and facilitates the mono-ubiquitination of FANCD2 and FANCI, whereas phosphorylation of FANCI is even an absolute prerequisite for the mono-ubiquitination of FANCD2. Thereby, this posttranslational modification step functions as a switch to stimulate FANCD2 mono-ubiquitination in order to activate the FA pathway.

5.4. Step 4: Recruitment of the FA core and Bloom’s protein complexes by FANCM

Besides 1) sensing ICL damage, 2) remodeling ICL-stalled replication forks and 3) checkpoint activation, the fourth role of FANCM is the recruitment of two distinct protein complexes: the FA core complex and the Bloom’s complex (Fig. 2). The FA core complex is responsible for mono-ubiquitination of FANCD2 and FANCI. Although FANCM and MHF are part of this large complex, the majority of FANCM-MHF does not associate with the FA core complex. This further suggests that besides the recruitment of other FA proteins and stimulating FANCD2 mono-ubiquitination, FANCM and MHF1/2 have additional roles. Indeed, as mentioned before, FANCM and MHF1/2 are involved in both remodeling of stalled replication forks as well as replication traverse, which is independent of the FA core complex. Besides the FA core complex, FANCM can also interact with RMI1 and Topoisomerase IIIa to recruit the Bloom’s complex (RMI1, RMI2, BLM and TopoIII) to the site of DNA damage. This complex is also known as the dissolvasome as it is involved in the separation
of Holliday junctions that can arise as DNA intermediates during DNA repair\cite{159}. BLM is a multifunctional protein involved in 1) DSB repair (e.g. via 5’ end resection and resolving double Holliday junction (dHJ) structures)\cite{160}, 2) the suppression of sister chromatid exchanges\cite{161} and 3) stimulating replication restart by promoting fork regression\cite{162}. Interestingly, FANCM and BLM share similar functions as both, BLM deficiency and FANCM deficiency caused an increased frequency of sister chromatid exchanges\cite{159,164,165} and both proteins have been implicated in replication fork progression. Therefore, the Bloom’s protein complex might be recruited to ICL-induced stalled replication forks by FANCM to stimulate fork regression, followed by replication fork stabilization, or to mediate downstream steps of ICL repair (e.g. repair of DSBs or dHJ resolution)\cite{159}.

In summary, ICLs are recognized by the FA and FA-associated proteins FANCM, MHF1/2 and FAAP24, which have cooperative as well as unique functions\cite{163}. All four proteins play an important role in the resistance to DNA crosslinking agents and the recruitment of the FA core complex. In addition, FANCM, MHF1 and MHF2 are also involved in replication fork remodeling and replication traverse, while FANCM-FAAP24 act in DNA damage-induced checkpoint activation.

5.5. Step 5: Mono-ubiquitination of FANCD2 and FANCI by the FA core complex

Mono-ubiquitination of the FANCD2-FANCI heterodimer is believed to be the central event in the FA pathway and is essential for downstream repair steps\cite{81}. ATR-mediated phosphorylation of FANCI initiates mono-ubiquitination of FANCI and FANCD2 (ref 158). The post-translational modification of these two proteins is executed by a multisubunit E3 ubiquitin ligase complex, also known as the FA core complex, and the E2 ubiquitin conjugating enzyme UBE2T\cite{166,167}. The FA core complex can be further divided into three subcomplexes: 1) the FANCA, FANCG and FAAP20 (A-G-20) subcomplex, 2) the FANCC, FANCE and FANCF (C-E-F) subcomplex and 3) the FANCB-FANCL-FAAP100 (B-L-100) subcomplex, which forms the catalytic module of which the RING domain protein FANCL bears the E3 ligase activity\cite{47,167–169}. Although all three proteins of the B-L-100 complex are absolutely essential for mono-ubiquitination of FANCD2, it is unclear how FANCB and FAAP100 contribute to this post-translational modification\cite{167,169}. Since the B-L-100 subcomplex is more active in FANCD2 mono-ubiquitination than FANCL alone, a conceivable role could be that FANCB and FAAP100 stimulate the E3 ligase activity of FANCL by stabilizing the protein or by supporting a conformation that enhances substrate recognition and positioning\cite{167,169}. Moreover, the interaction of the FA core complex with chromatin probably enhances the activity of the complex further as mono-ubiquitination of FANCD2 is augmented by the presence of DNA\cite{167}.

The work of Huang et al.\cite{169} and Rajendra et al.\cite{167} showed unexpectedly that not all FA core complex members contribute equally to mono-ubiquitination of
FANCD2, of which the amount correlated with ICL sensitivity. Residual FANCD2 mono-ubiquitination was observed in DT40 cells or gene-targeted mammalian cells depleted of members of the A-G-20 or C-E-F subunits. This is in contrast to FA patient-derived cell lines, in which FANCD2 mono-ubiquitination is absent in all FA core complex complementation groups except for complementation group FA-M. Despite these differences, the A-G-20 and C-E-F subunits are important and most likely contribute to activity, substrate recognition, specificity and localization of the complex to damaged chromatin. Indeed, FANCF has been shown to interact with FANCM, thereby facilitating the localization of the FA core complex to ICL-induced stalled replication forks, whereas the A-G-20 subcomplex might be recruited via RNF8-mediated ubiquitination of histone H2A. Besides stabilizing FANCA, FAAP20 contains an UBZ (ubiquitin binding zinc finger) domain capable of interacting with K63-linked ubiquitin chains on histone H2A, which are catalyzed by RNF8 and UBC13. Thus, the A-G-20 and C-E-F subcomplexes might be independently recruited to ICLs via RNF8 or FANCM, respectively. However, the recruitment of the FA core complex could also be cell cycle dependent in which the RNF8-FAAP20 cascade plays a role in the recruitment of the FA core complex in a replication-independent manner, while FANCM, MHF and FAAP24 operate during S phase (replication-dependent recruitment).

Finally, not only mono-ubiquitination of FANCD2 and FANCI is an essential step in the FA pathway, but also the deubiquitination of these proteins is important as disruption of either ubiquitination or deubiquitination results in abnormal DNA ICL repair. The deubiquitination enzyme (DUB) USP1 (ubiquitin-specific protease 1) together with its binding partner UAF1 are responsible for deubiquitinating FANCD2 and FANCI. Depletion of these proteins causes elevated levels of FANCD2 and FANCI mono-ubiquitination as well as cellular sensitivity to ICL agents. Moreover, Usp1 knockout mice (of which a large percentage die soon after birth) recapitulate many phenotypical aspects of FA, including growth retardation, gonadal dysfunction, haematopoietic defects and ICL sensitivity. An FA-like phenotype in terms of growth retardation, reduced fertility and ICL induced chromosomal instability was also observed in Uaf1−/− mice, whereas Uaf1−/− mice die during embryogenesis. Taken together, protein ubiquitination as well as deubiquitination are important regulatory mechanisms for the repair of ICLs.

5.6. Step 6: Unhooking of the crosslink by structure-specific endonucleases

Mono-ubiquitination of the FANCD2-FANCI heterodimer is followed by incisions at both sides of the ICL in one of the cross-linked strands, which is also known as unhooking (Fig. 2). In the dual convergence and replication traverse models (see section 5.2.), the DNA structure around the ICL would be an X-shaped form. In the dual fork convergence model, the 3’ ends of both converged leading strands initially
stall 20-40 nucleotides from the ICL, followed by the release of the MCM DNA replicative helicase mediated by BRCA1 (refs. 131 and 180). Subsequently, one leading strand advances to within 1 nucleotide of the ICL and the opposite parental strand is incised on either side of the ICL by structure-specific endonucleases\textsuperscript{180}. Depletion of FANCD2 in *Xenopus* egg extract or failure to mono-ubiquitinate FANCD2 prevents unhooking of the crosslink, demonstrating the importance of mono-ubiquitinated FANCD2 to promote incisions near the ICL\textsuperscript{181}. Six nucleases (XPF-ERCC1, MUS81-EME1, SLX1, FAN1, SNM1A and SNM1B) have been implicated in ICL repair as deficiency in any of these six nucleases leads to cellular hypersensitivity to ICL-inducing agents\textsuperscript{129,182–192}. XPF-ERCC1, MUS81-EME1, SLX1 and FAN1 have structure-specific endonuclease activity and might be responsible for the incisions near the crosslink, while SNM1A and SNM1B possess exonuclease activity, which might be required after the primary incisions at the crosslink are made\textsuperscript{192}. Besides endonuclease activity, FAN1 also exhibits 5\textsuperscript{\prime}→3\textsuperscript{\prime} exonuclease activity\textsuperscript{112}.

Through direct interaction, the scaffold protein SLX4/FANCP acts as a docking platform for the recruitment of three endonucleases (XPF-ERCC1, MUS81-EME1 and SLX1) to the site of DNA damage\textsuperscript{185,189,191}. Since SLX4 contains two tandem ubiquitin-binding zinc finger (UBZ) domains, which can bind to ubiquitin, it is tempting to speculate that SLX4 binds to mono-ubiquitinated FANCD2 and thereby, regulates the unhooking step by the recruitment of structure-specific endonucleases. This has indeed been shown in chicken DT40 cells, in which the UBZ domain of SLX4 is responsible for the interaction with ubiquitinated FANCD2\textsuperscript{193}, but could not be recapitulated in human cells\textsuperscript{194}. Nevertheless, one of the UBZ domains of SLX4 is important to localize SLX4 to the site of damage, but the ubiquitinated ligand in question seems not to be FANCD2 in human cells\textsuperscript{194}.

Based on DNA substrate preferences, XPF-ERCC1 and MUS81-EME1 are likely candidates to cut on the 3\textsuperscript{\prime} side of an ICL-associated X-shaped DNA structure, while SLX1 and FAN1 might prefer the 5\textsuperscript{\prime} side\textsuperscript{112}. Accumulating evidence suggests that XPF-ERCC1 is the most likely nuclease to unhook a DNA crosslink in the context of the FA pathway, while MUS81-EME1 probably cleaves ICL-induced stalled replication forks when normal fork processing is disrupted, resulting in an accumulation of double strand breaks\textsuperscript{192,195,196}. First, the interaction of SLX4 with XPF-ERCC1 is important for ICL repair, whereas the interaction of SLX4 with MUS81-EME1 is dispensable for repairing crosslinks\textsuperscript{197}. Moreover, the interaction of XPF-ERCC1 with recombinant mini-SLX4 (encompassing the N terminal domain, only capable of binding to XPF-ERCC1), enhances the nuclease activity of XPF-ERCC1 towards splayed arm and ICL-containing structures\textsuperscript{198}. Secondly, MUS81 depleted cells are less sensitive to ICL-inducing agents compared to XPF-depleted cells\textsuperscript{112}. Thirdly, work based on replication of a crosslinked plasmid in *Xenopus* egg extract indeed showed that depletion of XPF, but not of MUS81 prevented the incisions near the crosslink\textsuperscript{69}. 

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Since neither of the two incisions (3’ or 5’ incision) occur in XPF-depleted *Xenopus* egg extracts, XPF might be responsible for both incisions. Alternatively, the 5’ incision cannot take place without the 3’ incision and is actually made by a different endonuclease (e.g. SLX1 or FAN1), which requires prior incision by XPF-ERCC1 on the 3’ side of the ICL-associated X-shaped structure before it can cut the 5’ side. Of note, FAN1 contains an UBZ4-type ubiquitin binding domain which can interact with mono-ubiquitinated FANCD2 (refs. 186–188 and 190). Although FAN1 deficiency results in ICL sensitivity, FAN1 inactivation does not lead to FA but to chronic kidney disease. Collectively, these data suggest that FAN1 might function in ICL repair, but the precise role of FAN1 as well as of SLX1 is unclear.

5.7. Step 7: translesion synthesis

An intact as well as a broken sister chromatid are generated as a consequence of flanking incisions near an ICL. The intact sister chromatid is repaired by DNA translesion synthesis (TLS) (Fig. 2). This is a mechanism in which specialized low fidelity polymerases bypass bulky damaged lesions that block the processivity of normal replicative polymerases. By using the *Xenopus* replication system, it was shown that in addition to the nucleolytic incisions, FANCD2 mono-ubiquitination is required for facilitating the TLS step in ICL repair. Several TLS polymerases, including polymerase eta (Pol η/ POLH), polymerase iota (Pol ι/ POLI), polymerase kappa (Pol κ/ POLK), REV1, polymerase zeta (Pol ζ/ POLZ) and polymerase nu (Pol ν/ POLN), have been implicated in ICL repair. Based on biochemical studies, these polymerases are capable of inserting a base opposite the unhooked ICL and/or are responsible for extension of the nascent DNA beyond the crosslinked base pair, though the efficiency is dependent on the structure and the extent of endo- or exonucleolytic processing of the ICL. REV1 and polymerase ζ, which consists of the catalytic subunit REV3L and accessory subunit REV7, are key players in ICL repair as cells deficient in either one of these proteins are hypersensitive to ICL-inducing agents. REV1 is a deoxycytosine monophosphate (dCMP) transferase that can insert a cytosine opposite an unhooked ICL, which is then extended by polymerase zeta. The bypassed crosslink is subsequently removed by nucleotide excision repair (Fig. 2). Of note, similar to Rev1-deficient cells, FA patient cells are hypomutagenic in response to UV and a lower frequency of spontaneous point mutations was observed in FANCC-depleted chicken DT40 cells. These results further suggest that the FA pathway is important for mediating error prone TLS. The role of Polymerase zeta in replication-dependent ICL repair was supported by studying ICL repair in Rev7-depleted *Xenopus* egg extract. In the absence of Rev7, ICL repair was defective due to absence of the TLS extension step, while the nucleotide opposite the ICL was still inserted, most likely by Rev1 (ref. 180). By using a non-replicating plasmid-based reporter assay, it was also demonstrated...
that REV1 and Pol ζ are involved in bypass of psoralen, MMC or cisplatin site-specific ICLs in a replication-independent manner\textsuperscript{213,219}. In the Go/G1 phase of the cell cycle, TLS polymerases are recruited to ICLs by ubiquitinated proliferating nuclear antigen (PCNA)\textsuperscript{202,220–222}, whereas the coordination of TLS polymerases to the site of damage in S phase is less clear-cut, though a role of FAAP20 in recruiting REV1 has been suggested\textsuperscript{173}. Of note, Pol η, Pol κ or Pol ν have also been implicated in ICL repair and cells deficient in one of these polymerases display an FA phenotype in terms of sensitivity to ICL-inducing agents\textsuperscript{204,223–232}. However, the precise role of these polymerases in ICL repair is unclear, but these polymerases might play a minor role in the repair of crosslinked DNA depending on the structure of the ICL or in specific pathways or situations\textsuperscript{109}.

5.8. Step 8: homologous recombination

The intact sister chromatid, which is generated by the incision events, is repaired by translesion synthesis, whereas the broken sister chromatid is repaired by homologous recombination (HR) using the sister that has been repaired by TLS as a template\textsuperscript{81,109}. Several FA proteins that operate downstream of FANCD2 monoubiquitination are implicated in HR, including FANCD1 (BRCA2), FANCJ, FANCN (PALB2), FANCO (RAD51C) and FANCS (BRCA1). Homologous recombination repair (HRR) is initiated by end resection of the double strand break by nucleolytic degradation of the 5’ strand\textsuperscript{233,234}. This is performed by the combined action of CtIP and the MRN (MRE11-RAD50-NBS1) complex, together with BLM, EXO1 and DNA2\textsuperscript{235,236}). Remarkably, CtIP can interact with two FA proteins: monoubiquitinated FANCD2 (refs. 237–239) and when phosphorylated with BRCA1 (ref. 240). These interactions aid in the recruitment of CtIP to DSBs generated during ICL processing\textsuperscript{237–239}, accelerates CtIP-mediated end resection\textsuperscript{241} and in turn favor repair of these breaks by HR\textsuperscript{234,242}. As a consequence of end resection, the 3’ single-stranded stretch of DNA is initially coated by replication protein A (RPA), and subsequently displaced by the key facilitator of HR, RAD51, to form long helical polymers wrapped around the ssDNA tail\textsuperscript{243,244}. The assembly of RAD51 nucleofilaments specifically onto ssDNA protected by RPA requires the help of a variety of mediator proteins, including several FA proteins, such as BRCA1 (FANCS), BRCA2 (FANCD1), PALB2 (FANCN) and RAD51C (FANCO)\textsuperscript{245–247}. Besides the interaction with CtIP, BRCA1 can also directly interact with PALB2 (refs. 248 and 249) and FANCJ\textsuperscript{77}. Since PALB2 binds in addition directly to BRCA2 and RAD51 (refs. 247, 250 and 251), PALB2 not only mediates the localization of BRCA2 and RAD51 to the site of damage, but it also bridges BRCA1-BRCA2 interactions\textsuperscript{248,249}. Evidence also supports a role for FANCJ in homologous recombination, but it is still unclear at what step and how FANCJ precisely participates in HRR. BRCA2 and PALB2 are both essential for the specific assembly and stability of RAD51 nucleofilaments, thereby stimulating RAD51-
mediated strand exchange\textsuperscript{250–260}. This strand invasion into an intact homologous DNA molecule (in case of ICL repair: the sister chromatid that has been repaired by TLS) results in the formation of a displacement D-loop, followed by DNA synthesis of the ssDNA ends to generate a double Holliday junction (HJ) intermediate\textsuperscript{244}. Finally, this double HJ intermediate is dissolved by the BLM-RPM-TOP3α complex, which is plausibly recruited to the site of damage by FANCM, or resolved by structure-specific endonucleases (\textit{e}\textit{g}. SLX4-SLX1, MUS81-EME1 or GEN) that cleave symmetrically HJs\textsuperscript{261}.

**Table 3 Function of FA proteins**

<table>
<thead>
<tr>
<th>Protein</th>
<th>Function</th>
</tr>
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<tbody>
<tr>
<td>FANCA</td>
<td>Part of FA core complex; mono-ubiquitinates FANCD2-FANCI</td>
</tr>
<tr>
<td>FANCB</td>
<td>Part of the catalytic module of the FA core complex, mono-ubiquitinates FANCD2-FANCI</td>
</tr>
<tr>
<td>FANCC</td>
<td>Part of FA core complex; mono-ubiquitinates FANCD2-FANCI</td>
</tr>
<tr>
<td>FANCD1 (BRCA2)</td>
<td>Homologous recombination; loads RAD51 onto DNA</td>
</tr>
<tr>
<td>FANCD2</td>
<td>Coordinates downstream repair events</td>
</tr>
<tr>
<td>FANCE</td>
<td>Part of FA core complex; mono-ubiquitinates FANCD2-FANCI</td>
</tr>
<tr>
<td>FANCF</td>
<td>Part of FA core complex; mono-ubiquitinates FANCD2-FANCI</td>
</tr>
<tr>
<td>FANCG</td>
<td>Part of FA core complex; mono-ubiquitinates FANCD2-FANCI</td>
</tr>
<tr>
<td>FANCI</td>
<td>Coordinates downstream repair events</td>
</tr>
<tr>
<td>FANCI</td>
<td>Homologous recombination, 3’-5’ helicase; unwinds G-quadruplex structures</td>
</tr>
<tr>
<td>FANCL</td>
<td>Part of the catalytic module of the FA core complex (FANCL bears E3 ligase activity), mono-ubiquitinates FANCD2-FANCI</td>
</tr>
<tr>
<td>FANCM</td>
<td>Part of FA core complex, recruits FA core and BLM complex to site of damage; 5’-3’ translocase and checkpoint activation</td>
</tr>
<tr>
<td>FANCN (PALB2)</td>
<td>Homologous recombination; localizes and promotes BRCA2 function</td>
</tr>
<tr>
<td>FANCO (RAD51C)</td>
<td>Homologous recombination</td>
</tr>
<tr>
<td>FANCP (SLX4)</td>
<td>Scaffold protein for several endonucleases: XPF-ERCC1, MUS81-EME1 and SLX1</td>
</tr>
<tr>
<td>FANCQ (XPF)</td>
<td>Structure-specific endonuclease involved in unhooking ICL, role in nucleotide excision repair</td>
</tr>
<tr>
<td>FANCS (BRCA1)</td>
<td>Homologous recombination, DNA repair pathway choice, unloading replicative helicase complex</td>
</tr>
<tr>
<td>FAAP10-FAAP16 (MHF1-MHF2) complex</td>
<td>Stimulates branch migration and replication fork reversal activities of FANCM Recruitment of FANCM to chromatin Stabilizes FANCM and FAAP24 Required for optimal FANCD2 mono-ubiquitination</td>
</tr>
<tr>
<td>FAAP20</td>
<td>Part of FA core complex; mono-ubiquitinates FANCD2-FANCI, stabilizes FANCA</td>
</tr>
<tr>
<td>FAAP24</td>
<td>ATR-dependent checkpoint activation upon ICL damage</td>
</tr>
<tr>
<td>FAAP100</td>
<td>Part of the catalytic module of the FA core complex, mono-ubiquitinates FANCD2-FANCI</td>
</tr>
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</table>
6. **HNSCC**

Bi-allelic germline mutations in FA genes predispose to head and neck cancer: FA patients have a more than 500 to 700 times higher risk to develop HNSCC than an unaffected individual. These tumors are often located within the oral cavity, *e.g.* at the edge of the tongue and the gingival areas. Other etiological factors in the development of head and neck cancer and the molecular carcinogenesis of HNSCC are described in this section.

6.1. **Smoking, alcohol consumption and HPV infection as underlying cause of HNSCC**

Despite familial predisposition to HNSCC, most head and neck tumors seem to occur sporadically. These tumors account for more than a half million new cases a year, making HNSCC the sixth common cancer by incidence worldwide. The two most important risk factors for the development of head and neck cancer are tobacco use and excessive alcohol consumption, which have a synergistic effect. In addition to these exogenous risk factors, infection with high-risk types of human papilloma virus (HPV) is causative for a subset of head and neck cancers. The presence of HPV is particularly common in tumors that arise in the oropharynx, which are associated with a favorable outcome. The incidence of these tumors is increasing in most European countries including the Netherlands, as well as the United States. Given that infection with high-risk HPV is also a well-known etiologic factor in cervical carcinogenesis, a change in sexual behavior (*e.g.* increase in practice of oral sex) might explain the observed increase. Of note, an early study showed that many head and neck tumors in FA patients were HPV-positive. However, the assay that was used to detect HPV was likely too sensitive, which may have resulted in many false positive cases. The association between HPV and HNSCC in individuals affected with FA could not be confirmed in other studies, suggesting that HPV might not be the main cause of head and neck cancer in FA patients. Notwithstanding, the data on the role of HPV in anogenital tumors in FA patients are concordant, and the large majority of these tumors are HPV-positive. Hence, preventive vaccination is still encouraged. FA patients are advised not to smoke or use alcohol, but it is often difficult for young adults to follow this. The danger of alcohol consumption for FA patients is now better understood by recent evidence showing that FA-deficient cells are hypersensitivity to acetaldehyde, which is a byproduct of alcohol metabolism. Thus, alcohol consumption may increase genomic instability in these cells, and thereby might promote carcinogenesis in FA patients.

6.2. **Genetic predisposition to head and neck cancer**

Besides FA, there are several other syndromes associated with an increased risk of
Chapter 1

head and neck cancer. Patients with Dyskeratosis congenita (DC), an inherited bone marrow failure syndrome clinically similar to FA, have a relative risk of more than 1100-fold for HNSCC. Currently, mutations in nine genes (DKC1, TINF2, TERC, TERT, WRAP53 (TCAB1), CTC1, RTE1, NHP2 and NOP10) leading to aberrant telomere biology have been reported to be causative of DC. A second syndrome associated with an increased risk of HNSCC, but also of melanoma and pancreatic cancer, is familial atypical multiple mole melanoma (FAMMM), which is caused by an inactivating germline mutation in CDKN2A, encoding the p16 protein involved in cell cycle regulation. The CDKN2A gene is often mutated or deleted in a wide variety of malignancies, emphasizing its important role as a tumor suppressor gene.

The third gene in which a mutation leads to an unusually high risk of head and neck cancer is ATR. In this gene, a specific heterozygous missense mutation was found in individuals affected with familial cutaneous telangiectasia and increased cancer risk. This mutation predominantly predisposes to oropharyngeal cancer as 10 out of 24 affected individuals (all part of one family) developed this tumor. Rare germline mutations in ATR have also been reported in Seckel syndrome-1 (SCKL1), which is an autosomal recessive disorder characterized by growth retardation, dwarfism, microcephaly, mental retardation and the characteristic “bird-headed” facial appearance, but not by an increased cancer risk. However, one specific mutation and only five individuals with SCKL1 belonging to two related families have been described, making it uncertain whether the clinical phenotype of ATR deficiency has been fully identified. Furthermore, germline mutations in TP53 or BLM are associated with Li-Fraumeni syndrome or Bloom syndrome, respectively, and predispose to a variety of cancers, including HNSCC. Finally, a role for hereditary susceptibility factors in the development of HNSCC is suggested by the observation that first-degree relatives of patients with HNSCC have an increased incidence to develop head and neck cancer as well. This might be explained by the inheritance of genetic polymorphisms in genes affecting the function of carcinogen activating or detoxifying enzymes (e.g. cytochrome P450 group, GSTM1, ALDH2), or functioning in the apoptotic pathway (e.g. BIRC5) or DNA repair pathways (e.g. XRCC1 and XPD).

6.3. Treatment of patients with head and neck cancer

HNSCCs are staged according to the tumor dimensions and growth patterns as well as the dimension and number of lymph node metastasis in the neck and at distant sites. Depending on the stage and site of the disease, patients with sporadic HNSCC are treated with surgery, radiotherapy, chemotherapy or a combination of these modalities. About one third of HNSCC patients presents with early stage disease and is treated either by surgery or radiotherapy alone. Patients with advanced disease stages, representing two third of HNSCC patients, require a
multi-modality treatment approach, in which surgery is frequently combined with postoperative radiotherapy and/or chemoradiation. The latter consists of systemic cisplatin administration combined with locoregional radiotherapy and is, combined with surgery for salvage, increasingly performed to achieve organ-preservation. Cisplatin is very toxic and causes nausea and vomiting, myelosuppression, renal failure and deafness. Particularly when combined with concomitant radiotherapy also severe mucositis and tissue damage may occur leading to long term sequelae such as feeding tube dependence. For patients who are unfit to receive cisplatin, bioradiation can be applied, which consists of the concomitant application of cetuximab (anti-EGFR antibody) and radiotherapy. In contrast to patients with early-stage tumors, those with advanced malignancies have a poor prognosis and only 40-50% will survive for 5 years after treatment. These survival rates are poor because patients often develop locoregional recurrences, distant metastases and second primary tumors. The latter relates to the phenomenon of “field cancerization” and incomplete resection of genetically altered mucosal changes. HPV-induced head and neck cancers have a very favorable prognosis, and several clinical trials to de-intensify therapy have been initiated.

The survival outcomes for FA patients with HNSCC are relatively poor due to limited treatment options. Chemoradiation is excluded for the treatment of HNSCC in FA patients as all cells of these patients are very sensitive to cisplatin. Even reduced cisplatin dosing results in very high toxicities and organ failure followed by a fatal outcome. Severe complications are also observed after radiotherapy. Therefore, the mainstay of treatment for FA HSNCC patients is surgery, preferably at an early stage, eventually combined with postoperative radiotherapy. FA patients seem not to have additional toxicity profiles for cetuximab, indicating that bioradiation or adjuvant cetuximab treatments are reasonable alternatives. The lack of peer reviewed meta-analyses on the treatment results of HNSCC in FA patients hampers the development of international guidelines for this specific patient group.

6.4. Molecular carcinogenesis of HNSCC

A better understanding of the molecular pathogenesis of sporadic as well as FA head and neck cancer may lead to novel strategies for early diagnosis and anti-cancer therapies as well as improved tailoring of existing treatment options for the individual patient. Because bone marrow transplantation outcomes have substantially improved over the past decades, more FA patients survive long-term and face the very high risk of developing head and neck cancer.

A plethora of studies, including genome-wide exome sequencing projects and a comprehensive multi-platform characterization of hundreds of head and neck tumors performed by The Cancer Genome Atlas, has appeared on the identification of genetic and epigenetic changes causing HNSCC. These changes lead to
cancer-related phenotypes, the so called hallmarks of cancer, which encompass limitless replicative potential, self-sufficiency in growth signals, insensitivity to anti-growth signals, ability to evade apoptosis, invasion and metastasis, angiogenesis, deregulation of cellular energy metabolism and evasion of immune destruction\textsuperscript{296,297}. Most genomes of head and neck tumors are highly unstable with many copy number alterations (CNA) (deletions or amplifications) and structural aberrations (chromosomal fusions). Chromosome regions 3p, 8p and 18q are frequently lost in HNSCC, whereas 3q, 5p and 8q are often gained\textsuperscript{288,292,294,295}. In contrast to CNAs of whole chromosome arms, changes in copy number of small regions were also observed: co-amplifications of 11q13 and 11q22 (harboring genes implicated in cell cycle regulation (\textit{CCND1}), cell death (\textit{BIRC2}) and Hippo pathway signaling (\textit{YAP1})), focal amplifications of 3q26/28 (involving \textit{TP63}, \textit{SOX2}, two squamous lineage transcription factors and the oncogene \textit{PIK3CA}, which plays a role in cell growth, survival, proliferation and differentiation in response to various growth factors), focal amplifications of 7p11.2 and 17q12 (containing growth factor receptors \textit{EGFR} and \textit{ERBB2}) and focal deletions of 9p21.3 (which is the location of the cell cycle regulator \textit{CDKN2A})\textsuperscript{288,292,294,295}. In addition, numerous genes were significantly found mutated in head and neck cancer, such as \textit{TP53}, \textit{CDKN2A}, \textit{HRAS}, \textit{PTEN}, \textit{PIK3CA}, \textit{SMAD4}, \textit{NOTCH1}, \textit{CASP8}, \textit{FAT1}, \textit{FBXW7}, \textit{AJUBA} and \textit{NSD1} (for an overview of gains and losses and mutations, see Table 4)\textsuperscript{288,292,294,295}. Of note, many mutated genes were located in regions of CNAs, often leading to loss of heterozygosity\textsuperscript{292,295}.

Although more than 95% of sporadic head and neck cancers are squamous cell carcinomas, the disease is heterogeneous and several subclasses of HNSCC exist at the histological and genetic level\textsuperscript{288}. Different genetic changes as well as distinctive prognostic implications were observed between HPV-infected and non-infected tumors, whereas a second divergence is based on karyotyping and ploidy analysis. This results in three subgroups of HNSCC: 1) HPV positive tumors; 2) HPV negative tumors, which are aneuploid and have many CNAs; and 3) a small group of (near)-diploid tumors without CNAs\textsuperscript{288,292,298–300}. The last subgroup is likely driven by mutation rather than CNAs and contain a three-gene pattern: activating mutations in \textit{HRAS} (role in cell division in response to growth factor stimulation), inactivating mutations in \textit{CASP8} (role in apoptosis) and wild type \textit{TP53}\textsuperscript{292,300,301}. Although \textit{TP53} is the most commonly mutated gene in HNSCC, mutations in \textit{TP53} are rare in HPV-positive tumors\textsuperscript{288}. This is not surprising as inactivation of the tumor suppressor protein p53 in HPV-infected HNSCC is achieved by the expression of viral oncoprotein E6, which binds p53 and targets the protein for degradation\textsuperscript{302,303}. Likewise, inactivation of \textit{CDKN2A} by mutations, methylation or loss of 9p21 and/or amplification of \textit{CCND1}, leading to deregulation of the RB pathway and driving the G1-S phase transition of the cell cycle, is also rare in HPV-positive tumors, because the viral oncogene E7 destabilizes the retinoblastoma protein (pRb)\textsuperscript{288}. 
## Table 4 Common genetic changes in HNSCC

<table>
<thead>
<tr>
<th>Gene</th>
<th>Affected pathway</th>
<th>Chromosome location</th>
<th>Predominant in tumor type</th>
<th>Genetic change</th>
</tr>
</thead>
<tbody>
<tr>
<td>CASP8</td>
<td>Apoptosis</td>
<td>2q33.1</td>
<td>(Near)-diploid</td>
<td>Mutations</td>
</tr>
<tr>
<td>TP63</td>
<td>Squamous lineage transcription factor</td>
<td>(3q26/28) 3q</td>
<td>(Focal) amplification</td>
<td></td>
</tr>
<tr>
<td>SOX2</td>
<td>Squamous lineage transcription factor</td>
<td>(3q26/28) 3q</td>
<td>(Focal) amplification</td>
<td></td>
</tr>
<tr>
<td>PIK3CA</td>
<td>PI3K/AKT signaling pathway</td>
<td>3q</td>
<td>Amplification</td>
<td>Activating mutations</td>
</tr>
<tr>
<td>FBXW7</td>
<td>E3 ubiquitin protein ligase, involved in the degradation of MYC and NOTCH1</td>
<td>4q31.3</td>
<td>Mutations</td>
<td></td>
</tr>
<tr>
<td>FAT1</td>
<td>WNT/β-catenin signaling; role in cell migration and invasion</td>
<td>4q35.2</td>
<td>HPV-</td>
<td>Mutations</td>
</tr>
<tr>
<td>NSD1</td>
<td>Histone 3 lys 36 (H3K36) methyltransferase</td>
<td>5q35.2</td>
<td>Mutations</td>
<td></td>
</tr>
<tr>
<td>EGFR</td>
<td>Growth factor receptor</td>
<td>7p11.2</td>
<td>Focal amplification</td>
<td></td>
</tr>
<tr>
<td>MYC</td>
<td>Transcription factor, role in cell cycle progression</td>
<td>8q</td>
<td>HPV-</td>
<td>Amplification</td>
</tr>
<tr>
<td>CDKN2A</td>
<td>Cell cycle inhibitor/regulated</td>
<td>9p21.3</td>
<td>HPV-</td>
<td>Focal deletionMutations Methylation</td>
</tr>
<tr>
<td>NOTCH1</td>
<td>Squamous cell differentiation</td>
<td>9q34.3</td>
<td>Mutations</td>
<td></td>
</tr>
<tr>
<td>PTEN</td>
<td>PI3K/AKT signaling pathway</td>
<td>10q23.31</td>
<td>Mutations</td>
<td></td>
</tr>
<tr>
<td>HRAS</td>
<td>MAPK/ERK pathway</td>
<td>11p15.5</td>
<td>(Near)-diploid</td>
<td>Activating mutations</td>
</tr>
<tr>
<td>CCND1</td>
<td>Cell cycle regulation</td>
<td>11q13 and 11q22</td>
<td>HPV-</td>
<td>Co-amplification</td>
</tr>
<tr>
<td>BIRC2</td>
<td>Cell death</td>
<td>11q13 and 11q22</td>
<td>HPV-</td>
<td>Co-amplification</td>
</tr>
<tr>
<td>YAP1</td>
<td>Hippo pathway signaling</td>
<td>11q13 and 11q22</td>
<td>Co-amplification</td>
<td></td>
</tr>
<tr>
<td>AJUBA</td>
<td>WNT/β-catenin signaling, role in cell division and implicated in Hippo pathway signaling</td>
<td>14q11.2</td>
<td>HPV-</td>
<td>Mutations</td>
</tr>
<tr>
<td>TRAF3</td>
<td>Role in innate and acquired anti-viral responses, involved in NF-κβ activation</td>
<td>14q32.32</td>
<td>HPV+</td>
<td>Mutations</td>
</tr>
<tr>
<td>TP53</td>
<td>Role in genome maintenance</td>
<td>17p13.1</td>
<td>HPV-</td>
<td>Mutations</td>
</tr>
<tr>
<td>ERBB2</td>
<td>Growth factor receptor</td>
<td>17q12</td>
<td>Focal amplification</td>
<td></td>
</tr>
<tr>
<td>SMAD4</td>
<td>TGF-beta signaling</td>
<td>18q 18q21.2</td>
<td>Loss</td>
<td>Mutations</td>
</tr>
<tr>
<td>E2F1</td>
<td>Cell cycle regulation (HPV associated)</td>
<td>20q11.21</td>
<td>HPV+</td>
<td>Focal amplification</td>
</tr>
</tbody>
</table>
Moreover, HPV-positive tumors were distinguished by focal amplifications of E2F1 (also involved in the G1-S phase transition) and deletions and truncating mutations in TRAF3, which plays a role in innate and acquired anti-viral responses and of which loss deregulates NF-κB signaling.

Taken together, by (epi)genetic changes many pathways are affected resulting in deregulated cell cycle progression (HPV infection, inactivation of TP53 and CDKN2A, overexpression of CCND1 and E2F1), evasion of apoptosis (deregulated NF-κB signaling, inactivation of BIRC2, CASP8 or TRAF3), aberrant cell polarity and differentiation (inactivation of NOTCH1, AJUBA and FAT1 causing aberrant Wnt/β-catenin signaling), and increased cell growth and proliferation in the absence of growth signals (activating mutations in PIK3CA and HRAS and amplifications of EGFR).

7. Aim and outline of this thesis

Fanconi anemia patients are strongly predisposed to develop head and neck squamous cell carcinomas. Although our knowledge of FA is increasing, it is still not clear whether inactivation of the FA-BRCA pathway also contributes to the pathogenesis of head and neck tumors in non-FA patients. The aim of this study is to examine the role of the FA-BRCA pathway in sporadic HNSCC and whether a defect in this pathway can be exploited as a target in cancer therapy.

To explore the occurrence of FA-BRCA pathway inactivation in sporadic HNSCC, it is important to know all proteins involved in this DNA repair pathway. Therefore, part of the work in this thesis has focused on a better understanding of the FA-BRCA pathway by discovering mutations in genes that cause Fanconi anemia. In Chapters 2-4, we describe two newly identified FA genes: SLX4 (FANCP) and XPF (FANCQ). Bi-allelic germline mutations in these genes were found in individuals diagnosed with Fanconi anemia. In total, 17 FA and several FA-associated genes are known now. In Chapter 5, we examined whether inactivation of any of these 17 genes occur in sporadic head and neck tumor cell lines. Only a minority of cell lines had a defective FA-BRCA pathway: one cell line contained a homozygous nonsense mutation in FANCM, and in another cell line FANCF methylation was observed.

The last part of this thesis concerns the therapeutic exploitation of the FA-BRCA pathway. Bi-allelic mutations in the well-known breast cancer susceptibility gene BRCA2 cause Fanconi anemia. Since BRCA2-deficient cancer cells are hypersensitive to PARP inhibition, we investigated whether defects in other FA genes also sensitize cells to PARP inhibition (Chapter 6). The data suggest that PARP inhibitors might be beneficial in the treatment of tumors with FANCM mutations. To find additional drugable gene targets in FA-HNSCC cells, we generated cell models and performed genome wide siRNA screens in FA-deficient and corrected cells (Chapter 7).

Finally, the results described in this thesis are discussed in Chapter 8.
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