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Impact of mutated p53 on homologous recombination and the FA/BRCA pathway in NSCLC H1299 cells in response to DNA cross-linking drugs

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Contents

List of Figures

List of Tables

1. Hypothesis

Cancer is essentially a dysfunction of the cell signaling pathways, caused by genetic mutations. Knowledge of sensitivity or resistance to chemotherapeutics is essential for individualizing cancer treatment. The genetic profile of a tumor is a major predictor for chemo- or radio-therapy response. This study aims to elucidate the interactions between tumor-suppressor p53, the Homologous recombination (HR) repair pathway and the Fanconi anemia and BRCA (FA/BRCA) network.

p53 is a major regulator in DNA repair and the most frequently mutated gene found in human cancers. Wildtype p53 is a transcription factor for many genes and interacts directly with a multitude of proteins. The question of how p53 interacts specifically with proteins of the HR and FA/BRCA pathway, led to the selection of transactivation-inactive variants of p53. The motivation herefor is to create protein-protein interactions without the interference of p53-dependent transcription.

Prior research has shown that HR is required for the repair of DNA double-strand breaks (DSB), interstrand cross-links (ICL) and for restarting stalled replication forks (Haber 2000; Thompson and Schild 2001). DNA cross-linkers, such as Mitomycin C, or inhibitors of replication elongation, such as Thymidine, thus activate the HR pathway. The FA/BRCA pathway is essential for the HR repair mechanism of ICLs and stalled forks.

Transactivation-inactive p53 was found to suppress HR activity upon DNA damage (Sirbu et al. 2011). Rad51 is the main protein of the HR pathway and a commonly used marker for HR activity. This study aims to show how Rad51 focus formation, and thus HR activity, is reduced upon Thymidine treatment in NSCLC (non-small-cell lung carcinoma) cells expressing p53, compared to NSCLC cells without functioning p53. The inhibition of HR requires interaction of the p53 protein with the ATR (Ataxia Telangiectasia and Rad3 related) kinase and Replication protein A (RPA). p53 variants with mutations that hinder interaction with ATR and RPA are not able to suppress HR in response to DNA damage. As HR is required to repair damage caused by cross-linking agents and transactivation-inactive p53 is known to suppress HR, the presence of p53 should sensitize NSCLC H1299 cells to Mitomycin C. As a possible target for HR regulation this study further elucidates the role of FANCD2 within the repair of ICL and stalled replication forks and investigates a potential interaction with p53.

2. Introduction

2.1. Lung cancer

Among all human malignancies, lung cancer is one of the leading causes of cancer fatalities each year. Lung cancer is more common in men, but due to changing tobacco-smoking habits, numbers keep rising for women. It is widely known that tobacco smoking is the number one risk factor for developing lung cancer. About 90% of the cases in men and 70% in women can be linked to tobacco smoking, though these numbers vary between different countries. The highest incidence for lung cancer can be found in Europe and North America, whereas in Australia, New Zealand and eastern Asia it is moderately high. Lung cancer can be histologically divided into small-cell lung carcinoma (SCLC) and non-small-cell lung carcinoma (NSCLC). About 87% of lung cancer patients are diagnosed with NSCLC, which includes adenocarcinoma, squamous cell carcinoma and large cell carcinoma. The histological distribution varies between races, sexes and tobacco smoking histories and has been changing over the last decades (Peto et al. 1996; Shopland 1995; Parkin, Bray, and Devesa 2001; Parkin et al. 2002; Parkin et al. 1994; Jemal et al. 2005; Bray, Tyczynski, and Parkin 2004; Jemal, Chu, and Tarone 2001; López-Abente et al. 1995; Tyczynski et al. 2004; Travis, Travis, and Devesa 1995; Valaitis, Warren, and Gamble 1981).

The prognosis is usually poor; the survival after 5 years is about 16% in the United States, 10% in Europe and 8.9% in developing countries (Parkin, Pisani, and Ferlay 1999; Parkin et al. 2002; Howlader et al. 2014). The poor survival rates are due to late diagnosis (most patients are diagnosed in stage III or IV), aggressive growth, early metastases and insufficient treatment options. The response rates for chemotherapy for example are only around 20%, and the survival benefit in responding patients is rather small. At the same time, side effects of chemotherapy can be severe for all patients (Schiller et al. 2002; Hotta et al. 2007). Therapy selection and success strongly depend on factors such as stage, prior treatments, histology and genetic profile of the tumor (Turner, Tutt, and Ashworth 2004; Novello, Longo, and Levra 2007; Stinchcombe, Grilley-Olson, and Socinski 2010; Hirsch et al. 2008; Giachino et al. 2007; Wibmer et al. 2013; Soria et al. 2012).

Knowledge of predictive markers, e.g. certain genetic mutations in the tumor, could help in providing patients with individualized therapy that has a better chance of success. p53 is one of the most commonly mutated genes in cancer. p53 mutations are very common in NSCLC as well as in SCLC. p53 status has prognostic relevance, since tumors with altered p53 have been found to have a worsened prognosis (Tomizawa et al. 1999; Laudanski et al. 2001). Additionally, impaired p53 was found to be associated with increasing resistance to several chemotherapeutic drugs, making it a marker for chemo-resistance (Rusch et al. 1995; Higashiyama et al. 1998).

2.2. DNA damage response

Cells in the human body are continuously exposed to different exogenous and endogenous stressors that cause DNA damage. Examples are irradiation, alkylation of bases, reaction with oxidative free radicals, replication errors, cold- or heat-shock conditions, hypoxia, cellular ageing resulting in short telomeres, inflammation, viral infection and more conditions that may alter the genomic integrity (d'Adda di Fagagna, Teo, and Jackson 2004; Loft and Poulsen 1996). When DNA is damaged, the DNA damage response (DDR) is triggered. Several protein cascades are involved in damage recognition and organization of subsequent cellular pathways. These include activation of transcription, cell cycle control, apoptosis, senescence or DNA repair processes.

The main DNA repair pathways in eukaryotes are mismatch repair (MMR), base excision repair (BER), nucleotide excision repair (NER), non-homologous end-joining (NHEJ) and homologous recombination (HR) (Cline and Hanawalt 2003; Zhou and Elledge 2000; Kinsella 2009; van Gent, Hoeijmakers, and Kanaar 2001). The different pathways may overlap or work synergistically to repair DNA damage and protect genomic integrity.

The principal regulators of the DDR are the damage sensors ATM (Ataxia Telangiectasia mutated) and ATR (Ataxia Telangiectasia and Rad3 related). ATM and ATR, together with DNA-PK (DNA-dependent protein-kinase), belong to the family of PIKKs (phosphatidyl inositol 3' kinase-related kinases). PIKKs regulate the cellular response through phosphorylation of many downstream proteins involved in DNA repair (O'Neill et al. 2000; Kim et al. 1999), reviewed in (Kurz and Lees-Miller 2004; Matsuoka et al. 2007).

DNA DSBs are among the most destructive DNA lesions. If not repaired, DSBs may lead to permanent cell cycle arrest, apoptosis or mitotic cell death. DSBs can be caused by ionizing radiation (IR) or chemotoxic agents but also occur frequently within common cellular pathways as DNA replication, meiosis and immunoglobulin gene recombination (Michel, Ehrlich, and Uzest 1997; Guidos et al. 1996; Toyoizumi and Tsubouchi 2012; Albino et al. 2006; Noda et al. 2012; D. S. Lim and Hasty 1996), reviewed in (O'Driscoll and Jeggo 2006; Lieber et al. 2003; Jackson 2002). It is important to draw a distinction between two-ended and one-ended DSBs. One-ended DSB typically evolve when replication is stalled and replication forks break and require modified repair.

Some chemotoxic agents, such as Cisplatin and Mitomycin C, induce bifunctional adducts, which lead to ICLs and cause stalled replication forks in the S-Phase of the cell cycle. If the stalled forks persist, they collapse into one-ended DSBs and can cause permanent growth arrest or cell death (Iyer and Szybalski 1963; Al-Minawi et al. 2009; Palom et al. 2002; Kennedy et al. 1980; García Sar et al. 2012). Eligible repair mechanisms for DSBs are HR or NHEJ. HR requires the sister chromatid as a homologue template, and thus is limited to late S and G2-Phase. NHEJ repairs DSBs throughout all phases of the cell cycle. Since the DNA ends have to be modified before they can be joined, potentially resulting in deletions or insertions, NHEJ is more error-prone compared to the relatively error-free HR. Studies showed that both pathways may repair DSBs synergistically and communicate through regulation by ATM and ATR (Mansour et al. 2008; Rothkamm et al. 2003; Mills et al. 2004; Couëdel et al. 2004; Liang et al. 1998; Sargent, Brenneman, and Wilson 1997; Sonoda et al. 1999), reviewed in (Valerie and Povirk 2003).

2.3. Homologous Recombination Repair

The HR pathway is highly conserved and required to repair damages such as DSBs, ICLs and stalled replication forks. Improper repair of these lesions results in genomic instability through translocations, deletions and inversions and thus may result in carcinogenesis (Yu et al. 2000; Reliene, Bishop, and Schiestl 2007). Congenital defects in HR can lead to embryonic lethality (shown in Rad51, BRCA1 or BRCA2 knock out mice/knock down experiments) or cancer predisposition disorders. Popular examples are Ataxia Telangiectasia (defect ATM), the Nijmegen Breakage Syndrome (defect in Nijmegen Breakage Syndrome protein 1 (NBS1)) or Breast and Ovarian

Cancer connected to mutated BRCA1 or BRCA2 (Connor et al. 1997; Shen et al. 1998; D. S. Lim and Hasty 1996; Varon et al. 1998; Gatei et al. 2000; Miki et al. 1994; Tsuzuki et al. 1996), reviewed in (Moynahan 2002; Pierce et al. 2001).

When a cell experiences a DNA DSB, many things start happening. The main molecular sensor for DSBs is the MRN complex. Components of the MRN complex are MRE11, Rad50 and NBS1. Once the MRN complex detects a DSB, ATM is activated and recruited to the damage site (Williams et al. 2009; Falck et al. 2005; Lee & Paull 2004). At the damage site, ATM phosphorylates a plethora of downstream proteins. These include checkpoint kinases 1 (Chk1) and 2 (Chk2), p53 (Canman et al. 1998; G. C. Smith et al. 1999), NBS1 (Gatei et al. 2000; D.-S. Lim et al. 2000; S. Zhao et al. 2000), MRE11, BLM (Bloom's syndrome protein), BRCA1, WRN (Werner's syndrome protein), H2AX and ATM itself (autophosphorylation) (Matsuoka et al. 2007; Kim et al. 1999; Burma et al. 2001). All those proteins are crucial participants in DNA repair. The phosphorylated H2AX has emerged as a commonly used marker for existing DSBs in DNA repair research. It is referred to as γ-H2AX and can be visualized in sub-nuclear foci, which are surrogate markers for DSBs (Sedelnikova et al. 2002; Rogakou et al. 1998).

The HR repair process is complex and involves plenty molecular details. It can roughly be divided into a presynaptic, synaptic and postsynaptic phase. Firstly, the MRN complex catalyzes the 5'–3' resection of the DNA ends to form long 3' single-stranded DNA (ssDNA) tails. It then activates ATR and recruits RPA to the damage site (Liao et al. 2012; Buis et al. 2009; Olson et al. 2007). RPA coats the ssDNA tails and stabilizes the structure (Sugiyama, Zaitseva, and Kowalczykowski 1997; X. Wang and Haber 2004). Subsequently Rad51, the central protein of HR, replaces RPA and forms a nucleoprotein filament at the 3' tail, referred to as the presynaptic filament (Sung and Robberson 1995).

In the synaptic phase the Rad51 filament connects the 3' tail to the respective homologous sequence on the sister chromatid (Prasad, Yeykal, and Greene 2006; Sung and Robberson 1995). The complementary DNA strand of the template DNA is displaced and forms a D-loop structure (Petukhova, Sung, and Klein 2000).

The postsynaptic phase includes the formation of Holliday junctions (HJ) by branch migration and elongation of the invading strand through DNA polymerases. When the newly synthesized strand reaches the end of the second DSB, it creates a

double Holliday junction, in which two double-stranded DNA (dsDNA) duplexes are intertwined resulting in a four-way DNA junction (Sugiyama et al. 2006).

Holliday junctions can be resolved in several ways, leading to different outcomes. One solution is mediated by the BLM-TOPOIII-RMI1 complex and resolves into non-crossover products (Raynard et al. 2008; L. Wu and Hickson 2003), while a solution mediated by MUS81-EME1 leads to crossover products (Gaillard et al. 2003; Osman et al. 2003; Gaskell et al. 2007). A third solution is accomplished by Holliday junction resolvases as GEN1 or SLX1-SLX4 and results in either crossover or non-crossover products (Muñoz et al. 2009; Ip et al. 2008). Another possible solution is the process of synthesis-dependent strand annealing (SDSA), where the new strand dissociates from the template and anneals with the ssDNA overhangs on the other end of the DSB. SDSA leads to non-crossover repair products (Zou, Willers, and Pfaeffle 2012).

Within the HR process, Rad51 is supported by Rad52, Rad54, RPA, BRCA1, BRCA2 and the Rad51 paralogs (Rad51B, Rad51C, Rad51D, XRCC2 and XRCC3). All of those proteins are required to perform HR properly, as cells with defects of either one of them show impaired HR and develop sensitivity to IR and cross-linking agents (Mills et al. 2004; Shinohara and Ogawa 1998; Moynahan, Pierce, and Jasin 2001; Yonetani et al. 2005; H. Yang et al. 2002; Pellegrini et al. 2002; Jensen, Carreira, and Kowalczykowski 2010; Rodrigue et al. 2006; Sugiyama et al. 2006; Scully et al. 1997; Petukhova, Stratton, and Sung 1998; Sugiyama and Kowalczykowski 2002). The process of Holliday junction migration specifically requires Rad54, FANCM and the RecQ helicases (BLM, WRN, RECQL1 and RECQ5b) (see Fig. 1) (Gari et al. 2008; Bugreev, Mazina, and Mazin 2006; Constantinou et al. 2000; Bugreev, Brosh, and Mazin 2008; L. Wu and Hickson 2003; Garcia et al. 2004).

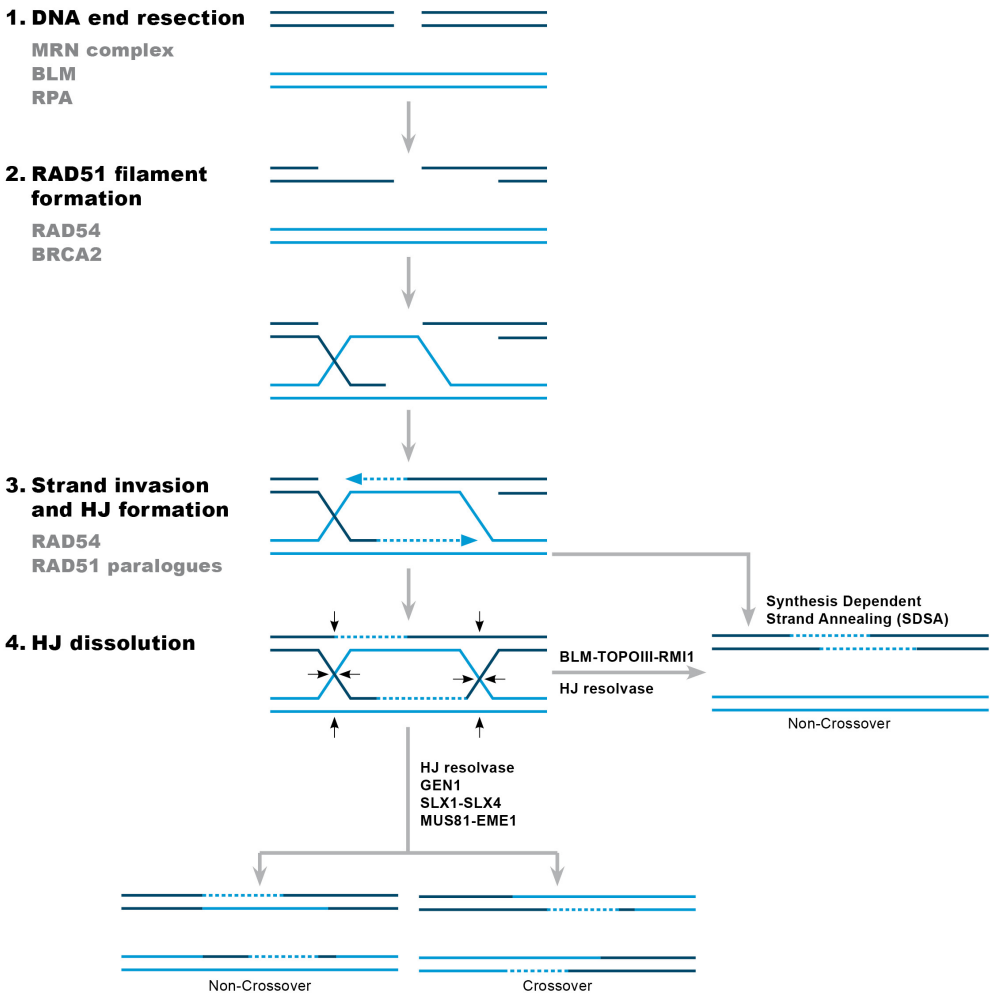

Figure 1.: Principal steps of HR repair and Holliday junction resolution
as proposed in (Zou, Willers, and Pfaeffle 2012); (1) DNA end resection with the help of the MRN complex, BLM, RPA and others; (2) Rad51 filament formation, supported by Rad54 and BRCA2; (3) Strand invasion and HJ formation; (4) HJ dissolution. HJ can be resolved via SDSA, the BLM-TOPOIII complex or HJ resolvases, resulting in non-crossover or crossover products.

2.4. Homologous Recombination at stalled replication forks

Stalled replication forks occur during the S-Phase of the cell cycle and can be caused by inter- or intra-strand cross-links, reduction of deoxyribonucleotide triphosphates (dNTPs) levels, bulky DNA adducts, repetitive DNA sequences or compacted chromatin structures. Persisting stalled forks hinder the replication and eventually collapse into one-ended DSBs. The molecular details of stalled fork reactivation and repair of DSBs resulting from collapsed forks remain unclear and might differ upon the primary lesion. Data has shown that HR is the main repair pathway for these scenarios but needs to work synergistically with other pathways to resolve the damage (Michel, Ehrlich, and Uzest 1997; Minca and Kowalski 2011; Petermann et

al. 2010; Vare et al. 2012; Clemente-Ruiz and Prado 2009; Fouché et al. 2006; Couëdel et al. 2004; Rothkamm et al. 2003; Sargent, Brenneman, and Wilson 1997).

The DDR upon stalled replication forks varies from the response to DSBs of other genesis. ATR plays a more prominent role in response to stalled forks while ATM is the main kinase activated through two-ended DSBs (Gottifredi et al. 2001). Stalled forks caused by ICLs activate the FA/BRCA pathway, which is one branch of the HR pathway. Many FA proteins are activated by ATR and are involved in detection and stabilization of stalled forks, promotion of HR proteins and dissolution of Holliday junctions (Ho et al. 2006; Matsuoka et al. 2007; Lomonosov et al. 2003; Taniguchi, Garcia-Higuera, Andreassen, et al. 2002).

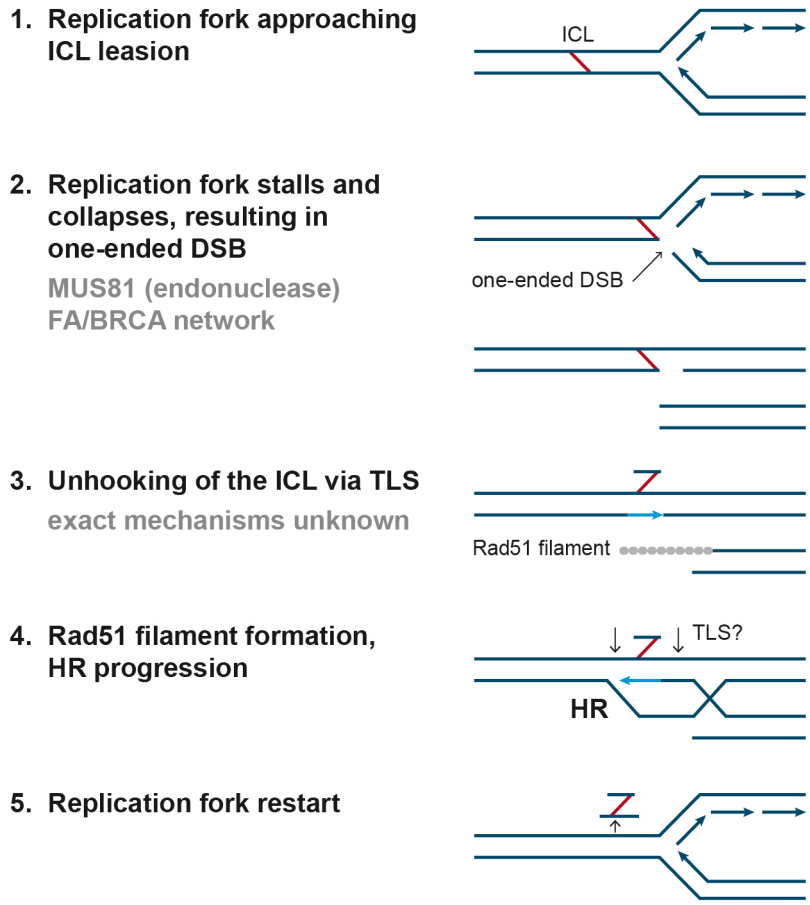

Figure 2.: Model for ICL repair steps
as proposed in (Zou, Willers, and Pfaeffle 2012); (1) a replication fork is approaching an ICL; (2) the replication fork collapses upon approach of the ICL, creating an one-ended DSB, activating endonuclease MUS81 and the FA/BRCA pathway; (3) unhooking of the ICL via TLS, exact mechanisms unknown; (4) Rad51 filament formation and HR progression; (5) continuation of replication.

2.5. Regulation of Homologous Recombination

Although HR is seen as relatively safe and error-free, it can increase genomic instability. Excessive and defective HR is associated with higher cancer susceptibility and an impaired HR pathway can be found in many tumors. Ill-regulation may lead to execution of HR in the wrong phase of the cell cycle or with wrong partners, resulting in translocations or loss of heterozygosity (LOH), thus predisposing the organism for cancer development (Pedersen et al. 2013; Abkevich et al. 2012; Reliene, Bishop, and Schiestl 2007). Therefore, a tight regulation of HR is required to protect genomic integrity (Klein 2008; Akyüz et al. 2002).

Cell cycle phase coordination, posttranslational modifications and many accessory actors that promote or inhibit interactions between HR proteins assure an appropriate regulation. Several proteins have been found to modulate HR fidelity, but the exact mechanisms remain unclear for many of them. Gene products that were found to be involved are: bcl-2, bcr-abl and bcl-x(L) (Saintigny et al. 2001; Wiese et al. 2002; Slupianek et al. 2001) and the DNA repair proteins: ATM, GADD45, MSH2 (Elliott and Jasin 2001; de Wind et al. 1995), BRCA2 (Marmorstein, Ouchi, and Aaronson 1998; Powell, Willers, and Xia 2002), BLM (Traverso et al. 2003; Sengupta et al. 2003), REcQ5 (Hu et al. 2007), WRN (Otterlei et al. 2006) and p53 (Arias-Lopez et al. 2006; Saintigny et al. 1999; Boehden et al. 2003; Bishop et al. 2003).

Especially p53 plays an important and complex role in HR regulation (Willers et al. 2000; Yun, Lie-A-Cheong, and Porter 2004). While p53, as a transcription factor, down-regulates the transcription of the HR key protein Rad51, the main regulation through p53 depends on direct protein-protein interactions (Arias-Lopez et al. 2006). Those occur between p53 and several crucial participants of HR, as Rad51 (Stürzbecher et al. 1996; Yoon et al. 2004), Rad54 (Linke et al. 2003), BLM (Sengupta et al. 2003), RPA (Romanova et al. 2004), BRCA1 (Chai et al. 1999; H. Zhang et al. 1998) and BRCA2 (Marmorstein, Ouchi, and Aaronson 1998; Rajagopalan et al. 2010). Most studies indicate an inhibiting effect of p53 upon HR (Mekeel et al. 1997; Wiesmüller, Cammenga, and Deppert 1996). Interestingly, there are also hints that p53 can promote HR under certain circumstances and seems to discriminate between different kinds of DNA damage and variations of HR (Dudenhöffer et al. 1998; Akyüz et al. 2002; Yun, Lie-A-Cheong, and Porter 2004). The molecular details and determining factors remain to be elucidated.

2.6. p53

The p53 gene encodes for the p53 protein and is one of he most frequently mutated genes in human cancers (Hollstein et al. 1991; Vogelstein 1990; Harris 1996; Greenblatt et al. 1994; Hollstein et al. 1999). It was discovered in 1979 by Lane and Crawford and is also referred to as “the guardian of the genome” (Lane 1992). p53 was found to regulate pathways as cell cycle control, apoptosis, cell differentiation, DNA repair and gene transcription. The consequences of a defective p53 are seen in patients suffering from the Li-Fraumeni syndrome, which is an autosomal dominant hereditary disorder, characterized by a mutation in the p53 gene. The patients are prone to developing several tumors during their lifetime, beginning at a young age (Li et al. 1988), reviewed in (Strong, Williams, and Tainsky 1992; Chompret 2002).

Experiments have shown in vitro and in vivo that cells with defective p53 are highly susceptible to cancer development. The oncogenic effect of viruses, as for example human papilloma virus 16 and 18, hepatitis B virus and simian virus 40, is originated in their ability to target the p53 protein (El-Mahdy et al. 2000; Unger et al. 1992; Fields and Jang 1990; Parkin, Bray, and Devesa 2001; Ueda et al. 1995; Zimmermann et al. 1999; Werness, Levine, and Howley 1990; Bargonetti et al. 1991).

The p53 gene is located on chromosome 17 p13. It encodes for the p53 protein, which consists of 393 amino acids and is named after its molecular mass, 53 kDa (Mcbride, Merry, and Givol 1986). The protein can be divided into different functional domains: The N-terminal domain, the core-domain and the C-terminal domain. The N-terminal domain (residues 1-43) includes the transactivation site, which is target to various protein-kinases that regulate p53’s transcriptional activity through phosphorylation (Unger et al. 1992; Siliciano et al. 1997). The core-domain (residues 100-300) contains sequence-specific DNA binding sites, which are indispensable for p53’s role as a transcription factor (Y. Wang et al. 1993; Bargonetti et al. 1993). The majority of p53 mutations found in cancer cells are located in this region and cluster in four so called “hot-spots”. These findings underline the core domain’s importance for tumor-suppression (Nigro et al. 1989; Baker et al. 1989; Cho et al. 1994; Vogelstein 1990; Hollstein et al. 1994; Pavletich, Chambers, and Pabo 1993; Kern et al. 1991). The C-terminal domain (residues 325–393) consists of nuclear localization signals, a tetramerization domain (residues 325–356) and the C-terminal regulatory domain. Short, single-stranded DNA binds to the regulatory

domain on the transactivation site to manage p53 activity (Jayaraman and Prives 1995; Hupp and Lane 1994; L. Wu et al. 1995; Pavletich, Chambers, and Pabo 1993; El-Deiry et al. 1992; Y. Wang et al. 1993; Jeffrey, Gorina, and Pavletich 1995; Bakalkin et al. 1995), reviewed in (Prives 1994; Levine 1997).

p53 is a transcription factor for a multitude of proteins that are involved in apoptosis, cell cycle regulation, growth regulation and cell proliferation and differentiation (R. Zhao et al. 2000). Additionally it binds directly to various proteins to regulate cellular pathways. Reviewed in (Bertrand, Saintigny, and Lopez 2004; Green and Kroemer 2009). p53 concentration rises in response to genotoxic stress, as for example DNA damage, viral infection or hypoxia (Graeber et al. 1996; Kessis et al. 1993; Lu and Lane 1993). p53 can launch DNA repair, apoptosis or senescence, depending on the character and severity of the damage (Lu and Lane 1993). p53 activation results in an increased protein half-life and enhanced production of p53 messenger RNA (mRNA), leading to increased protein concentrations in the cell and an improved capability of p53 in binding to specific DNA sequences (Takagi et al. 2005; Jayaraman and Prives 1995; Waterman et al. 1998; Ashcroft, Taya, and Vousden 2000).

Considering p53's strong impact on fundamental cellular processes that may decide about life and death of the cell, it is reasonable to have sound regulatory mechanisms. p53 is involved in several autoregulatory-positive and autoregulatory-negative feedback loops to provide a tight control. The central protein of p53 regulation is mouse double-minute protein 2 (MDM2). MDM2 binds to the p53 transactivation domain and thus abrogates transcription of p53-regulated genes (Oliner et al. 1992; Oliner et al. 1993; Momand et al. 1992). Through continuous ubiquitylation of p53, MDM2 mediates constant protein degradation (Haupt et al. 1997). p53 activates the transcription of MDM2 itself and therefore provides its own degradation in an auto-regulatory manner (X. Wu et al. 1993; Barak et al. 1993; Kubbutat, Jones, and Vousden 1997).

Posttranslational modifications channel further p53 activity. Potential modifications are phosphorylation, acetylation, ubiquitylation and sumoylation at different amino-acid residues (Oliner et al. 1992; Canman et al. 1998; M. Li et al. 2002; Y. Liu and Kulesz-Martin 2001). The protein kinases ATM, ATR and DNA-PK activate p53 through phosphorylation on serine 15 and 37 (only DNA-PK) in response to genotoxic stress, such as IR, ultraviolet radiation (UV) or DNA cross-

linking agents. The phosphorylation results in higher concentrations of p53 in the cell and improves the ability to activate downstream genes (Stokes et al. 2007; Siliciano et al. 1997; Canman et al. 1998; Ashcroft et al. 2000; Woo et al. 1998; Shieh et al. 1997).

Upon DNA damage, the activated p53 is able to maintain a G1 or G2 cell cycle arrest, so the cell is able to launch repair pathways. If no sufficient repair is possible, p53 can induce apoptosis via the intrinsic as well as the extrinsic pathway (Chen et al. 1996; Kuerbitz et al. 1992; Miyashita and Reed 1995; R. Zhao et al. 2000; Miyashita et al. 1994).

2.7. p53 and DNA repair

p53 is involved in regulation of the main DNA repair pathways in eukaryotes. NER, HR, MMR, NHEJ and BER are influenced by transcriptional and transcription independent interactions of p53. The repair of DNA DSBs requires either HR or NHEJ, depending on the cell cycle phase. While NHEJ is more error-prone than the generally safe HR, increased or ill-regulated HR can still lead to LOH and genomic instability (Mansour et al. 2008; Rothkamm et al. 2003; Pedersen et al. 2013; Abkevich et al. 2012). p53 was found to regulate both pathways in a more complex manner.

As a protector of genomic integrity, p53 inhibits undesired HR fidelity (Mekeel et al. 1997; Wiesmüller, Cammenga, and Deppert 1996; Dudenhöffer et al. 1998). Besides the inhibition of Rad51 transcription, p53 mainly regulates HR through transcription independent protein-protein interactions (Arias-Lopez et al. 2006). p53 directly binds to several proteins involved in HR, such as BRCA2, RPA and Rad51 (Romanova et al. 2004; Marmorstein, Ouchi, and Aaronson 1998; Rajagopalan et al. 2010; Yoon et al. 2004). Through the direct interaction with RPA for example, p53 abrogates the binding of RPA to ssDNA and thus hinders the onset of replication (Dutta et al. 1993). p53 is also involved in the regulation of the RecQ helicases, which belong to the family of 3′→5′ DNA helicases, are highly conserved and are involved in protection of genomic integrity (Q. Yang et al. 2002). Three of the helicases, BLM, WRN and RecQ4 are known for their association with the cancer-predisposition syndromes Bloom syndrome (BLM), Werner syndrome (WS) and Rothmund–Thomson syndrome (RTS) when altered by mutations (German 1969; Bloom 1966; S. Kitao et al. 1999; C.-E. Yu et al. 1996). p53 was found to interact with BLM and WRN and modulate their ability to unwind Holliday junctions. Both, BLM

and p53 co-localize together with Rad51 at stalled forks within ICL repair (Garkavtsev et al. 2001; Q. Yang et al. 2002; Sengupta et al. 2003; L. Wu et al. 2001).

How the transactivation-independent protein-protein interactions fit into p53's complex role as guardian of the genome and contribute to its multiple ways to protect genome stability requires further investigation.

2.8. FA/BRCA pathway

Fanconi Anemia (FA) is an autosomal recessive, heterogeneous disorder that was first described by the pediatrician Guido Fanconi in 1927. Affected patients experience symptoms as anemia, leading to bone marrow failure, congenital abnormalities and are prone to developing several cancers at a very young age. Acute myeloid leukemia (AML), head and neck cancers, gynecological cancers and gastrointestinal cancers are commonly observed (Alter 2005; Alter 2003; Xie et al. 2000).

Analysis of FA patient's cells resulted in a distinction of an increasing number of FA groups assigned to defects in different genes. Currently, 16 subgroups and their complementary genes are known and connected to the FA disorder. Namely FANCA, FANCB, FANCC, FANCD1/BRCA2, FANCD2, FANCE, FANCF, FANCG/XRCC9, FANCI, FANCJ/BRIP1/BACH1, FANCL/PHF9, FANCM, FANCN/PALB2, FANCO/Rad51C, FANCP/SLX4 and FANCQ/XPF (Lo Ten Foe et al. 1996; Strathdee, Duncan, and Buchwald 1992; Joenje et al. 1997; Levitus et al. 2004; Meetei et al. 2005; Smogorzewska et al. 2007; de Winter, Léveillé, et al. 2000; Xia et al. 2007; Meetei et al. 2003; Stoepker et al. 2011; Reid et al. 2007; de Winter et al. 1998; Timmers et al. 2001; Howlett et al. 2002; Vaz et al. 2010; Bogliolo et al. 2013; Youds et al. 2008)

Many of those proteins are known for their important roles in DNA repair. Some of the FA proteins are found to be defective or inactivated in several types of sporadic cancers. For example, FA protein FANCF has been found to be inactivated through a promoter methylation in ovarian tumors, cervical tumors, head and neck squamous cell carcinomas (HNSCC) and NSCLC (Marsit et al. 2004; Taniguchi et al. 2003; Narayan et al. 2004). Down-regulation of FANCB, FANCC, FANCF, FANCG, FANCJ, FANCL and FANCM was found to be common in HNSCC (I. M. Smith et al. 2010; Wreesmann et al. 2007).

Characteristics of FA cells are chromosomal instability and hypersensitivity to cross-linking agents like Mitomycin C and Cisplatin (Sasaki & Tonomura 1973;

Sasaki 1975; M. Koomen et al. 2002; García Sar et al. 2012). The FA proteins are involved in the healthy replication process, DSB repair and especially in the repair of ICLs at stalled replication forks (Garcia-Higuera et al. 2001; Niedzwiedz et al. 2004; Yamamoto et al. 2005). Important regulators and participants of the FA pathway are the previously introduced DNA damage-response proteins ATM, ATR, BRCA1 and BRCA2 (FANCD1) (Kim et al. 1999; X. Wang, Andreassen, and Andrea 2004; Garcia-Higuera et al. 2001).

The network around the FA proteins is also referred to as the FA/BRCA pathway (Howlett et al. 2002). The FA/BRCA pathway is activated upon DNA damage and 8 of the 16 proteins form the FA core complex. The core complex is a central element of the pathway and consists of FA proteins A, B, C, E, F, G, L and M. It has E3 ubiquitin ligase activity, which is located in the FANCL protein (Meetei, Yan, and Wang 2004; Meetei et al. 2003; Medhurst et al. 2006; Garcia-Higuera et al. 2001). The key element of the active FA/BRCA pathway is the mono-ubiquitylation of FANCD2 at K561 and FANCI at K523 through the FA core complex, specifically through FANCL. The mono-ubiquitylated FANCD2 and FANCI form the ID complex and are able to load onto chromatin (Smogorzewska et al. 2007; de Winter, van der Weel, et al. 2000; Meetei et al. 2005). The FA-associated-proteins FA-AP100 and FA-AP24 were found to be associated with the core complex and required for sufficient FANCD2/FANCI mono-ubiquitylation. Defective FA-AP100 or FA-AP24 were found to cause increased sensitivity to cross-linking agents (Ling et al. 2007; Ciccia et al. 2007). The mono-ubiquitylation occurs within general replication during S-phase and in response to DNA damage (Garcia-Higuera et al. 2001; Howlett et al. 2005).

Cross-linking agents cause ICLs and subsequently stalled replication forks. The FA/BRCA network is involved in damage recognition, fork stabilization and promotion of HR, as well as in Holliday junction dissolution towards the end of DSB/ICL repair. Particularly FANCD1 (BRCA2) was found to stabilize stalled replication forks. The mono-ubiquitylated ID complex and the FA core complex bind to chromatin, interact with several DNA repair proteins and are involved in insertion and incision steps of ICL repair (Lomonosov et al. 2003; Mi and Kupfer 2005; Knipscheer et al. 2009; Montes de Oca et al. 2005; Gari et al. 2008). FANCD2 foci are commonly used as a marker for pathway activity, as they form in response to DNA damage. Mono-ubiquitylated FANCD2 was found to accumulate in sub-nuclear

foci and to co-localize with BRCA1, BRCA2, Rad51, γ-H2AX and proliferating cell nuclear antigen (PCNA) at the DNA damage site. Absence of FANCD2 foci in response to cross-linking agents can be a hint towards a defect FA/BRCA pathway and thus predict possible sensitivity to cross-linking drugs in cancer cells (Taniguchi, Garcia-Higuera, Andreassen, et al. 2002; Howlett et al. 2005; Hussain et al. 2004; X. Wang, Andreassen, and Andrea 2004; Garcia-Higuera et al. 2001). FANCD2 was found to participate in another FA complex, forming independently from ID mono-ubiquitylation, indicating an important role in ICL repair. It consists of FANCD1, FANCD2, FANCG and XRCC3 and might specifically be involved in replication fork restart (Wilson et al. 2008; Wilson et al. 2010). FANCD2 deficient cells are defective in HR mediated DSB repair and thus extremely sensitive to cross-linking agents as Mitomycin C and Cisplatin (Sasaki 1975; Sasaki and Tonomura 1973). Additionally, these cells show signs of genetic instability, such as elevated levels of sister chromatid exchange (SCE) (Yamamoto et al. 2005; García Sar et al. 2012).

Within the DDR, several FA proteins are phosphorylated and thus activated by ATM and ATR (Smogorzewska et al. 2007; Kim et al. 1999; Matsuoka et al. 2007). The mono-ubiquitylation of FANCD2 is promoted by phosphorylation through ATR and ATM at two sites of FANCD2, T691 and S717. The phosphorylation of FANCD2 by ATR was found to be pivotal for mono-ubiquitylation and resistance to cross-linking agents, as ATR suppressed cells failed to build FANCD2 foci in response to Mitomycin C treatment (Ho et al. 2006; Andreassen, D'Andrea, and Taniguchi 2004).

Summed up, functions of the FA/BRCA pathway are believed to be detection, stabilization and repair of stalled replication forks through synergistic interaction with other repair processes as translesion synthesis (TLS) and HR (Nakanishi et al. 2005; Huang et al. 2010; X. Wang, Andreassen, and Andrea 2004; Renaud and Rosselli 2013). The particular mechanisms of this complex network in DNA repair need further investigation.

3. Methods and Material

3.1. Cell lines

Table 1.: Genetic alterations in cell lines used

Cell Line	Origin	p53 status	Other mutations
H1299	NSCLC	homozygous deletion	
H1299 p53-QS		p53 L22Q/W23S	
H1299 p53-QM		p53 L22Q/W23S +W53S/F45S	NRAS mut
H1299 p53-S15A		p53 L22Q/W23S + S15A	
H1299 p53-237		p53 237	
A549	NSCLC	wildtype	K-RAS mut
H1792		p53 mut	K-RAS mut
Calu-6		p53 mut	K-RAS mut
PC-9		p53 mut	EGFR-activating
MCF7	Breast Cancer	wildtype	ERBB2 amplification
MDA-MB-436		p53 mut	BRCA1 mut

The NSCLC cell lines NCI-H1299, A549, Calu-6, H1792, PC-9, as well as the breast cancer cell lines MCF7 and MDA-MB-436 were used for the experiments. H1299 cells are p53 null due to a partial homozygous deletion of both alleles of the p53 gene. The H1299 cells contained a stable integrated FRT target site for the FLP recombinase and a Zeocin resistance gene and are referred to as H1299 p53-null. Those cells were transfected with N-terminal transactivation-inactive mutant versions of p53 (L22Q/W23S, L22Q/W23S + W53S/F45S and L22Q/W23S + S15A) referred to as H1299 p53-QS, H1299 p53-QM and H1299 p53-S15A. Besides having the transactivation-disabling mutation at L22Q/W23S, H1299 p53-QM cells have another mutation at the RPA binding site that disables RPA from binding. The H1299 p53-S15A cells harbor an additional mutation that inactivates the main phosphorylation target site for ATR and ATM. H1299 p53-237 cells have been stably transfected with a mutation in the DNA-binding core-domain. A549 cells harbor wildtype p53. Calu-6 and H1792 (mutant p53) are K-RAS-mutant cell lines. PC-9 (mutant p53) cells harbor

an activating mutation in the EGFR-kinase domain. MDA-MB-436 cells carry an inactivating BRCA1 mutation. MCF7 cells express wildtype p53 and were used as a repair-proficient positive control (Sunaga et al. 2011).

3.2. Cell culture

Table 2.: Cell line media and additives

Cell Line	Medium	Additives
H1299 p53-null	RPMI-1640	
H1792		
PC-9		
H1299 (p53-QS, -QM, -S15A)	RPMI-1640	Hygromycin B 200 µg/ml
H1299 p53-237	RPMI-1640	G418 200 µg/ml
A549	DMEM	
MCF7		
MDA-MB436	DMEM/F12	
Calu-6	alpha-MEM	

All cells were kept in an incubator at 37° C in a humidified, 5% CO2 containing atmosphere. All media were supplemented with 10% bovine serum, 1x HEPES buffer, 1% Penicillin-Streptomycin and 2 mmol/L L-Glutamine. Cells were handled under sterile conditions under a cell culture hood and kept in T25 or T75 cell culture flasks. Depending on the cell line and growth rate the cells were split every 2-5 days at about 70-80% confluence. When splitting the cells, the old medium was removed, the cells were washed once with 3 ml 1x PBS and trypsinized 3-5 minutes at 37°C and 5% CO2. About 1 million cells were seeded into a new T75 cell culture flask. Fresh medium was added afterwards. A haemocytometer with 4 chambers was used to count the cells during passaging. The cells were counted manually under a microscope. The cells were not passaged more than 20 times after they were thawed to prevent the risks of aging and spontaneous mutations. They were centrifuged at 1200 rpm after passaging and counting for freezing and storage in liquid nitrogen. The cells were re-suspended in medium containing 10% DMSO to a concentration of

1 Million cells/ml. 1 ml was pipetted in each 1.5 ml cryo-vial. They were placed in a -20°C freezer for 1-2 hours, then transferred to an -80°C freezer for at least one night and then transferred to liquid nitrogen tanks. When new cells were needed, they were thawed from the storage in liquid nitrogen tanks. The frozen cells were placed in a 37°C water bath, added to about 5 ml fresh medium and centrifuged at 1200 rpm. The pellet was re-suspended in 5 ml fresh medium and filled into a T25 cell culture flask. If a selection medium was required it was added after two passages.

3.3. Drug treatment

3.3.1. Mitomycin C

Cells were treated with Mitomycin C from Streptomyces caespitosus in different concentrations (0.1 µg/ml – 2 µg/ml). First, the cells were seeded on 8-well chamber slides, $4x10^4$ per 0.5 ml in four chambers. They were then left in an incubator overnight and treated for 1 h the next morning. Therefore the medium was removed from each chamber and 0.5 ml medium containing Mitomycin C was added. After 1 h the medium was removed and 0.5 ml fresh medium was added to each chamber. Finally they were incubated at 37° for 4 h or 24 h until they were fixed.

3.3.2. Thymidine

Cells were treated with 5 mM Thymidine. The cells were prepared as described above for Mitomycin C treatment. After removing the complete medium Thymidine was added in a 5 mM concentration. The cells were exposed to Thymidine for 24 h until the fixing process was started.

3.4. Fixing and immunostaining

After incubating 4 or 24 h the treated cells and untreated control slides were taken out of the incubator and maintained on a workbench, as sterile environment was not necessary anymore. The medium was disposed of and the cells washed once with 1x PBS. They were fixed for 5 minutes with 0.5 ml fixing buffer (3% paraformaldehyde (PFA), 2 % Sucrose, 1x PBS) in each chamber and then washed three times with 1x PBS. For the next step, 0.5 ml permeabilization buffer (0.5% Triton-X, 20 mM HEPES, pH 7.9, 50 mM NaCl, 3 mM KCl, 300 mM Sucrose) was pipetted into each chamber and incubated for 3 minutes. After two more washes with 1x PBS, the slides were kept in blocking buffer (0.5% NP40, 10% BGS, 0.3% NaN3, 1x PBS) for 1-5 days at 4°C. For the staining process, the blocking buffer was disposed of and the

slides washed with 1x PBS. Thereafter they were incubated with 100 µl/well of the primary antibody dilution for 3 h at 37°C in a humidified chamber. After the incubation time the primary antibody was discharged and the slides were washed three times for 5 minutes with 100 µl/well of 0.1% Triton X/1x PBS. Cells were incubated with 100 µl/well of the secondary antibody in a humidified chamber at room temperature. To prevent the fluorescence from fading, the cells were handled in the dark from now on. All antibodies were diluted in 2% BSA/0.1% Triton X/PBS. After the incubation time the secondary antibody was disposed of and the slides were washed three times for 5 minutes with 100 µl/well of 0.1% Triton X/PBS. Cells were incubated for 2 minutes at room temperature with 100 µL/well of DAPI ([1 µg/ml ddH_20]) and washed two times for 2 minutes with 100 µl/well of 0.1% Triton X/PBS afterwards. Slides were covered with cover slides after adding one drop of Antifade on each well and then sealed with nailpolish. Slides were stored at 4°C.

3.5. Fluorescence microscopy

Slides were analyzed 1-10 days after staining by means of an Olympus BX51 Fluorescence microscope, using oil immersion and a magnification of 100x. 100 cells were counted on each slide, respectively 50 in two different chambers. Foci were only counted in non-apoptotic cells and only in the nucleus. The counts were documented in ranges from 0, 1-4, 5-9, 10-14, 15-20 and 20+ foci per nucleus.

3.6. Western Blotting

3.6.1. Lysate preparation

To obtain cell samples for western blotting, cells were plated in p60 dishes (60x15 mm tissue culture dishes) at a concentration of about $25x10^4$ cells/ml. The next day one dish of each cell line was treated with 0.5 µg/ml Mitomycin C and one dish remained untreated as a control. After 4h the lysate preparation continued with the cells cooled on ice. The medium was removed and the cells were washed with 5 ml ice cold 1x PBS twice. After the last wash the 1x PBS was removed as thoroughly as possible and 25 µl cell lysis buffer (1 ml Cell Extraction Buffer, 10 µl protease inhibitor and 5 µl PMSF) were added for an incubation time of approximately 3 minutes. The cells were scraped off with a cell scraper and pipetted into ice cold 1.5 ml eppendorf vials. The samples were centrifuged at 4°C at 1200 rpm for 12 minutes. The protein containing supernatant was collected into a new 1.5 ml eppendorf tube and placed on ice. The samples were stored at -80°C. The protein concentration was

measured with a GeneQuant pro photometer at a wavelength of 595 nm. One reference sample was prepared with 800 µl ddH2O and 200 µl protein dye, the other samples contained 798 µl ddH2O, 200 µl protein dye and 2 µl lysate.

3.6.2. Preparation of western blot samples

The samples for 1 mm 4-12% Bis-Tris gradient gels were mixed with 2.5 µl 10x reducing agent, 6.25 µl 4x sample buffer and a maximum of 16.3 µl protein sample. If a lower amount of the protein sample was needed to gain the required concentration, the difference was filled with ddH2O, so each sample was 25 µl and contained the same amount of protein. The samples for 1.5 mm 10% Bis-Tris gels were generated with 3.7µl 10x reducing agent, 9.25 µl 4x sample buffer and 24.05 µl protein to a total volume of 37 µl. The amount of loaded protein depended on the lowest concentrated lysate and varied from 45,6 µg – 100 µg. For a 1 mm 4% Tris-Glycine gel of the Novex system (Invitrogen), samples were mixed with 2.5 µl reducing agent, 12.5 µl sample buffer and 10 µl protein for a total of 25 µl. The samples for 1.5 mm 4% Tris-Glycine gels of the Novex system were mixed with 3.7 µl reducing agent, 18.5 µl sample buffer and 14.8 µl protein to a total volume of 37 µl. The samples were denaturated at 70°C in the heatblock, then placed on ice and recollected by pulse spinning at 12000 rpm, before pipetting into the wells of the gel.

3.6.3. Running of the gel

The Invitrogen box was assembled, put on ice and the gel placed inside. The chambers of the box were filled with running buffer. A 20x MOPS running buffer was used for gels from the NuPage system (Invitrogen). A 10x Tris-Glycine SDS running buffer was used for gels from the Novex system. The left outer lane was loaded with 17 µl protein standard when using 1 mm gels, the following lanes loaded with 25 µl sample. The volumes for 1.5 mm gels were 27 µl and 37 µl. The box was closed and connected to the power device. The running voltage varied from 50-200 V, the running time varied between 1-6 h.

3.6.4. Transfer

The PVDF membrane was first washed briefly in methanol, then in ddH2O and then kept in chilled transfer buffer. The gel was taken out of the gel box and the protective case by placing it in chilled transfer buffer. The transfer cassette was assembled by placing the membrane on the gel between two filter papers and two sponges soaked in transfer buffer. The closed cassette was placed in the transfer box that was

prepared with a cooling block and chilled transfer buffer before. Transfers ran 40-90 minutes (depending on the protein size) at 100 V. The transfer buffer used for the NuPage system contained 840 ml ddH2O + 10 ml 10% SDS solution + 50 ml 20x Transfer Buffer + 100 ml MeOH. The transfer buffer used for the Novex System contained 850 ml ddH2O + 10 ml 10% SDS solution + 40 ml 25x Transfer Buffer + 100 ml MeOH. After dissembling the transfer box the membrane was rinsed in 0.1% TBS-T (100 ml TBS-T + 900 ml ddH2O) and blocked for 1 hour in 10 ml 5% milk solution on a shaker at room temperature. The milk solution was prepared by mixing 0.5 g non-fat dry milk with 10 ml 0.1% TBS-T.

3.6.5. Probing for FANCD2, FANCF and Rad51

After blocking the membrane it was rinsed with 0.1% TBS-T and blotted with a specific primary antibody for FANCD2, FANCF or Rad51. For detection of FANCD2 an anti FANCD2 mouse monoclonal antibody was used in an 1:200 dilution in 5% BSA/TBS-T. The solution was prepared by mixing 0.5 g Albumin powder with 10 ml 0.1% TBS-T. First, the antibody was diluted in a 5% milk solution, which yielded poor results. Blotting in 5% BSA/TBS-T improved the signal. Blotting for FANCF was accomplished with a rabbit polyclonal antibody at a 1:500 dilution in 5% milk/TBS-T. First, the antibody was diluted in a 5% BSA/TBS-T solution, but the background signal was very high and could be reduced by using the milk solution. An anti-Rad51 mouse monoclonal antibody was used in a 1:200 dilution in 5% BSA/TBS-T to blot for Rad51. The membrane was placed in a dish, covered with the primary antibody solution and incubated overnight on a shaker at 4°C. The next morning the membrane was rinsed with 0.1% TBS-T three times and then washed three times for ~15 minutes with 0.1% TBS-T on a shaker at room temperature. Then the membrane was incubated with a horseradish peroxidase (HRP) linked secondary antibody, specific for the primary antibody. A goat anti-mouse IgG secondary antibody was used for the mouse anti-FANCD2 and mouse anti-Rad51 primary antibodies. A chicken anti-rabbit IgG secondary antibody was used for the rabbit anti-FANCF primary antibody. The secondary antibodies were diluted at a 1:10000 ratio in 5% dry milk/TBS-T and blotted on a shaker at room temperature for 1 h. Afterwards the membrane was rinsed with 0.1% TBS-T three times and washed on the shaker three times for at least 15 minutes with 0.1% TBS-T.

3.6.6. Loading control

Filamin was used as a loading control for membranes blotted with FANCD2 antibody. The membrane was either cut before blotting with the primary antibody, so Filamin and FANCD2 primary could be applied simultaneously, or stripped after blotting for FANCD2 and then blotted for Filamin. The anti-Filamin primary antibody was diluted at a 1:2000 ratio in 5% milk/TBS-T. For membranes blotted with FANCD2 after using 4-12% gradient gels, β-actin was also used as loading control. The primary anti β-actin antibody was diluted at a 1:1500 ratio in 5% milk/TBS-T. β-actin was used as loading control for membranes blotted with FANCF, as described above. For membranes blotted with Rad51 antibody, β-actin was used as loading control after the membrane was stripped. Therefore primary β-actin antibody was diluted at a 1:1000 ratio in 5% milk/TBS-T. The membranes were incubated on a shaker either at room temperature for at least 3 h or overnight at 4°C. After the incubation, the membrane was handled as described above for FANCD2/FANCF/Rad51 primary antibodies. As a secondary antibody a goat anti mouse antibody was used.

3.6.7. Visualization

To visualize the bands of the blotted protein, ECL reagents were mixed at a ratio of 1:1. The membrane was placed in a dish and covered with the prepared ECL reagents immediately and incubated for ~1 minute. Afterwards the membrane was placed in a radiation therapy cassette. In the dark room a chemiluminescence film was placed on the membrane and exposed to the appropriate (10 seconds - overnight) time to show a signal. The film was developed in a developing machine.

3.7. Depletion of FANCD2 using siRNA

In preparation for the protein depletion by siRNA the cells were plated in T25 cell culture flasks. The amount of cells plated depended on the doubling time, the goal was to approach a ~50% confluence the next day. Approximately 5x 10^5 H1299 and MCF7 cells were seeded in each flask and left in the incubator overnight. Four flasks were prepared for each cell line, so two could be transfected with siRNA and two with scramble RNA as a control. An antibiotic-free medium was used. It contained neither selection antibiotics nor Penicillin-Streptomycin. The master mixtures were generated by mixing X-tremeGENE reagent with Opti-MEM by a ratio of 2:8 and diluting the siRNA and scramble RNA to a concentration of 200 nM. 100 µl of both master mixes were pipetted together in a tube for each T25 flask and incubated at room

temperature for 15 minutes. During the incubation time the medium was removed from the T25 flasks, the cells rinsed with 1 ml Opti-MEM and then covered with 2 ml Opti-MEM. The 200 µl Mastermix was applied dropwise into the Opti-MEM of each flask. The flasks were incubated at 37°C for 4 h until 2 ml medium containing 20% BGS, 1x HEPES buffer, and 2 mmol/L L-Glutamine were added into each flask and then incubated at 37°C overnight. The next morning the cells were counted (as described previously) and seeded for lysate preparation and a colony survival assay. The lysates were collected 48 h and 72 h after the transfection to confirm the success of the FANCD2 depletion in a western blot.

3.8. Colony survival assay

A colony survival assay was processed after FANCD2 depletion with siRNA. Two T25 flasks for each concentration were plated for the FANCD2 depleted and scramble RNA transfected cells. One of the flasks was treated with Mitomycin C the next day and one remained untreated as a control. They were incubated overnight and treated for 1 h with Mitomycin C the next morning. Thereafter the old medium was removed and 5 ml Mitomycin C containing medium was added to each flask. The Mitomycin C concentrations were 5 µg/ml or 1 µg/ml. The amount of cells in the flasks varied between 500 and 10000. Concentrations were determined by considering the plating efficiency that was provided through previous colony formation assays. After 1 h the medium was removed and the cells were rinsed with 1xPBS. 5 ml fresh medium were added and the flasks remained in the incubator for colony formation. After about 14 days the colonies were fixed and stained. Therefore the medium was disposed of and the cells washed with 1xPBS. After pouring out the 1xPBS they were washed with 1 ml 100% MeOH and air dried for 5 minutes. The cells were exposed to methylene blue (500 ml 100% Ethanol 2 g Methylene Blue) for 5-15 minutes for staining. After exposure the cells were washed with tap water three times. They were left at the air to dry overnight. The colonies were counted on an illuminated surface. Colonies that contained at least 50 cells, counted under a light microscope, were considered survivors.

3.9. Material

Table 3.: Lab equipment

Lab Equipment	
Haemozytometer	Hausser Scientific 0.100 mm deep Levy Haemozytometer
Incubator	Forma Scientific Water jacketed Incubator
Nitrotank	Forma Scientific Cryomed CM290
Centrifuge	Thermo Electron Corporation IEC Central CL2 Centrifuge
-20°C, -70°C Freezer	VWR Scientific NINTA
Photometer	Amersham Biosciences Gene Quant pro
Weigh	Denver Instrument Company TL104 Fisherbrand Weighing Paper
Fluorescense Microscope	Olympus America BX51
Source of Fluorescence	Olympus BX-URA2
Microscope Camera	Zeiss AxioCam MR
Centrifuge	Beckman Coulter Microfuge®R Centrifuge
Film Developing Machine	Kodak X-OMAT 2000 Processor
Digital Camera	Fuji Finepix E900
Scanner	Epson Perfection 2480 Photo

Table 4.: General lab supplies

General lab supplies			
PBS (phosphate buffered saline)	137 2,7 2 10 mM Na2HPO4 x 2H_2O / 4°C	mM mM mM	NaCl KCl KH2PO4
Automatic Pipet	Sigma		

Pipets 2 ml	Falcon	357507
Pipets 5 ml	Fisherbrand	13-678-27F
Pipets 10 ml	Fisherbrand	13-678-27E
Pipets 25 ml	Costar	4489
Pipets 50 ml	Costar	4490
Microcentrifuge tubes 1.5 ml	Fisherbrand	02-681-10
Microcentrifuge tubes 1.5 ml	Sealrite, USA Scientific	1615-5500
Tubes 50 ml	Falcon	352070
Tubes 15 ml	Falcon	352099
Pipet tips 1-10 µl	Corning Incorporated	4826
Pipet tips 1-200 µl		4863
Pipet tips 100-1000 µl		9032
Tissue Culture Flasks 25 cm^2	Falcon	353109
Tissue Culture Flasks 75 cm^2		353136

Table 5.: Cell culture supplies.

Cell culture supplies		
RPMI 1640 medium	Sigma /4°C	
DMEM medium		
Alpha-DMEM		
Eagle's Minimum Essential Medium		
L-Glutamine	Sigma /aliquoted at -20°C	
Penicillin/Streptomycin	Cellgro, /-20°C	#30-002CL
Hepes	Siena /4°C	
BGS (bovine growth serum)	Hyclone /4°C	30541.03
Trypsin	Sigma Trypsin EDTA solution /4°C	#T4174
Hygromycin B	Invitrogen /4°C	10687-010
Zeocin	Invitrogen /-20°C	#46-0509
G418		
DMSO	Sigma /RT	#D4540-100ML

Cryotubes	NUNC CryoTube Vials	#377267
Thymidine	Sigma	T1899-5G
Mitomycin C from Streptomyces caespitosus	Sigma	M4287-2MG

Table 6.: Fixing and staining supplies

Fixing and staining supplies		
Sucrose 99%	Sigma /RT	S0398-500G
PFA 4% (paraformaldehyde)	Boston Bioproducts /-20°C	BM154
BSA (bovine serum albumine)	Sigma /-20°C	A 9647-50G
Triton X-100	Sigma /RT	T-9 284
Fixing Buffer	3% PFA, 2 % Sucrose 1X PBS	
Permeabilization Buffer	0.5% Triton-X 20mM HEPES, pH 7.9 50mM NaCl 3mM KCl 300mM Sucrose	
Blocking Buffer	0.5% NP40 10% BGS 0.3% NaN3 1X PBS	
Mounting Medium	Vector, Vecatshield /4°C	H-1000

Table 7.: Western blot supplies

Western blot supplies		
Cell Extraction Buffer	Invitrogen	FNN0011
Protease Inhibitor Cocktail	Sigma	P8340
PMSF	Sigma	P7626
Celllifter	Fisher Scientific Fisherbrand Disposable CellLifter	
Protein Dye	BioRad Protein Assay Dye Reagent Concentrate	500-0006
H2O Machine	Continental Water Systems MILLIPORE MILLI-Q	H2O Machine

Heatblock	VWR Scientific Products Select Heatblock	13259-050
NuPAGE 4-12% Bis-Tris Gel, 1mmx 10wells	Invitrogen	
NuPAGE 10% Bis-Tris Gel, 1,5mmx, 10 wells	Invitrogen	NP0315
Novex 4% Tris-Glycine gel, 1mmx, 10 wells	Invitrogen	
Novex 4% tris-Glycine gel, 1,5mmx, 10 wells	Invitrogen	EC6058
Western Blot Box	Invitrogen Novex Mini-Cell and XCell Surelock lid	
Powersource	Bio-Rad Powerpac 200	
NuPAGE MOPS SDS Running Buffer (20x)	Invitrogen	NP0001
Novex 10x Tris-Glycine SDS running buffer	Invitrogen	LC2675
Reducing Agent	Invitrogen NuPage Sample Reducing Agent (10x)	NP0009
Sample Buffer	Invitrogen NuPage LDS Sample Buffer (4x)	NP0007
Sample Buffer	Invitrogen Tris-Glycine SDS Sample Buffer	LC5800
Stripping Buffer	Thermo Scientific Restore Plus Western Blot Stripping Buffer	46430
Molecular Weight Ladders	Invitrogen Novex Sharp PreStained Protein Standards	
Transfer Cell	Bio-Rad Mini Trans-Blot Cell	
Transfer Sandwich	Bio-Rad Mini PROTEAN 3Cell Sandwich	
Transfer Membranes	Invitrogen PVDF Membrane Filter Paper Sandwich, 0.45µm Pore Size	LC2005
NuPAGE Transfer Buffer (20x)	Invitrogen	NP0006-1
Methanol		
Novex Transfer Buffer	Invitrogen Tris-Glycine Transfer Buffer	LC3675

10% SDS-Solution ultraPURE,	Invitrogen GIBCO	15553
FANCD2 Antibody Mouse	Santa Cruz Mouse Monoclonal Antibody	FI17 sc 20022
FANCF Antibody Rabbit	Sigma Rabbit Polyclonal Antibody	SAB1101098
Rad51 Antibody Mouse	Abcam Mouse Monoclonal Antibody	ab213
Loading Control Antibody	Sigma Monoclonal Mouse Anti-ß-actin Antibody (Clone AC-15)	
Filamin	Santa Cruz	sc17749
Secondary Antibodies	Santa Cruz ThermoScientific Goat Anti-Mouse IgG HRP-Linked ImmunoPure Antibody	sc 2031
	Santa Cruz Anti-Rabbit IgG, HRP-Linked, Cell Signaling	sc 2030
TBS-T		
Non-Fat Dry Milk Powder	BioRad	170-6404
ECL-Reagents	Invitrogen Novex ECL HRP Chemoluminescent Substrate Reagent Kit	WP20005
Film	Amersham Hyperfilm Ecl 5x7 inches	28906835

Table 8.: siRNA deplention supplies

SiRNA depletion supplies		
Custom siRNA FANCD2	Ambion	4390827
Silencer negative control siRNA	Ambion	AM4611
SiRNA transfection reagent	Roche X-tremeGENE	4476093001
Reduced Serum Medium	Invitrogen Opti-MEM 1x	11058-021

4. Results

4.1. Visualization of Thymidine (TdR) - induced HR activity via Rad51 foci induction

HR activity was monitored in Thymidine-treated NSCLC cell lines in an attempt to find differences between repair responses. HR was visualized by the assembly of Rad51 protein in sub-nuclear foci as a surrogate marker for repair sites and activity. Rad51 is the principal recombinase and central actor of the HR pathway. Several research groups have found p53 to suppress HR activity (Akyüz et al. 2002; Romanova et al. 2004; Boehden et al. 2003). Studies showed p53-conducted inhibition of HR upon treatment with agents that lead to DNA cross-links and stalled replication forks (Sirbu et al. 2011; Saintigny and Lopez 2002).

A recent study from our lab showed how Thymidine treatment is potent to induce Rad51 focus formation in response to treatment and visualizes increased HR activity. In cells expressing transactivation-inactive p53 a decrease in HR activity upon Thymidine or Hydroxyurea (HU) treatment could be observed. Thymidine and HU are both cross-linking agents that lead to stalled replication forks (Sirbu et al. 2011).

These findings stand in line with p53's role as "the guardian of the genome" (Lane 1992). Even though HR is seen as a high fidelity repair mechanism, enhanced HR activity, especially at stalled replication forks, has been found to lead to genomic instability (Yu et al. 2000; Reliene, Bishop, and Schiestl 2007). The NSCLC cell line H1299 p53-null and the transfected versions H1299 p53-QS, H1299 p53-QM and H1299 p53-S15A were used to confirm the findings published by Sirbu et al. and to evaluate the impact of different p53 mutants on HR activity (Figure 3). p53-QS, p53-QM and p53-S15A are N-terminal mutants of wildtype p53. The L22Q/W23S mutation in p53-QS cells results in largely reduced transactivation-activity and allows the observation of direct interactions between p53 and other proteins. p53-QM is additionally (to L22Q/W23S) mutated at W53S/F54S, abrogating the ability to bind to RPA. p53-S15A harbors the L22Q/W23S mutation and a mutation at the phosphorylation site S15A. This phosphorylation site is target of the kinases ATM and ATR. Recent studies showed that the HR suppressing effect of p53 depends on phosphorylation through ATR rather than ATM (Sirbu et al. 2011).

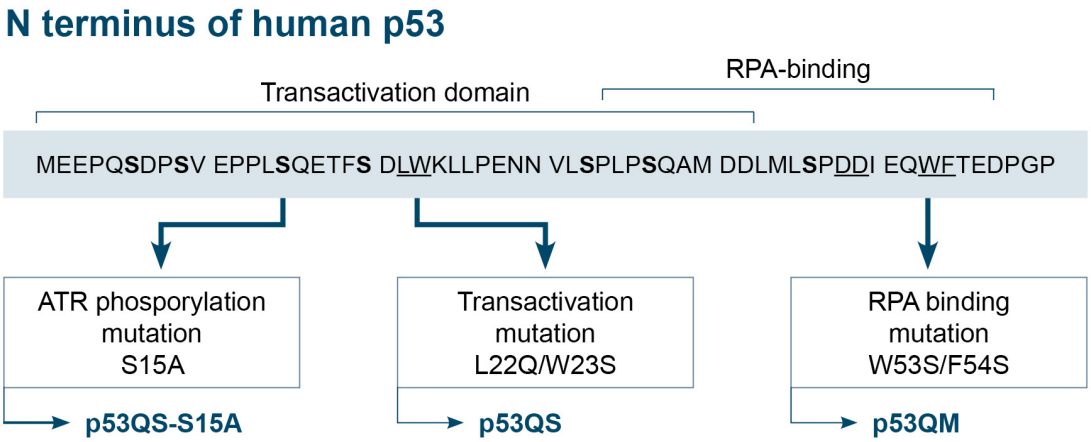

Figure 3.: N-terminal p53 mutations
The mutant constructs were stably expressed in H1299 FRT cells.

All cell lines were treated with Thymidine and Rad51 foci were subsequently counted under a fluorescence microscope. The baseline Rad51 activity, measured by foci number in untreated cells, was subtracted from the foci counts in treated cells. The presence of more than 15 additional foci in one cell was interpreted as Rad51 foci induction. Thymidine inhibits replication elongation through depletion of the nucleotide triphosphate dCTP (deoxycytidine triphosphate). Lack of nucleotide triphosphates thus stalls replication forks and arrests cells in S-phase. These stalled forks require HR repair to restart replication and cell cycle progression (Eriksson, Thelander, and Akerman 1979; Bolderson et al. 2004; Bjursell and Reichard 1973).

Figure 4 compares Rad51 foci induction in response to Thymidine treatment in H1299 cell lines with different p53 status (foci induction classified as described above). The results indicate a decrease in Rad51 foci induction in H1299 cells expressing p53-QS in response to Thymidine treatment (28.5 %), compared to H1299 p53-null cells (45.5 %). These results support the hypothesis that p53 inhibits HR in response to replicative stress confirming our previous experiments (Sirbu et al. 2011). Neither p53-QM nor p53-S15A showed the ability to suppress Rad51 foci formation. Instead, the Rad51 foci levels in p53-QM and p53-S15A expressing cells were un-affected or slightly increased, compared to p53-null expressing cells. Since p53-QM lacks the ability of RPA binding but otherwise equals p53-QS, RPA binding is likely required for HR inhibition. These findings are consistent with prior publications, showing that HR inhibition by p53 requires RPA binding to be efficient

(Romanova et al. 2004). p53-S15A harbors a mutation at the S15 phosphorylation site. The phosphorylation site is target to ATM and ATR. Data from Sirbu et al. showed that in response to replicative stress ATR is the main regulator (Sirbu et al. 2011). Higher Rad51 foci levels in H1299 p53-S15A cells indicate increased HR activity in these cells and thus possibly implicate a role for ATR in HR regulation. The molecular details of how these different p53 mutants influence HR in particular need further investigation and are elaborated in the discussion of this study.

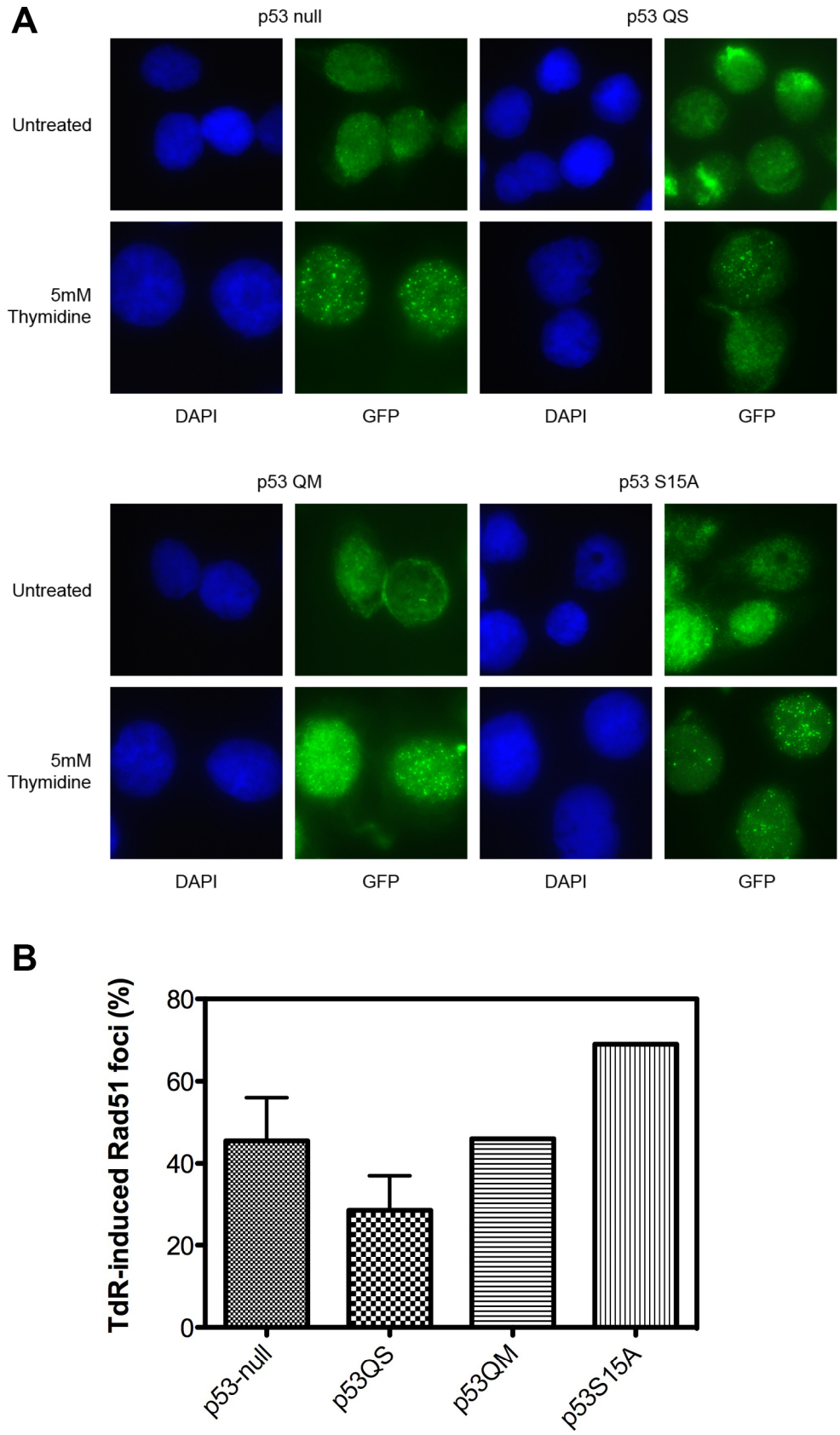

Figure 4.: TdR induced Rad51 foci in H1299 cell lines
(A) Images of sub-nuclear Rad51 foci induction in response to 5 mM Thymidine, applied for 24 h, in H1299 p53-null cells and H1299 cells stably expressing p53-QS, p53-QM or p53-S15A. (B) Effect of p53 status on Rad51 foci induction after TdR treatment. (T-test: p = 0,3 for p53-null vs. p53-QS)

4.2. Rad51 and γ-H2AX foci induction in response to Mitomycin C treatment

Many drugs frequently used in chemotherapy damage DNA by blocking replication. The above experiments (Figure 4) were repeated with Mitomycin C to further investigate how p53 status influences ICL repair. Mitomycin C is a bifunctional alkylator that causes ICLs, leading to stalled forks during replication (Kennedy et al. 1980). If the stalled forks persist, they collapse and turn into one-ended DSBs. Mitomycin C is commonly used as an anticancer drug in chemotherapy for the treatment of different cancer types (Palom et al. 2002). While Thymidine leads to replication stop and DSBs due to a lack of required components, Mitomycin C blocks replication physically.

Figure 5 shows Rad51 foci formation in all cell lines in response to Mitomycin C. However, the lowest numbers of foci were found in H1299 p53-null as well as in H1299 p53-QS cells. These findings differ from results in Thymidine-treated cells (Figure 4) and indicate that p53-QS does not suppress Rad51 foci formation in response to Mitomycin C treatment. Since Rad51 foci levels are similar in H1299 p53-QS and H1299 p53-null cells, p53-QS might not have a regulatory impact on HR in response to Mitomycin C treatment.

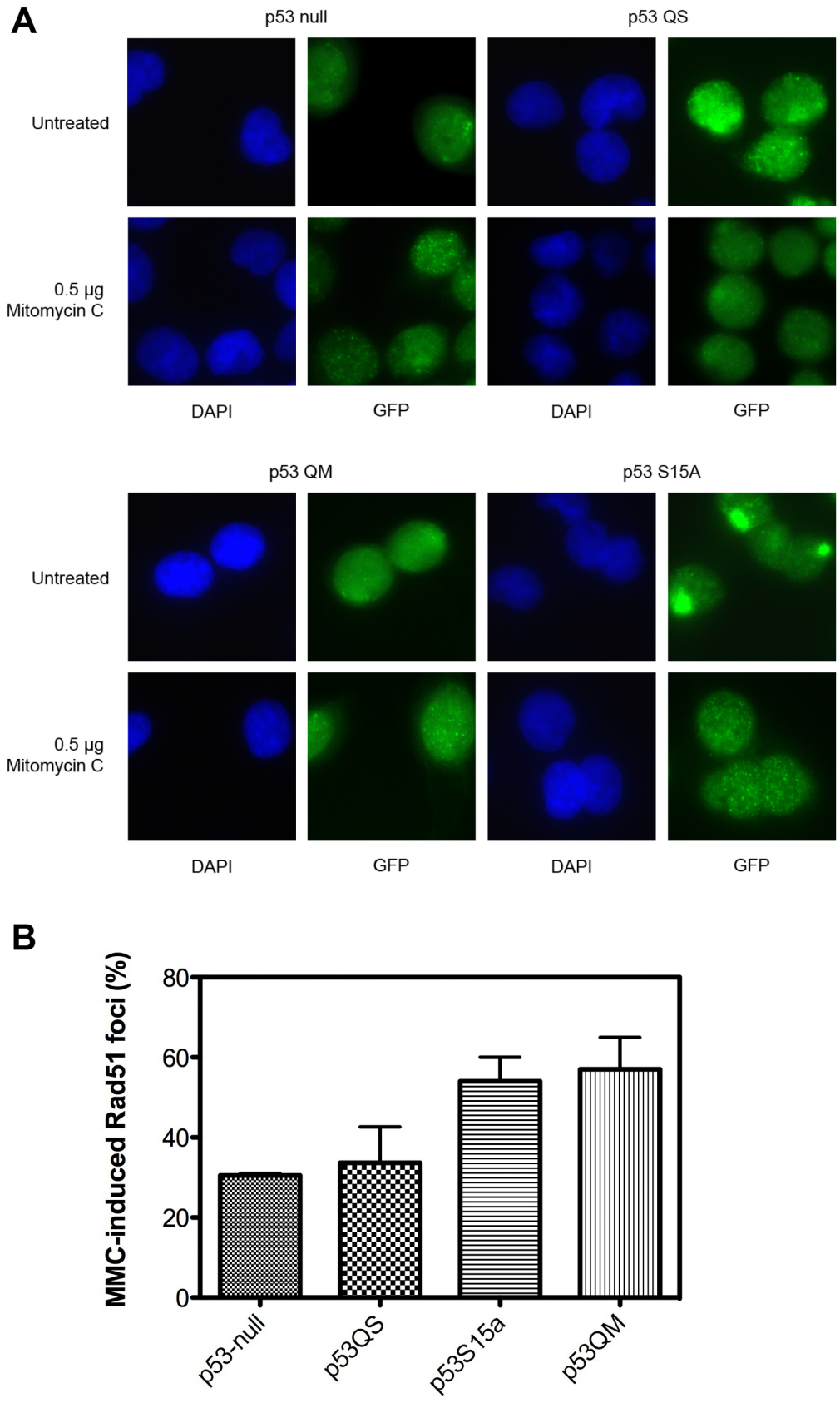

Figure 5.: Rad51 foci formation in response to Mitomycin C treatment
(A) Images of sub-nuclear Rad51 foci induction in response to Mitomycin C (0.5 µg/ml applied for 1 h and stained after 4 h) in H1299 p53-null cells and H1299 cells stably expressing p53-QS, p53-QM or p53-S15A. (B) Effect of p53 status on Rad51 foci induction after Mitomycin C treatment. (T-test p = 0,08 for p53-null vs. p53-QM)

Although statistically not significant, these results show reproducibly a slightly higher induction of Rad51 foci in H1299 cells expressing p53-S15A or p53-QM compared to p53-null. These findings are comparable to those in reaction to Thymidine treatment and suggest that p53-S15A and p53-QM are either incapable of HR inhibition or might even act as gain-of-function mutants that promote HR activity in an unknown manner. As the transactivation-inactive p53-QS does not show a suppressing influence on HR in response to Mitomycin C, it becomes obvious that p53 has a differentiated effect and a complex role upon HR regulation. For further investigation of differences in management of DNA damage between H1299 p53-null and H1299 p53-QS cells DSBs upon Mitomycin C treatment were quantified.

Observation of γ-H2AX foci induction can serve as a marker for number of DSBs and as a marker for chemo-sensitivity and repair efficiency. Analysis of γ-H2AX foci can show how HR activity is related to frank DSBs and not only stalled replication forks. γ-H2AX is the phosphorylated form of H2AX and is involved in recruitment of further repair proteins and activation of the HR process. The phosphorylation is pursued by ATM, ATR and DNA-PK in response to DNA damage (Sedelnikova et al. 2002; Rogakou et al. 1998; Paull et al. 2000; Burma et al. 2001). γ-H2AX foci were analyzed 24h after Mitomycin C treatment to visualize Mitomycin C induced DSBs. Foci induction was compared in H1299 p53-null and H1299 p53-QS cells (Figure 6).

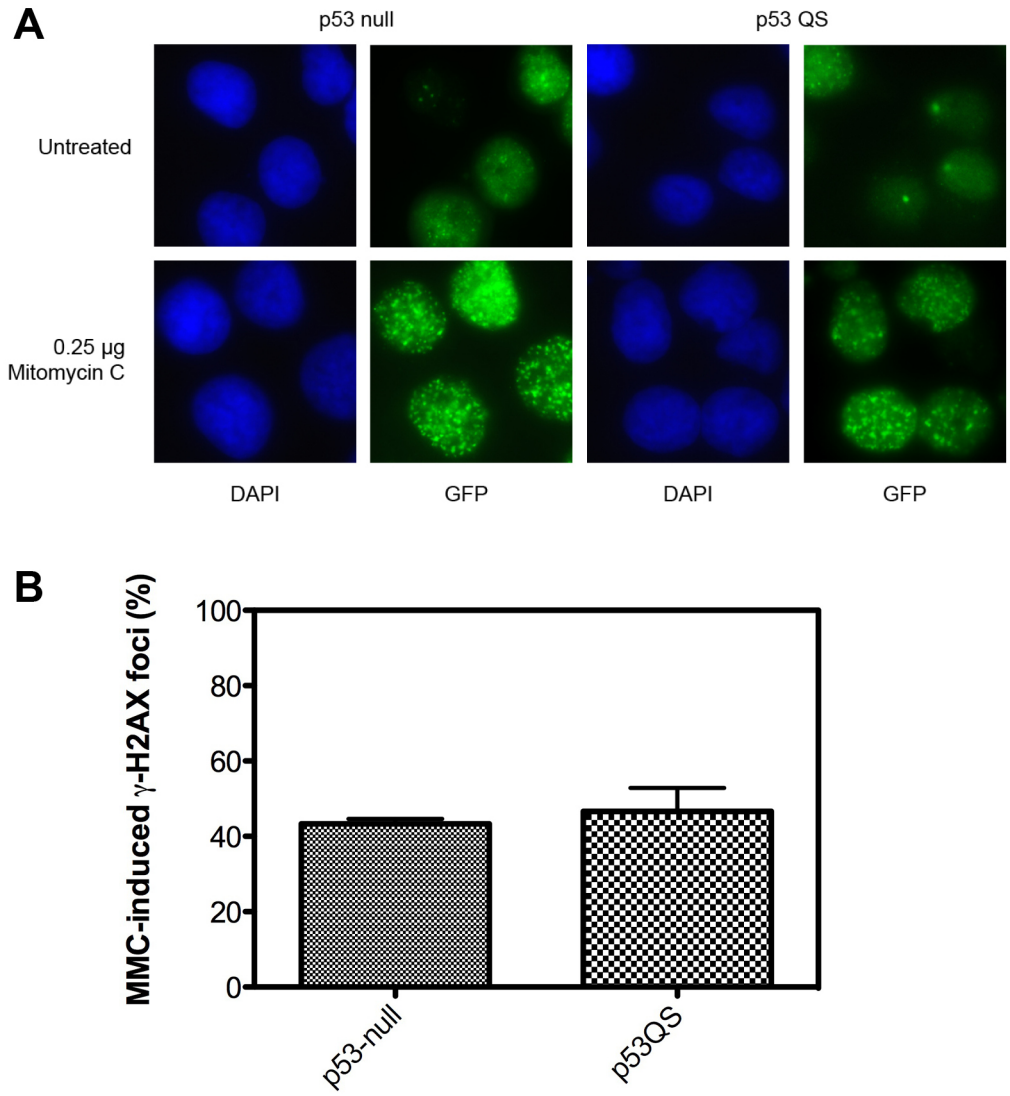

Figure 6.: γ-H2AX foci formation in response to Mitomycin C treatment
(A) Images of sub-nuclear γ-H2AX foci induction in response to Mitomycin C treatment (0.25 µg/ml applied for 1 h and stained after 24 h) in H1299 p53-null cells and H1299 cells stably expressing p53-QS. (B) Effect of p53 status on sub-nuclear γ-H2AX foci induction after Mitomycin C treatment.

The results show no difference in γ-H2AX foci amount after 24h of Mitomycin C exposure, indicating that there is no discrepancy between DSB induction and repair after Mitomycin C during the first 24 h in these two cell lines.

4.4. FANCD2 foci induction in response Mitomycin C treatment

Upon ICLs or stalled replication forks, the FA/BRCA pathway is activated. It is required for detection and repair of ICLs or stalled forks and for recruitment of HR proteins (Knipscheer et al. 2009). The fact that FA deficient cells are generally

hypersensitive to DNA cross-linking agents underlines the pivotal role of the FA proteins within ICL repair and replication restart. Mitomycin C treatment leads to collapsed replication forks and one-ended DSBs and thus activates the FA/BRCA pathway. The FA/BRCA pathway is considered indispensable for repair of ICLs, therefore it is reasonable to visualize FA/BRCA activity in response to Mitomycin C treatment.

The central reaction of the active FA/BRCA pathway is the mono-ubiquitylation of FANCD2 by the FA core complex. Mono-ubiquitylated FANCD2 can be visualized in sub-nuclear foci, presumably reflecting the activation of the pathway. Mono-ubiquitylated FANCD2 interacts with several other repair proteins and is essential to start the HR pathway. FANCD2 foci induction has been monitored before to acquire information about chemo-sensitivity and functional efficiency of the FA/BRCA pathway (Burkitt and Ljungman 2007; Willers et al. 2008). H1299 p53-null, H1299 p53-QS, H1299 p53-QM and H1299 p53-237 cells were treated with Mitmomycin C and subsequently analyzed for FANCD2 foci induction 4 h later.

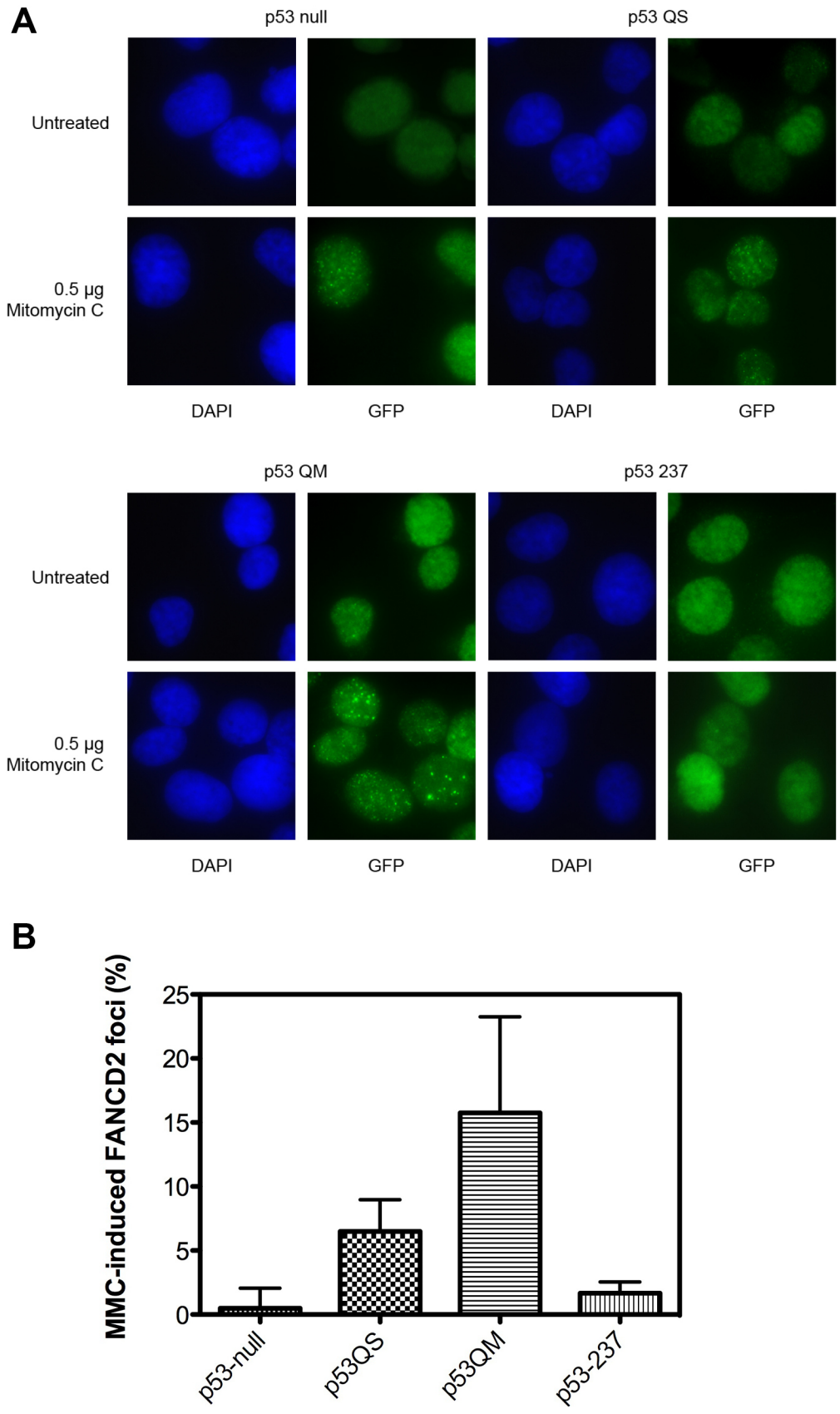

Figure 7.: FANCD2 foci formation in response to Mitomycin C treatment
(A) Images of sub-nuclear FANCD2 foci induction in response to Mitomycin C treatment (0.5 ug/ml for 1 h and staining after 4 h) in H1299 p53-null cells and H1299 cells stably expressing p53-QS, p53-QM or p53-237. (B) Effect of p53 status on FANCD2 foci induction after Mitomycin C treatment. (T-test: p = 0,09 for p53-null vs. p53-QM)

Results in Figure 7 show remarkably low FANCD2 foci induction levels in all H1299 cell lines. Especially if compared to Rad51 foci induction shown in Figure 6. Prior results from our lab (unpublished, Natalie Ferraiolo) showed that H1299 cells have one of the lowest FANCD2 foci induction levels in response to Cisplatin, another cross-linking agent, compared to other NSCLC cell lines. The highest levels of FANCD2 foci induction were found in H1299 p53-QM cells, followed by H1299 p53-QS. In H1299 p53-null and H1299 p53-237 cells, expressing p53 with mutated core region, FANCD2 foci induction was hardly detectable at all.

The pattern of FANCD2 foci induction seems to be similar to the pattern of Rad51 foci induction in response to Mitomycin C with the highest induction in H1299 p53-QM. A definite correlation cannot be made at this point though, especially in consideration of the very low FANCD2 induction overall. Taking in account the importance of the FA/BRCA pathway within ICL repair it remains to be elucidated how H1299 cells maintain the repair of ICLs with low FANCD2 activity. Despite the very low FANCD2 activity levels, the activity seen seems to be connected to p53 status. The results regarding H1299 p53-QM suggest that transactivation-inactive p53 might be able to promote the FA/BRCA pathway and thus HR, as p53-QM expressing cells showed the highest FA/BRCA activity as well as the highest RAD51 foci levels (Figure 5). In p53-QS expressing cells however, Rad51 levels were un-affected while FANCD2 levels seem to be slightly increased. Further experiments are required to find out whether the FA/BRCA pathway might be a possible target for p53 to regulate the repair of stalled forks.

4.5. Clonogenic survival of H1299 cells with different p53 status

Colony survival assays executed in our lab have shown differences in survival between Mitomycin C-treated H1299 cells with different p53 status. Data in Figure 8 show that H1299 cells expressing the p53 mutants QM, QS and S15A have a survival benefit compared to p53-null or p53-237 cells. These results suggest that the presence of transactivation-inactive p53 increases the resistance to Mitomycin C, compared to absence of p53 or expression of p53 with a mutated core domain at 237. H1299 p53-QM and S15A cells were most resistant to Mitomycin C. Considering the increased Rad51 and FANCD2 foci induction in response to Mitomycin C in H1299 p53-QM cells (Figure 6 and 7), the survival advantage might be due to promoted HR activity through transactivation-inactive p53 versions. These results are consistent with the hypothesis of a complex role for p53 within HR regulation. It

seems to have a repair promoting and thus resistance improving function upon stalled forks caused by Mitomycin C, while at the same time, p53-QS inhibits HR upon stalled replication in response to Thymidine treatment.

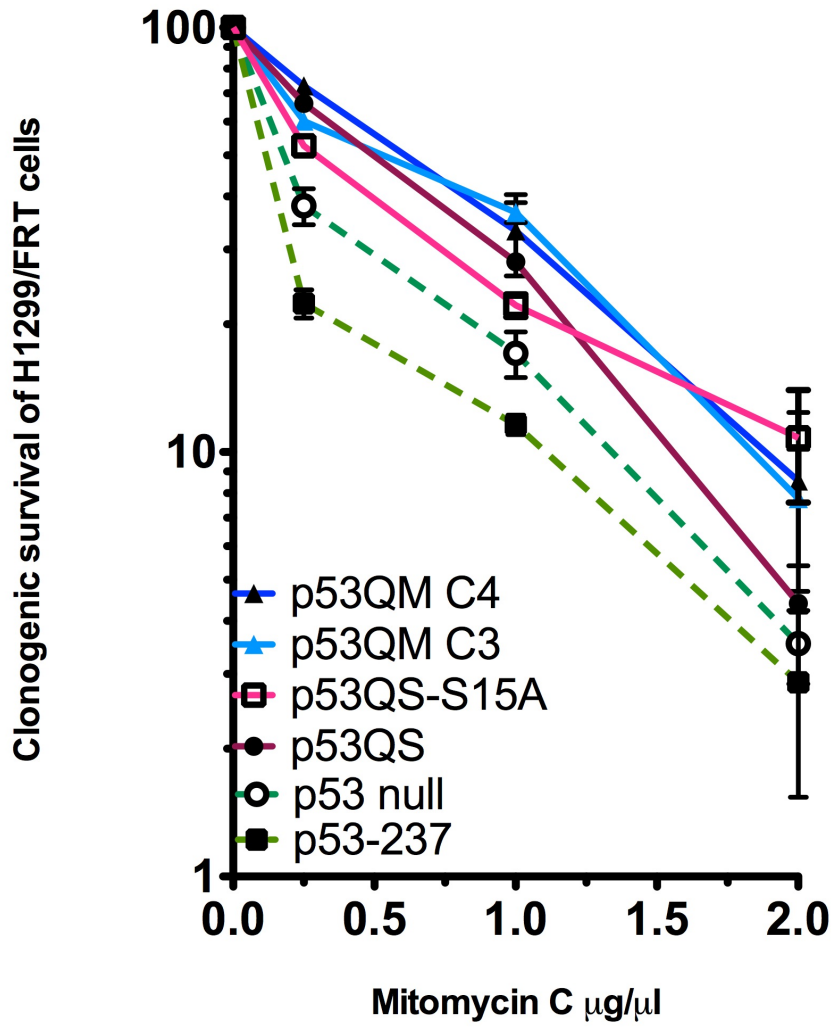

Figure 8.: Clonogenic survival of H1299/FRT cells
Influence of different N-terminal p53 mutants on the survival of Mitomycin C-treated H1299 cells (Including two different clones, C3 and C4 of p53-QM). The data shown in figure 8 was kindly provided by the Willers lab.

4.6. Visualization of FANCD2 mono-ubiquitylation in western blots

It remains unclear, how exactly the mutated p53 promotes survival upon Mitomycin C treatment. A possible target to promote repair is the FA/BRCA pathway, specifically the mono-ubiquitylation process of FANCD2, since it is the key element of activation and thus a reasonable target for regulation. A western blot was performed to further determine how the p53 status influences FANCD2 mono-ubiquitylation. NSCLC cell

line A549 served as a positive control for sufficient FANCD2 mono-ubiquitylation (Fig. 9A). FANCD2 and mono-ubiquitylated FANCD2 bands were compared in untreated and Mitomycin C-treated H1299 cells with different p53 status. The non-ubiquitylated FANCD2 (155 kDa) is 7kDa shorter than the mono-ubiquitylated FANCD2-ub (162 kDa) (Timmers et al. 2001).

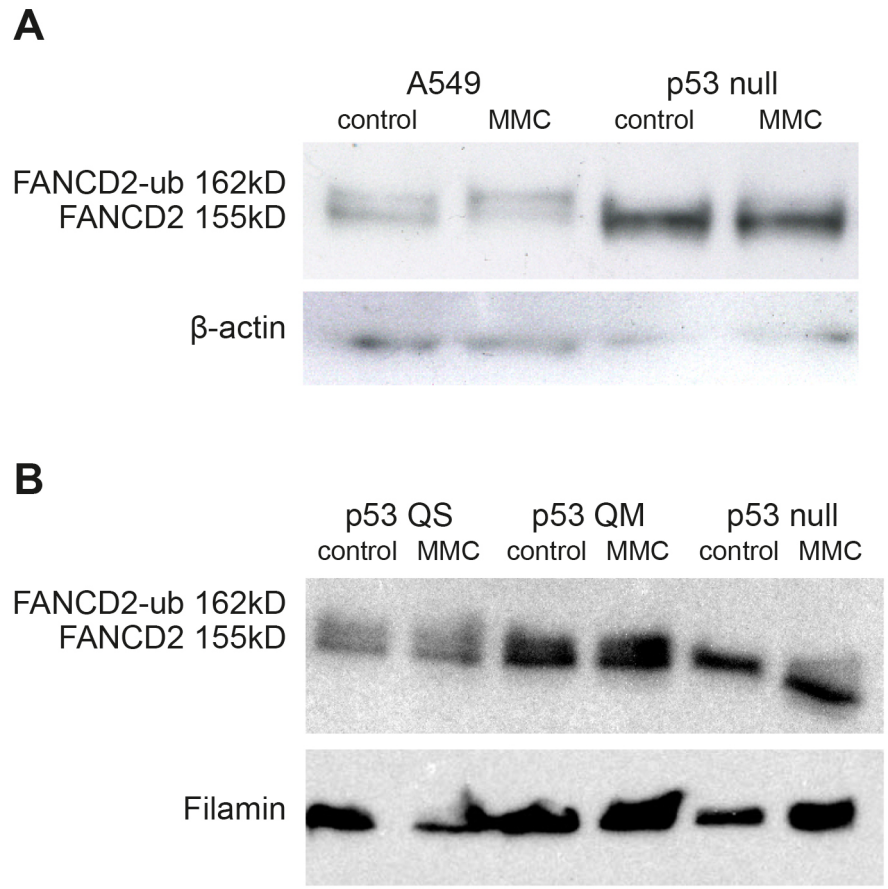

Figure 9.: FANCD2 mono-ubiquitylation in response to Mitomycin C
(A) Western blot illustrating the mono-ubiquitylation of FANCD2 after treatment with 1 µg/mL Mitomycin C for 5 hours continuously in NSCLC cell line A549 serving as a positive control compared to H1299 p53-null cells. (B) Mono-ubiquitylation of FANCD2 after Mitomycin C treatment in H1299 cells with different p53 status compared to A549 cells.

Figure 9A shows intact FANCD2 mono-ubiquitylation in A549 cells, as the FANCD2-ub band is brighter after Mitomycin C treatment. There is no visible FANCD2-ub band in H1299 p53-null cells. This finding could be reproduced in Figure 9B. Compared to the faint or missing FANCD2-ub band in H1299 p53-null cells, the mono-ubiquitylation seems to be at least partly rescued in H1299 cells expressing p53-QS or p53-QM (Figure 9B). H1299 p53-QM cells have a generally brighter band for

FANCD2 as well as for FANCD2-ub, compared to H1299 p53-null cells or H1299 cells expressing p53-QS. The brightness of bands for FANCD2 and FANCD-ub correlates with the amount of induced FANCD2 foci (Figure 7) and the surviving fraction after Mitomycin C treatment (Figure 8).

These findings suggest that transactivation-inactive p53 might support FANCD2 mono-ubiquitylation and consequently promote FANCD2 foci formation. A promotion of the FA/BRCA pathway might lead to more sufficient repair and thus explain increased resistance towards Mitomycin C. p53-QM seems to be more effective in promotion of FANCD2 than p53-QS. The only difference between p53-QS and p53-QM is the RPA-binding and thus inhibiting ability, which is lost in p53-QM. RPA is needed for HR, so higher RPA activity might lead to higher HR activity. Further experiments are required to elaborate whether p53 is directly or indirectly responsible for the increased FANCD2 activity in p53-QS or p53-QM expressing cells and how that impacts survival in response to Mitomycin C treatment.

4.7. siRNA depletion of FANCD2 in H1299 cells

Since the cell lines with the highest survival upon Mitomycin C treatment (Figure 8) were the same cell lines with the higher FANCD2 foci induction (Figure 7) and the brightest FANCD2 bands (Figure 9), a link between high FANCD2 and improved survival seemed possible. A siRNA-mediated depletion of FANCD2 in H1299 cells was accomplished to investigate whether FANCD2 is responsible for increased resistance towards Mitomycin C in H1299 cells expressing p53. If the small but consistent FANCD2 foci induction in response to Mitomycin C in H1299 p53-QM cells was connected to improved survival compared to H1299 p53-null cells, the difference in survival between the cell lines would disappear after the FANCD2 depletion.

H1299 p53-null and H1299 p53-QM cells were used for the siRNA-mediated depletion of FANCD2 while breast cancer cell line MCF7 served as a control cell line. The successful depletion was confirmed in FANCD2 western blots accomplished after 48h (not shown) and 72h (Figure 10A). There are no visible FANCD2 bands in the siRNA depleted H1299 cell lines. MCF7 cell lines showed initially a bright FANCD2 band, which was clearly reduced after siRNA treatment, confirming successful FANCD2 depletion. As a control, the cell lines were transfected with siRNA scramble. The survival fractions after Mitomycin C treatment were monitored with a colony survival assay.

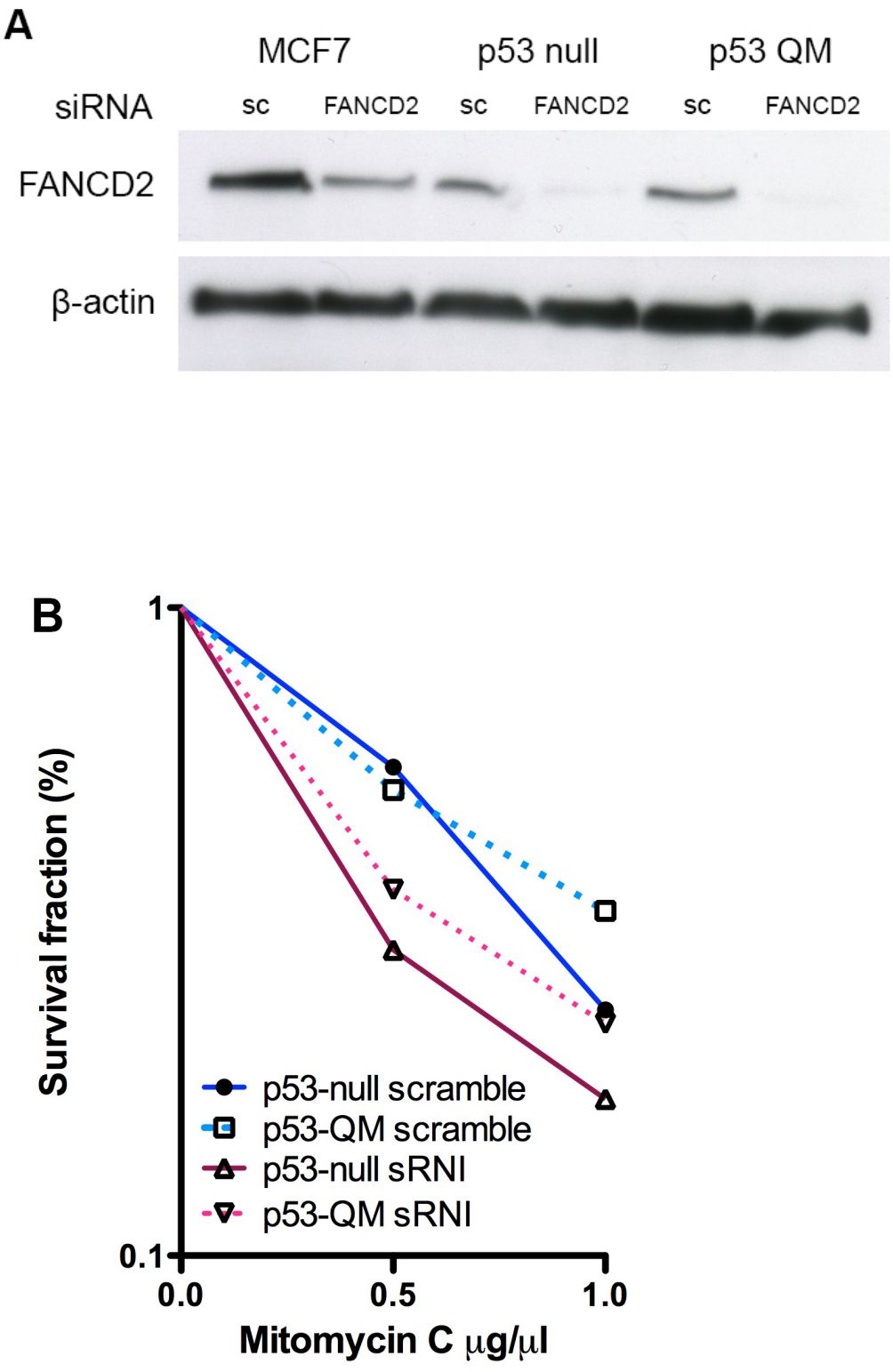

Figure 10.: siRNA depletion of FANCD2
(A) Western blot illustrating the successful depletion of FANCD2 after 48h. (B) Survival curves for FANCD2 depleted and control H1299 p53-null and H1299 p53-QM cells in response to Mitomycin C treatment.

Results revealed that both cell lines transfected with FANCD2 siRNA became more sensitive to Mitomycin C treatment compared to the scramble transfected cells. However, H1299 p53-QM cells depleted of FANCD2 were still more resistant to Mitomycin C treatment than H1299 p53-null cells depleted of FANCD2, comparable to survival curves seen in Figure 8. As cell lines depleted of FANCD2 seem to be more sensitive to Mitomycin C than cells with intact FANCD2, a connection between repair proficiency and FANCD2 activity becomes possible. The presence of p53-QM also seems to be connected to improved survival, as H1299 p53-QM cells with intact FANCD2 as well as FANCD2 depleted H1299 p53-QM cells were more resistant than the respective H1299 p53-null cells. A possible conclusion could be that both proteins promote survival in some way, which needs further investigation. These results deliver no support for the hypothesis of FANCD2 as a target to p53 yet.

4.8. FANCF expression levels in NSCLC

14% of NSCLC cell lines have been found to have a promoter methylation at the FANCF gene. FANCF is one of the crucial proteins involved in the FA core complex, which is responsible for FANCD2 mono-ubiquitylation. The promoter methylation was found to result in an inactive FA/BRCA pathway. The observation could be confirmed in tissue samples with methylation-specific polymerase chain reaction (PCR), but not in cell lines so far (Marsit et al. 2004). In a screening of 473 lung cancer cell lines (111 of them NSCLC) for FANCF gene expression levels, H1299 cells appeared to have a two-times lower expression of FANCF than the mean value of NSCLC cells (data provided by (Birkelbach et al. 2013). Figure 11.1. shows FANCF gene expression levels in H1299 cells compared to 111 other NSCLC cell lines.

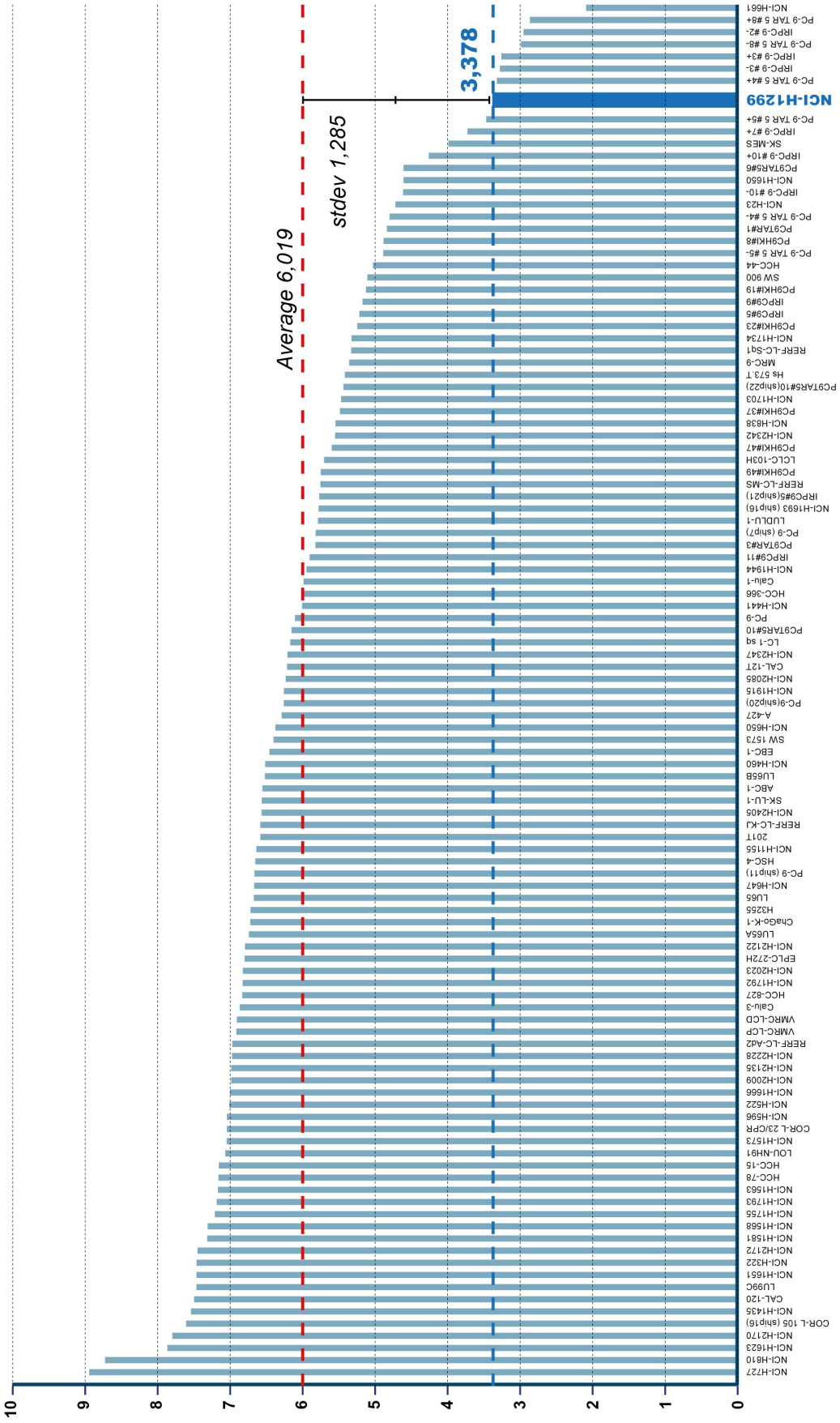

Figure 11.1: FANCF gene expression levels in NSCLC
H1299 cells show a two STDEV lower FANCF expression than the other 111 NSCLC lung cancer cell lines. Data drawn from (Birkelbach et al. 2013)

A western blot was accomplished to further investigate the FANCF expression levels in H1299 cells. H1299 p53-null, H1299 p53-QS and H1299 p53-QM cells were compared to NSCLC cell lines A549 and PC9.

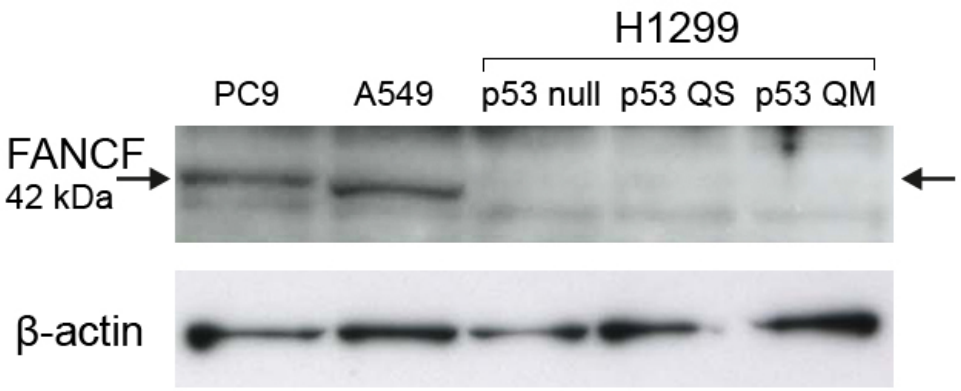

Figure 11.2: FANCF western blot
Western blot illustrating differences in FANCF expression between H1299 cells with different p53 status and NSCLC cell lines PC9 and A549.

The western blot in Figure 11.2. shows the absence of bands for FANCF in H1299 cells. The absence of FANCF bands is independent of the p53 status in H1299 cells. The other NSCLC cell lines, A549 and PC9, have clearly visible bands for FANCF. These findings suggest a very low FANCF expression, if not deficiency in H1299 cells. Considering that, it seems likely that the assembling of the FA core complex in H1299 cells is impaired. Therefore the performance of FANCD2 mono-ubiquitylation has to be affected as well. This constitution would explain the low levels of FANCD2 foci and weak FANCD-ub bands in H1299 cells. Apparently FANCD2 is still at least partly inducible, even if cells are FANCF deficient, as shown by FANCD2 foci induction and visible FANCD2/FANCD2-ub bands upon treatment with DNA cross-linker Mitomycin C (Figures 7 and 9). It remains open how H1299 cells achieve Mitomycin C resistance and if mutated p53 plays a role for the FANCD2 mono-ubiquitylation process.

4.9. Visualization of Rad51 expression

For further investigation of the differences between H1299 cells and other Mitomycin C resistant cell lines a closer look at Rad51 expression and activity was of interest. It is currently a matter of debate whether Rad51 over-expression or presence and especially persistence of foci reflect resistance or repair deficiency (Vispé et al. 1998; Martin et al. 2007; Schild and Wiese 2010; Klein 2008; Cherry et al. 2007; Harper, Anderson, and Neill 2010; Fuente et al. 2006). We thus asked whether H1299 cells might gain resistance towards DNA cross-linkers through Rad51 over-expression. A Rad51 western blot was accomplished in which H1299 cells were compared to Mitomycin C resistant and repair proficient NSCLC cell lines H1792 and Calu-6.

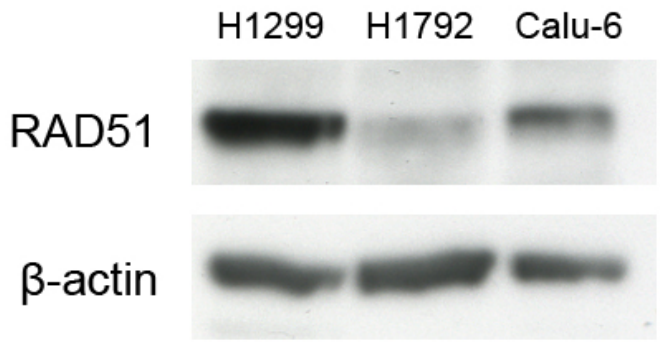

Figure 12.: Rad51 western blot
Western blot illustrating differences in Rad51 expression between NSCLC cell lines H1299, H1792 and Calu-6.

The western blot in Figure 12 shows a clearly brighter Rad51 band in H1299 cells than in H1792 or Calu-6 cells. These findings suggest that Rad51 over-expression is a possibility in H1299 cells. Further experiments are required for confirmation and relation to other results.

4.10. γ-H2AX foci induction in NSCLC cell lines in response to Mitomycin C treatment

γ-H2AX foci induction in response to Mitomycin C treatment was compared in H1299 and other NSCLC cell lines to achieve further clues about how H1299 cells achieve their resistance to cross-linking agents like Mitomycin C. γ-H2AX foci visualize the amounts of DSBs after Mitomycin C treatment and can also be interpreted as a marker for repair proficiency and chemo-sensitivity. The goal of this experiment was to elucidate whether H1299 cells are resistant to Mitomycin C because they are especially repair proficient, or because they experience less damage. In the latter case, the γ-H2AX foci induction would be expected to be lower in H1299 cells compared to other NSCLC cell lines. γ-H2AX foci formation in response to Mitomycin C was compared in H1299 p53-null, H1299 p53-QS, Calu-6, H1792 and PC9 cells (Figure 13). Calu-6 and H1792 are Mitomycin C resistant cell lines while PC9 is Mitomycin C sensitive.

The cell lines show no significant differences in γ-H2AX foci induction 24h after Mitomycin C treatment. The evaluation of γ-H2AX foci 24 hours after the treatment does not differentiate between DSB induction and repair. However, it seems like an underlying difference in DSB induction or repair is not what causes the inequality in Mitomycin C resistance.

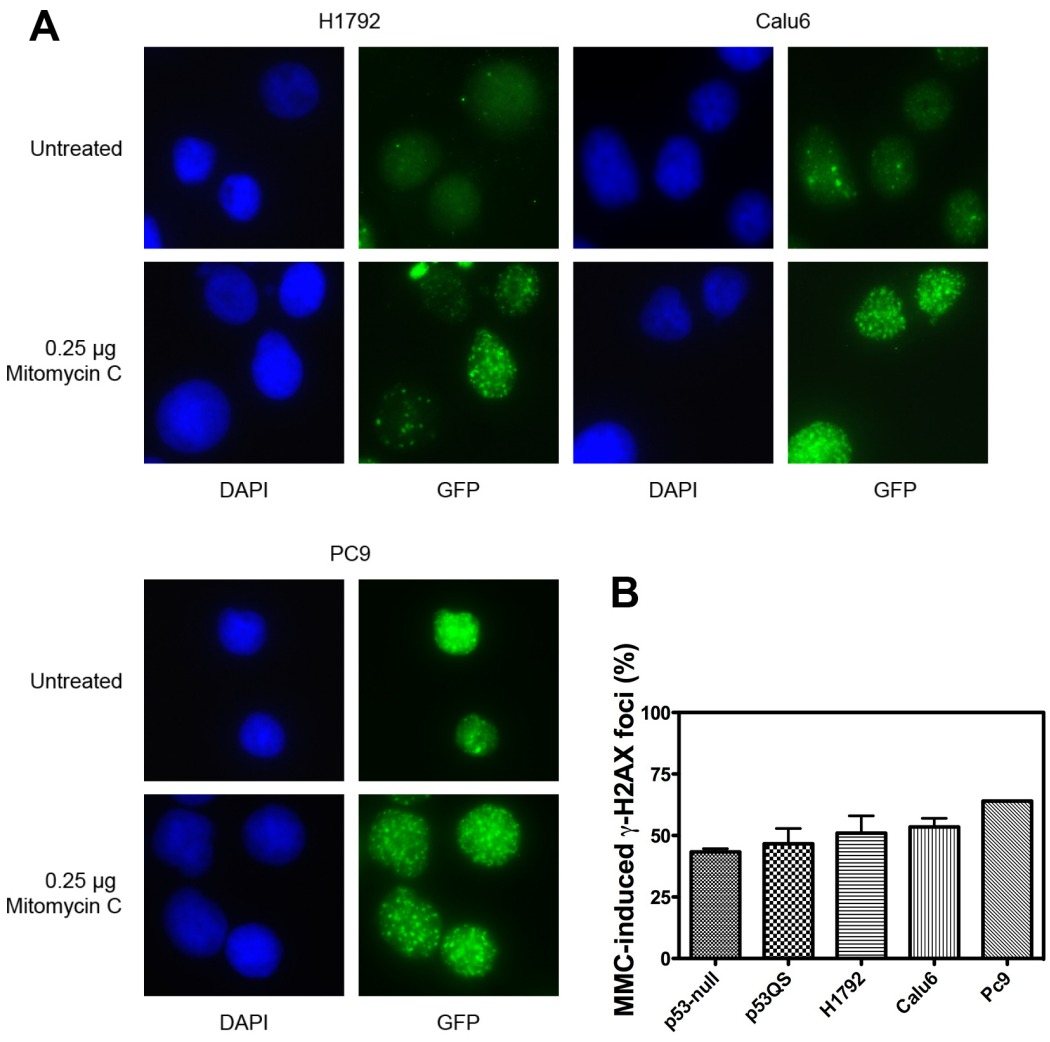

Figure 13.: γ-H2AX foci formation in response to Mitomycin C treatment
(A) Images of sub-nuclear γ-H2AX foci induction in response to Mitomycin C treatment in H1299 p53-null cells and H1299 cells stably expressing p53-QS, p53-QM or p53-237. (B) Effect of p53 status on γ-H2AX foci induction after Mitomycin C treatment (0.25 µg/ml applied for 1 h and stained after 24 h).

4.11. pATM foci induction in NSCLC cell lines in response to Mitomycin C treatment

For more insight in how H1299 cells manage their DSB repair, the visualization of pATM foci was accomplished. ATM is one of the central protein kinases in the DDR and becomes autophosphorylated as sign of activation. ATM is indispensable for the HR pathway (Golding et al. 2007). The phosphorylated ATM is referred to as pATM. pATM activity can be visualized in foci and is a marker for repair proficiency. pATM co-localizes with γ-H2AX at the DNA damage site but is not absolutely required for γ-H2AX foci formation. In the absence of ATM, H2AX could be phosphorylated by ATR or DNA-PK (Golding et al. 2007). It has been shown before that there are scenarios where phosphorylation of H2AX is rather performed by ATR, as for example in response to oxidative stress or one-ended DNA DSB as they occur from stalled replication forks (Katsube et al. 2014).

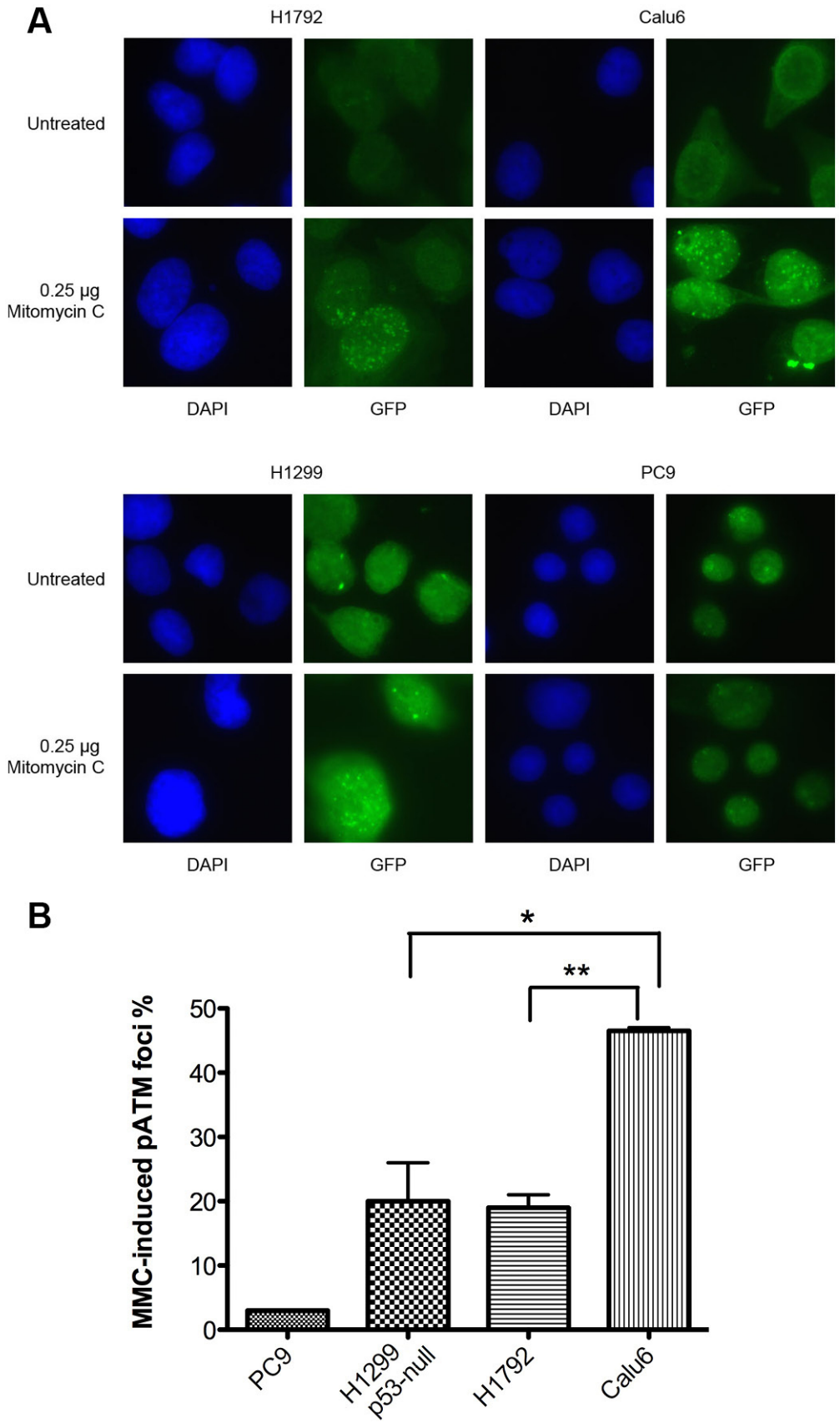

Figure 14.1.: pATM foci formation in response to Mitomycin C treatment
(A) Images of sub-nuclear pATM foci induction in response to Mitomycin C treatment in H1299 p53-null, PC9, H1792 and Calu6 cells. (B) Comparison of pATM foci induction after Mitomycin C treatment (0.25 µg/ml applied for 1 h and stained after 24 h) in different NSCLC cell lines. (P = 0.048 for Calu6 vs. H1299 p53-null is 0,0479, p = 0,0056 for Calu6 vs. H1792).

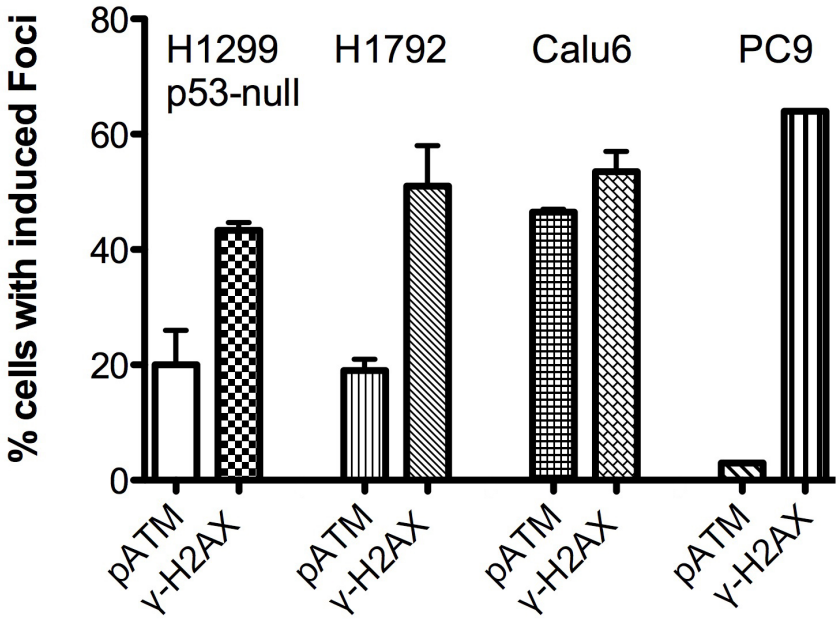

Figure 14.2.: Comparison of γ-H2AX foci induction and pATM foci induction

pATM foci formation in response to Mitomycin C was compared in H1299 p53-null, Calu-6, H1792 and PC9 cells. H1299 p53-null, Calu-6 and H1792 are Mitomycin C resistant and PC9 is Mitomycin C sensitive. While Figure 13 shows no differences in γ-H2AX foci induction between those cell lines, pATM formation clearly varied. Calu-6 cells show the highest amount of pATM foci induction while foci levels in H1299 were significantly lower ($p = 0,0479$) and highly significantly lower ($p = 0,0056$) in H1792 cells. In Mitomycin C sensitive PC9 cells pATM foci formation was hardly detectable. Comparing γ-H2AX and pATM foci induction in Figure 14.2. confirms pATM independent formation of γ-H2AX foci in PC9, H1299 and H1792 cells. The fact that H1299 and H1792 cells are rather repair proficient and have visible γ-H2AX foci upon DNA damage while pATM foci levels are low compared to Calu-6 suggests a relevant role for ATR in these cells. Clearly pATM foci are not a reliable predictor for repair proficiency or resistance to cross-linkers. Further experiments are required to clarify if ATR activity is indeed increased in repair proficient cells with low pATM levels.

5. Discussion

p53 is involved in countless pathways and interactions within the cell to protect genomic integrity. As the “guardian of the genome” it acts for one as a transcription factor, and again through direct protein-protein interactions. The focus here is set on transcription independent interactions of p53, while exclusively using mutated, transactivation-inactive p53, as seen in many tumors. These p53 mutants might differ from p53 wildtype in more qualities than just transactivation and therefore do not mirror the wildtype activity authentically.

p53’s role within DNA damage response depends on the reacting agent and the nature of the resulting alteration. The inhibiting influence of p53 on HR has been described in several studies. HR is required for repair of DSBs, ICLs and stalled replication forks. Recent publications have revealed further details and suggest a more complex role for p53 in HR regulation. In response to IR for example, p53 acts mainly as a transcription factor; in response to HU, however, where replication is blocked, far less transcriptional activation of p53 responsive genes could be observed (Gottifredi et al. 2001). These findings show that not only does p53 seem to be able to differentiate between various kinds of DNA damage; it also seems to act upon them differently, thus protecting genomic integrity.

Embracing its complex role, p53 is capable of promoting HR under very specific circumstances (Yun, Lie-A-Cheong, and Porter 2004). This aspect is further elaborated in this study. Findings in this work further indicate a repair promoting effect of transactivation-inactive p53 in response to the DNA cross-linker Mitomycin C. This effect is not necessarily transferable to other cross-linking agents though, as Thymidine treatment yielded different results. In fact, Mitomycin C and Thymidine both cause stalled replication forks through different mechanisms. Mitomycin C actively damages the DNA and causes ICLs, which stop the replication physically. Thymidine on the other hand depletes nucleotides, which are indispensable for an ongoing replication, leading to a replication stop through a lack of components.

The results further show an opposing influence of p53 on different kinds of replication blocks. The different outcomes underline the complexity of how p53 launches cellular responses to DNA damage. p53-QS was able to inhibit HR in response to Thymidine treatment. Findings in Figure 4 indicate that RPA binding, as

well as phosphorylation of p53 at S15 by ATR, are required for this inhibition of HR. A similar inhibiting effect is not present after Mitomycin C treatment in any tested p53 variants. Especially H1299 cells expressing p53-S15A and p53-QM showed higher Rad51 foci levels than H1299 p53-null and H1299 p53-QS cells in response to Mitomycin C. This implies a higher HR activity in H1299 p53-S15A and p53-QM, due to a possible HR promoting effect of these p53 variants. Therefore, p53-S15A and p53-QM might act as gain-of-function mutants.

There are countless p53 mutations that lead to an inactivated or impaired p53 protein and thus to a loss of function. It is also possible, that a mutation results in increased efficiency or empowers the protein to additional functions. Several types of cancer express mutated p53 that not only has lost its tumor suppressing features but also promotes tumorigenesis and drug-resistance through newly gained functions (Xu 2008; Blandino, Levine, and Oren 1999). H1299 cells expressing the transactivation-inactive p53 mutants became more repair proficient in response to cross-linker Mitomycin C opposed to H1299 p53-null cells. Here it is particularly notable that the p53 mutations used are transactivation-inactive. The hypothetical wild type p53 protein most likely stops the repair of an extensively damaged cell to protect genomic stability. Other studies have shown similar results in response to other cross-linkers like Etoposide or Cisplatin (Blandino, Levine, and Oren 1999).

Clonogenic survival of Mitomycin C-treated H1299 cells, shown in Figure 8, suggests a survival improving effect of p53 mutants QM, QS-S15A and QS compared to p53-null and p53-237. A possible reason for improved survival might be an increase in HR levels, which in turn implies up-regulated repair. Aside from the survival improving effect of transactivation-inactive p53, differences in terms of degree between the p53 variants emerged. H1299 cells expressing p53-S15A and p53-QM were most resistant to Mitomycin C. p53 binds to RPA to inhibit HR, so it is reasonable to assume that HR levels rise when the binding is abrogated (Dutta et al. 1993; Romanova et al. 2004). In response to Thymidine treatment the Rad51 foci levels in p53-QM expressing cells were similar to p53-null cells (Figure 4). In response to Mitomycin C treatment on the other hand, p53-QM expressing cells showed elevated Rad51 foci levels compared to p53-null cells (Figure 5). How p53 differentiates between the two agents and how exactly p53-S15A and p53-QM may promote Rad51 activity needs further experimental investigation.

The results suggest a connection between p53 status, Rad51 levels, FANCD2

activity and Mitomycin C resistance. A possibility for transactivation-inactive p53 to promote the repair of stalled replication forks could be an interaction with the FA/BRCA pathway. The FA proteins detect and stabilize ICLs caused by Mitomycin C. These steps are crucial for sufficient repair. Given their importance, they appear to be reasonable targets for HR regulation, as for example HR up-regulation through p53. The central step of FA/BRCA pathway activation is the mono-ubiquitylation of FANCD2. Studies have shown that FANCD2 mono-ubiquitylation is inducible by IR or Mitomycin C. Mono-ubiquitylated FANCD2 acts together with mono-ubiquitylated FANCI, building the ID complex. This complex is the central element of the active FA/BRCA pathway (X. Wang, Andreassen, and Andrea 2004; Smogorzewska et al. 2007). How exactly it supports the repair process is still enigmatic, but many theories are discussed. Knipscheer et al. for example suggested, that the ID complex stabilizes forks through activation of further proteins like FANCP and also promotes the TLS repair pathway, which is needed for sufficient ICL repair (Knipscheer et al. 2009).

FANCD2 foci levels in response to Mitomycin C were surprisingly low in all H1299 cell lines (Figure 7). These findings were consistent with prior results from our lab (Natalie Ferraiolo, unpublished), which indicate that H1299 cells have one of the lowest FANCD2 foci induction levels in response to the cross-linking agent Cisplatin, compared to other NSCLC cell lines. Even though the foci induction levels were very low, distinct differences depending on the p53 status could be noted. H1299 cells expressing p53-QM or p53-QS showed higher FANCD2 activity compared to cells expressing p53-null or p53-237 (Figure 7). p53-237 is mutated at the core and thus mostly inoperable. The inability of H1299 p53-237 cells to form any FANCD2 foci suggests that the DNA binding core domain of p53 must be essential for the activation of the FA/BRCA pathway. p53-QM expressing cells had the highest FANCD2 foci levels as well as the highest Rad51 foci induction, indicating higher HR activity. Accordingly, FANCD2 western blots showed the brightest bands in H1299 p53-QM cells (Figure 9). H1299 cells expressing p53-QM were also the most resistant to Mitomycin C, supporting the hypothesis of p53-QM being a HR promoting actor in response to Mitomycin C.

As a limitation of these results, it remains questionable to what extend the small amount of active FANCD-ub was capable to have any effect on repair and survival. Additionally, the observations made here were drawn from just one cell line

and might not be transferable to other cell lines. A parallel control cell system would have been useful for excluding cell line specific variations in DNA repair. A cell line should be studied that is FANCD2 proficient and allows the comparison of different p53 variants. Several publications have shown that it is common for some tumor cells to have promoter methylations in genes of the FA group and thus a compromised FA/BRCA pathway. A study by Marsit et al. showed a high rate of FANCF methylations in NSCLC tissues but could not reproduce these results in cell lines yet (Marsit et al. 2004). It has been shown before that FANCF deficiency can lead to a lack of FANCD2 foci formation, or, the other way around, FANCF over-expression is able to restore FANCD2 mono-ubiquitylation (Taniguchi et al. 2003; van der Heijden, Brody, and Kern 2004).

FANCF western blots (Figure 11.2) suggested that H1299 cells could be FANCD2 deficient as a consequence of the lack of FANCF expression. In support of these findings, H1299 cells were found to have two times lower FANCF expression levels than the average of NSCLC cell lines. Since FANCF is part of the FA core complex, which is needed to activate FANCD2, its absence could be an explanation for low FANCD2-ub activity in H1299 cells. H1299 cells, however, are resistant to Mitomycin C as well as to Cisplatin, despite their low levels of FANCD2 foci induction and apparent FANCF deficiency (Figure 8).

These findings stand in contrast to many studies, showing consistently that one of the main characteristics of FA deficient cells is their cross-linker hypersensitivity (Koomen et al. 2002; Sasaki 1975; Sasaki and Tonomura 1973). Since mono-ubiquitylation of FANCD2 by the FA core complex is the central element of the active FA/BRCA pathway, it would be a possible target for regulation by p53. There has been no evidence for a direct interaction of p53 with the proteins of the FA pathway so far, but especially the key protein FANCD2 and p53 share many binding partners (as BRCA1, BRCA2, Rad51, ɣ-H2AX, BLM), suggesting that a direct or indirect influence of p53 on FANCD2 is possible. In the contrary, a recent publication by Rego et al. indicates a strong influence of p21 on the FA/BRCA pathway and finds no impact of p53 on the FANCD2/FANCI mono-ubiquitylation process (Rego et al. 2012). p21 is a downstream actor of p53, activated through transcription. Since this study focused on transactivation-inactive p53, the promotion of the FA/BRCA pathway by p53 through p21 in the experiments here is unlikely. Interestingly though, p21 seems to be emerging as an important factor in repair of stalled replication forks.

Several studies found that p53 can promote Cisplatin resistance through activation of p21 (Fan et al. 1997; Fan et al. 1995; Hawkins, Demers, and Galloway 1996).

Even though foci studies are well established and widely used, their informative value is limited. Foci are insufficient to visualize the protein activity directly, but can give reliable hints and show trends for molecular mechanisms (Bhattacharyya et al. 2000; J. Zhang et al. 2004; Sørensen et al. 2005). For a better understanding of the FANCD2 mono-ubiquitylation process, FANCD2 western blots were conducted (Figure 9). FANCD2 is visualized in the mono-ubiquitylated and non-ubiquitylated form. The results support the proposal that p53 increases FANCD2 mono-ubiquitylation and thus up-regulates HR leading to improved survival. H1299 cells expressing p53-Q showed the brightest bands for FANCD-ub in response to Mitomycin C. These findings are in line with results in Figure 7, showing the highest FANCD2 foci levels (representing mono-ubiquitylated FANCD2) in p53-QM expressing cells. The western blots also support the impression of generally low FANCD2-ub concentrations in H1299 cells, since the bands are rather weak. However, FANCD-ub concentration seems to be connected to the p53 status. Despite the positive correlation between p53 and FANCD2 activity, a proof for direct physical interaction is still missing. Further investigations, as for example immuno-precipitation experiments would probably give an answer to that question.

siRNA depletion of FANCD2 (Figure 10) suggested that the survival promoting mechanism in H1299 cells is at least partly FANCD2 dependent, as cells depleted of FANCD2 became more sensitive to Mitomycin C. However, this effect could be seen in p53-QM expressing as well as in p53-null cells. Additionally, the presence of p53-QM seemed to have a survival promoting effect independently of FANCD2 status. It remains unclear how exactly these proteins promote survival and if they can act synergistically.

Not only FANCD2 but many other FA proteins were found to be involved in the repair response following DNA damage caused by cross-linkers. Taking a closer look at those, as for example; FANCD1, FANCJ, FANCE, FAN1, FA-AP100 and FA-AP24 and the evaluation of possible interactions with p53 could give new insight into how Mitomycin C resistance evolves (Ling et al. 2007; Ciccia et al. 2007; Cantor et al. 2001; Litman et al. 2005; MacKay et al. 2010; X. Wang et al. 2007). It has to be considered that FANCD2 likely has a more complex role in response to ICL causing agents, which might involve mono-ubiquitylation independent activity. Since FANCD2

foci only visualize mono-ubiquitylated FANCD2, this study fails to measure all aspects of FANCD2 activity. Previous studies showed that FANCD2 foci studies fail to visualize FANCD2 activity completely (Willers et al. 2008). Therefore, the actual FANCD2 activity might be higher than what was found in this work through conduction of foci studies and western blots.

One of the mono-ubiquitylation independent actions of FANCD2 is its function within a complex consisting of FANCD1, FANCG and XRCC3. This complex assembles independently from ID mono-ubiquitylation through the core complex but is also involved in replication fork restart (Wilson et al. 2008; Wilson et al. 2010). FANCD2 was found to promote resistance towards Topoisomerase II poisons, as for example Etoposide, independently from mono-ubiquitylation and the core complex (Kachnic et al. 2011).

Another notable aspect is the varying response of FANCD2 to different cross-linking agents, as for example Cisplatin/Oxaliplatin and Mitomycin C. Variation in dosage and exposure time of the agents also led to different outcomes (Kachnic et al. 2010). The molecular details remain unclear. Some actions of FANCD2, as for example the activation of an IR-inducible S-phase checkpoint, are rather dependent on phosphorylation and not mono-ubiquitylation (Smogorzewska et al. 2007; Taniguchi, Garcia-Higuera, Xu, et al. 2002). FA proteins FANCD1 (BRCA2) and FANCJ were found to be important for Mitomycin C resistance, as deficient cells have strongly reduced HR levels (Cantor et al. 2001; Litman et al. 2005). These two proteins are involved in formation of another FA complex, acting downstream of the ID complex. It consists of FANCJ (BACH1, BRIP1), FANCD1 (BRCA2) and FANCN (PALB2). FANCJ is a DNA helicase, binds to BRCA1 and contributes to BRCA1 function in DNA repair (Cantor et al. 2004). FANCJ is indispensable for cell cycle progression after ICL damage and ICL repair response, independently from the interaction with BRCA1 (Peng et al. 2007). A FANCD2-ub independent but cross-linker resistance promoting mechanism might be excecuted by phosphorylated FANCE. FANCE is required for Mitomycin C resistance, while disposable for DNA replication, normal cell cycle progression or FANCD2 mono-ubiquitylation (Q. Liu et al. 2000; X. Wang et al. 2007).

Besides all these interesting approaches of how the FA/BRCA network is able to promote ICL repair, it remains unclear how H1299 cells achieve their resistance towards cross-linking agents. A recent publication includes H1299 cells in a group

that is called “tumor initiating cells” (TICs). TICs were found to have characteristics of stem cells and are especially resistant to DNA-damaging agents. They show differences in their DDR, cell cycle checkpoint and apoptosis management and form spheres, leading to remarkable therapy resistance. Additional characteristics of TICs were diminished FANCD2 mono-ubiquitylation and ATM activity (Lundholm et al. 2013). These characteristics correlate with the findings in this work regarding H1299 cells. FANCD2 foci induction was low in response to Mitomycin C treatment as well as western blots showed weak FANCD2-ub bands. H1299 had significantly lower pATM levels than Calu-6 cells upon treatment with Mitomycin C. Not only do the low pATM levels stand in line with the hypothesis of H1299 cells belonging in the group of TICs, they also correlate with theories proposed by other studies, stating the importance of ATR and not ATM in response to DNA damage caused by cross-linking agents (Flynn and Zou 2011; Zou, Willers, and Pfaeffle 2012; Ward and Chen 2001).

The western blot shown in Figure 12 indicates high levels of Rad51 in H1299 cells, compared to other repair proficient NSCLC cell lines. Several publications found correlations between Rad51 over-expression and resistance to DNA-damaging agents. Presence of Rad51 has been shown to promote Mitomycin C, Cisplatin and IR resistance. One possible mechanism could be Rad51’s ability to restart stalled replication forks before they collapse into DSBs. It remains to be elucidated if H1299 are able to recover more stalled forks than cells expressing lower Rad51 levels (Petermann et al. 2010). On the other hand, Rad51 over-expression and persisting Rad51 foci can be hints for repair deficiency (Martin et al. 2007; Klein 2008). Repair deficiency would likely result in drug-sensitivity, which is not the case in H1299 cells. More experiments to exactly determine Rad51 levels and activity in H1299 cells are needed for further investigation in this direction.

6. Summary

In order to fully exploit cancer treatment possibilities, it is crucial to know exactly how DNA repair works. The concept of targeting DNA repair mechanisms has led to many advances in current therapeutic possibilities. Both p53 and the FA/BRCA pathway are recently trending targets for anti-cancer therapy (Han et al. 2015; Gurpinar and Vousden 2015). A strong correlation has been found between the FA/BRCA pathway and Cisplatin resistance for example, as an inhibition of this pathway leads to sensitization of Cisplatin resistant cells (Chirnomas et al. 2006). These experiments therefore reveal additional pieces in the puzzle of DNA repair mechanisms. It is becoming clear that p53 has an enormous range of functions through which it adapts to environmental factors. DNA damage as the occurrence of DSBs or stalled replication forks are collective terms for a wide variety of scenarios. This study aims to elucidate how transactivation-inactive p53 impacts HR and the FA/BRCA pathway upon the use of DNA cross-linking drugs.

The two drugs that were used, Thymidine and Mitomycin C, led to subtly different results. These results again varied with the use of different p53 mutants. p53-QS seemed to inhibit HR in response to Thymidine while having no effect upon Mitomycin C treatment. p53-S15A and p53-QM seemed to promote HR after Mitomycin C treatment and thus act as gain-of-function mutants. Overall, p53 status could be linked to HR activity level, FANCD2 mono-ubiquitylation and improved post-treatment survival in H1299 NSCLC cells.

Additionally, this study showed novel characteristics of the widely used H1299 cell system. Contrary to prior expectations, H1299 cells were found to be FANCF deficient and showed very low levels of mono-ubiquitylated FANCD2. Given the Mitomycin C resistant nature of this cell line, these findings were surprisingly contradictory, since one would expect FA deficient cells to be sensitive to DNA cross-linkers. Mitomycin C resistance was dependent on p53 status and remaining FANCD2 activity. Findings imply that p53 can interact with the FA/BRCA pathway in HR regulation after treatment with cross-linking agents in a repair-promoting manner. Taken together, the findings might lead to further insight into how tumor cells gain resistance to DNA cross-linkers and help in finding novel targets and strategies for individualized cancer therapy.

7. Summary (german)

Um alle Möglichkeiten der Tumortherapie vollkommen ausschöpfen zu können, ist es unumgänglich, die Prinzipien der DNA-Reparaturmechanismen genau zu verstehen. Das Konzept, DNA-Reparatur als ein Angriffsziel in der Krebstherapie zu sehen, hat zu großen Fortschritten bezüglich der Therapiemöglichkeiten geführt. Sowohl p53 als auch der FA/BRCA Signalweg spielen eine wachsende Rolle in individualisierten Therapiekonzepten. Es besteht zum Beispiel eine starke Korrelation zwischen dem FA/BRCA Signalweg und Cisplatin-Resistenz. Es konnte gezeigt werden, dass eine Hemmung des Signalweges zu einer Sensitivierung von zuvor Cisplatin-resistenten Zellen geführt hat. Diese Arbeit konnte neue Teile des großen Puzzles der DNA-Reparaturmechanismen aufzeigen. Es wird klar, dass p53 eine enorme Zahl an Funktionen erfüllt, welche es an die jeweilige Situation anpasst. DNA-Schädigung wie Doppelstrangbrüche oder angehaltene Replikationsgabeln können durch grundverschiedene Szenarien entstehen. Ein Ziel dieser Arbeit war es, neue Hinweise zu erlangen, wie genau transaktivierungs-inaktives p53 die Homologe Rekombination und den FA/BRCA Signalweg nach Behandlung mit DNA-vernetzenden Stoffen beeinflusst.

Die Verwendung der zwei DNA-vernetzenden Stoffen, Thymidin und Mitomycin C, führte zu unterschiedlichen Resultaten. Diese wurden wiederum durch die Kombination mit unterschiedlichen p53-Mutanten beeinflusst. p53-QS schien die HR nach Thymidin-Behandlung zu hemmen, während nach Behandlung mit Mitomycin C kein Effekt gesehen werden konnte. p53-S15A und p53-QM schienen die HR nach Mitomycin C-Behandlung heraufzuregulieren und somit als “gain-of-function” Mutanten zu agieren. Der p53-Status stand in Zusammenhang mit Aktivität der HR, Ubiquitinierung von FANCD2 und Überleben der H1299 NSCLC Zellen nach Behandlung mit DNA-vernetzenden Substanzen.

Außerdem zeigten die Ergebnisse neue Charakteristika des viel genutzten H1299 Zellsystems. Entgegen der Erwartungen waren H1299 Zellen FANCF-defizient und zeigten niedrige Level von ubiquitiniertem FANCD2. Angesichts der Mitomycin C-Resistenz der H1299 Zellen waren diese Ergebnisse eher überraschend, da eine FA defiziente Zelllinie erwartungsgemäß hochsensitiv gegenüber DNA-vernetzenden Substanzen wäre. Die Ausprägung der Mitomycin C-Resistenz war abhängig von dem jeweiligen p53-Status und der verbleibenden

FANCD2-Aktivität. Die Ergebnisse implizieren eine Interaktion zwischen p53 und dem FA/BRCA Signalweg im Sinne einer HR Hochregulation nach Behandlung mit DNA-vernetzenden Substanzen. Zusammenfassend liefern die Resultate neue Einsichten über die Entwicklung von Resistenzen gegenüber DNA-vernetzenden Stoffen in Tumorzellen und können helfen, neue Ansatzpunkte und Strategien für individualisierte Tumortherapie zu finden.

8. List of Abbreviations

AML	Acute myeloid leukemia	**ICL**	Interstrand cross-link
ATM	Ataxia telangiectasia mutated	**IR**	Ionizing radiation
ATR	Ataxia telangiectasia and Rad3 related	**LOH**	Loss of heterozygosity
BAX	Bcl-2-associated X protein	**MDM2**	Mouse double-minute protein 2
BER	Base excision repair	**MMC**	Mitomycin C
BLM	Bloom syndrome/Bloom syndrome protein	**MMR**	Mismatch repair
BRCA1	Breast cancer 1	**mRNA**	Messenger RNA
BRCA2	Breast cancer 2	**NBS1**	Nijmegen breakage syndrome protein 1
Chk1	Checkpoint kinase 1	**NER**	Nucleotide excision repair
Chk2	Checkpoint kinase 2	**NHEJ**	Non-homologous end joining
DDR	DNA damage response	**NSCLC**	Non-small-cell lung carcinoma
DNA	Deoxyribonucleic acid	**PCNA**	Proliferating cell nuclear antigen
DNA-PK	DNA-dependent protein-kinase	**PCR**	Polymerase chain reaction
dCTP	Deoxycytidine triphosphate	**PIKK**	Phosphatidyl inositol 3‘ kinase-related kinases
dNTP	Deoxyribonucleotide triphosphate	**RNA**	Ribonucleic acid
DSB	Double strand break	**RPA**	Replication protein A
dsDNA	Double-stranded DNA	**RTS**	Rothmund–Thomson syndrome
FA	Fanconi anemia	**SCE**	Sister chromatide exchange
FA/BRCA pathway	Fanconi anemia and BRCA pathway	**SDSA**	Synthesis-dependent strand annealing
FAN1	Fanconi anemia associated nuclease	**ssDNA**	Single-stranded DNA
GADD45	Growth arrest and DNA-damage inducible gene a45	**TdR**	Thymidine
HNSCC	Head and neck squamous cell carcinomas	**TIC**	Tumor-initiating cells
HJ	Holliday junction	**TLS**	Translesion synthesis
HR	Homologous recombination	**UV**	Ultraviolet radiation
HRP	Horseradish peroxidase	**WRN**	Werner syndrome protein
HU	Hydroxyurea	**WS**	Werner syndrome

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Stipendien

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12. Statutory declaration/Eidesstattliche Versicherung

Ich versichere ausdrücklich, dass ich die Arbeit selbständig und ohne fremde Hilfe verfasst, andere als die von mir angegebenen Quellen und Hilfsmittel nicht benutzt und die aus den benutzten Werken wörtlich oder inhaltlich entnommenen Stellen einzeln nach Ausgabe (Auflage und Jahr des Erscheinens), Band und Seite des benutzten Werkes kenntlich gemacht habe.
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