DNA damage signaling networks: from stem cells to cancer

Carreras Puigvert, J.

Chapter 1

General introduction

1. DNA damage, an overview

Genome instability is the source of many malignancies, ranging from developmental diseases to cancer, as well as aging. Every cell of the human body deals with thousands of exogenous and endogenous insults that can generate DNA lesions, daily. In order to maintain their genomic stability to ultimately deliver their genetic material unharmed, cells have developed a tightly orchestrated DNA damage response (DDR), which allows them to prevent the transmission of unwanted modifications in the genome (Jackson et al. 2009).

There are several types of DNA lesions that can be generated by a great diversity of exogenous agents, as well as endogenous metabolic intermediates. In order to repair these lesions, a complex recognition and response mechanism has evolved, including several DNA damage repair mechanisms. The DNA damage response consists of a network of interacting pathways that involves sensors, mediators, transducers and effectors. The amplitude and duration of these signal transduction pathways ultimately determines the response that may involve repair of the damage, or, if the damage is too severe, programmed cell death or senescence. Although repair strategies are specifically designed to target distinct types of damage, these mechanisms also operate collectively and share many components (Hoeijmakers 2009).

DNA lesions have mainly two sources of origin, endogenous and exogenous. The endogenous DNA damage is generated by the interaction of the DNA with several molecules present in the cell. The DNA is in an aqueous medium and therefore it reacts with water continuously. When this reaction occurs, hydrolysis takes place resulting in depurination, depyrimidation, and deamination of cytosine as well as 5-methylcytosine. Oxidation reactions within the cell result in the formation of oxygen free radicals that can react with the DNA and lead to base lesions such as 7,8-dihydro-8oxoguanine (8-oxo-G), ring-saturated pyrimidines and lipid peroxidation products. Finally in the replication process of the DNA, errors during the incorporation of new bases as well as the incorporation of damaged nucleotide precursors can create mismatches. In summary, the endogenous DNA damage can induce severe lesions in the DNA that can lead to mutations, which, if not properly repaired, can lead to malignant transformation or cell death (Friedberg et al. 2009).

The second main cause of DNA lesions is derived from exogenous or environmental sources. The human body is daily exposed to agents that can cause DNA damage. These agents are ionizing radiation, which can create lesions directly in the DNA molecules, or indirectly by generating reactive intermediates that target DNA. Additionally, ultraviolet radiation creates photoproducts, which lead to crosslinking between bases such as cyclobutane pyrimidine dimers, as well as cross-linking between DNA molecules to proteins. There are also various chemical agents that can induce DNA alterations. Among the interactions of chemicals with the DNA, the ones that are most harmful are alkylating reactions, which add an alkyl group to the DNA generating DNA adducts. Cross-linking reactions are produced by

bifunctional alkylating agents that as a result can create links between bases of the same strand of DNA (intrastrand) or from one strand to the opposite one (interstrand). Alkylation of the DNA can also lead to single strand breaks (SSBs), which in turn can become double strand breaks (DSBs) during the replication process due to uncoupled strands at the replication fork. These alkylating agents can also induce DNA-protein cross-links. Chemicals that cause DNA adducts, such as alkylating agents and cross-linking agents, form the basis for many chemotherapeutic anticancer drugs. An alternative strategy used successfully to cause DNA damage in cancer cells, involves targeting the enzymes

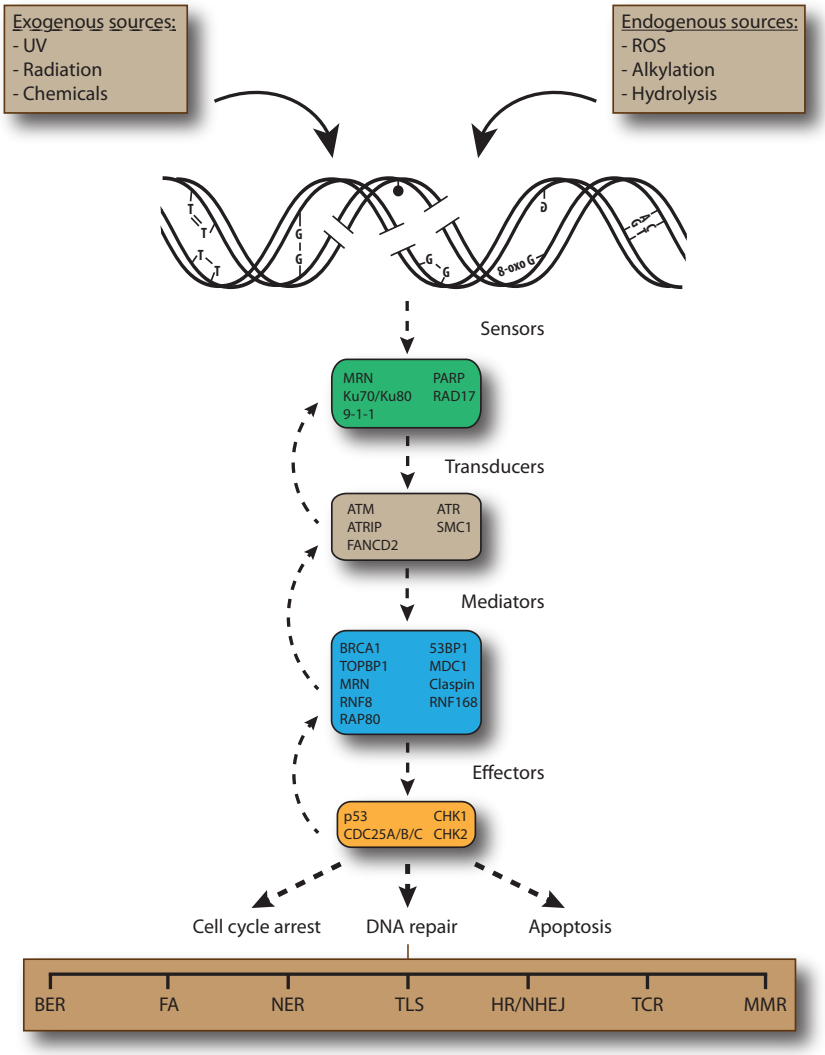

Figure 1. The DNA Damage: Sources, Response and Repair. DNA lesions can be induced from either exo- or endogenous sources. When the damage is detected, a cascade-like response involving sensors, transducers, mediators and effectors, is triggered. If the damage can be repaired, depending on the lesion, different DNA repair mechanisms are activated.

that normally regulate proper DNA replication. For instance, topoisomerase inhibitors prevent closing of DNA breaks that are generated by topoisomerases (Friedberg et al. 2009).

2. Single and double strand breaks

SSB and DSB activate distinct DDR pathways mediated by specialized DDR proteins from the phosphatidylinositol 3-kinase-like protein kinase (PIKK) family, as well as by members of the poly (ADP-ribose) polymerase (PARP) family. In the case of DSBs, the response is mediated by the ataxia-telangiectasia mutated protein kinase (ATM), the DNA-dependent protein kinase catalytic subunit (DNA-PKcs) and PARP; and the response to SSBs is mediated by the ataxia-telangiectasia mutated and Rad3-related protein kinase (ATR) as well as PARP (Falck, Coates, & Jackson 2005, Ciccia & Elledge 2010).

Double strand breaks (DSBs) and ATM activation: the sensing of DSBs occurs through the MRN (Mre11-Rad50-Nbs1) mediator complex, which is recruited by the phosphorylated histone H2AX (γH2AX), also involved in the recruitment of Rad51 and BRCA1, upon DNA damage (Celeste et al. 2002). The MRN complex binds to the DSB; unwinds the ends and recruits the transducer protein kinase ATM. Through an incompletely understood mechanism, inactive ATM dimers are then dissociated, resulting in the accumulation of monomeric active ATM molecules (Bakkenist et al. 2003; J.-H. Lee et al. 2005).

ATM phosphorylates a large variety of targets; amongst them is the tumor suppressor transcription factor p53, enhancing its function as transcription factor (Banin 1998). ATM also phosphorylates checkpoint kinase 2 (Chk2) and the negative regulator of p53, the E3 ubiquitin-protein ligase MDM2, which, in a non-stress status promotes the proteasome-mediated degradation of p53 by ubiquitination. Chk2 in turn phosphorylates p53 which interferes with the feedback loop regulation of p53 by MDM2 (Khosravi et al. 1999; Bartek et al. 2001; Ryan et al. 2001; Ruth Maya et al. 2001). P53 activation and stabilization leads to increased p21 transcription, which inhibits cyclin dependent kinase 2 (CDK2), disturbing the G1 to S phase transition, resulting in reduced proliferation (Boutros et al. 2006). Transcription of BAX, which is a Bcl-2 pro-apoptotic family member and target gene of p53, is also increased and in turn induces apoptosis (Yosef Shiloh 2003).

Single strand breaks (SSBs) and ATR activation: When SSBs are formed at stalled replication forks, they are bound by the ssDNA binding protein complex RPA which recruits ATR via its regulatory subunit ATR-interacting protein (ATRIP) (Zou et al. 2003; Cortez et al. 2001). However, in order for ATR to be activated, it requires the presence of the 9-1-1 complex (Rad9-Hus1-Rad1) that clamps onto the chromatin and is loaded onto the DNA by Rad17, allowing the phosphorylation of Chk1 by ATR. This process is similar to the one carried out by the proliferating cell nuclear antigen (PCNA), which is a clamp structurally similar to the 9-1-1 complex, and is loaded onto the DNA by the replication factor pl40-RFC during DNA replication (Parrilla-Castellar et al. 2004). The 9-1-1 complex recruits the

ATR activator topoisomerase binding protein 1 (TOPBP1) (J. Lee et al. 2007). Once ATR is activated it phosphorylates its substrates, including checkpoint kinase 1 (Chk1), which amplifies the DNA damage signaling (Walworth 1996). An important target of Chk1 is CDC25, which controls cell cycle transitions by dephosphorylating cyclin dependent kinases (CDKs) (Boutros et al. 2006). Chk1 inhibits CDC25 by phosphorylation, and consequently CDK activation is abrogated (Sanchez 1997), preventing the cells to enter mitosis, and allowing the repair of the damaged DNA.

3. Repairing the damage

The DNA damage response -or DDR- consists of several DNA repair pathways that contribute to the sensing of the lesions, signaling of the damage and ultimately their repair. Different types of DNA lesions require different types of repair mechanisms:

Base excision repair pathways serve to remove small DNA lesions like base alterations, which do not interfere with the helix, but might if not repaired lead to mutations. An – in most cases monofunctional - DNA glycosylase catalyzes the hydrolytic removal of the altered base, creating AP (apurinic/ apyrimidinic) sites. These AP sites can be recognized by an AP exonuclease that cuts the sugar backbone. One distinguishes two types of BER; in the short patch repair pathway only a single nucleotide is replaced; while in the long patch repair 2-10 new nucleotides are being synthesized (Fortini et al. 2010).

Homologous recombination (HR) and non homologous end joining (NHEJ) are activated when Double Strand Breaks - DSBs - occur. DSBs are the most deleterious DNA lesions; they can either be induced by the blockage of replication forks by other types of lesions or directly by ionizing radiation. The decision for one (HR) or the other (NHEJ) pathway is dependent on the organism, cell type, cell cycle status and the way the DSB was induced. While NHEJ is error prone and functions throughout the cell cycle, HR requires a homologous template and functions only in S and G2 phase where a sister chromatid is present. IR induced DSBs can be repaired by either NHEJ or HR; DSBs which arise from stalled replication forks are exclusively repaired by HR (Shrivastav et al. 2008).

In the NHEJ pathway the two ends of a DSB are ligated together. End binding involves the MRN complex which also functions in HR, as well as the Ku70-Ku80 protein heterodimer that forms a complex with DNA-PKcs. If required the DNA ends have to be processed, involving Artemis nuclease. The ligation requires the presence of DNA-PK that facilitates the recruitment of the DNA ligase 4- XRCC4 complex.

Homologous recombination is composed of 3 steps. During presynapsis the double strand break is sensed by the MRN complex and the DSB is processed to create a 3' overhang. Single stranded DNA is covered by RPA which is subsequently replaced by Rad51, a process that is facilitated by BRCA2. During synapsis Rad51 mediates homology search and strand invasion, with the help of different mediator proteins including five human Rad51 homologues. In the presynapsis the

junction is resolved involving different types of helicases, like BLM (Li and Heyer 2008). Mouse embryonic stem cells have been shown to have very low levels of DNA-PK, hence in this cell type HR is the predominant pathway for DSB repair (Bañuelos et al. 2008).

DNA-dependent protein kinase (DNA-PK) is activated upon DSBs induced by ionizing radiation or V(D)J recombination, and it is involved in the NHEJ DNA damage repair mechanism (Smith et al. 1999). DNA-PK contains a catalytic subunit (DNA-PKcs) as well as an accessory protein, the Ku antigen, which is a heterodimer composed by the units Ku70 and Ku80 (Gottlieb et al. 1993). The Ku heterodimer acts as sensor of DSBs and recruits the DNA-PKc subunit, which once at the damaged site, binds to the DNA and repairs the broken ends by joining them (DeFazio et al. 2002).

The poly (ADP-ribose) polymerase (PARP) family consists of 16 members, from which PARP1 and PARP2 have been implicated in the sensing of DNA damage (Schreiber et al. 2006) as well as in repair mechanisms such as Homologous Recombination (HR) (Hochegger et al. 2006) and Non Homologous End Joining (NHEJ) (M. Wang et al. 2006). PARP1 is involved in the recruitment of the MRN complex at the DSB sites, independently from the phosphorylation of histone H2AX (Haince et al. 2008). It is also involved in the recruitment of ATM, which contributes to the activation of γH2AX, playing an important role in the stabilization of the DDR complex (Haince et al. 2007).

Mismatch repair is a strand specific repair mechanism with the general function to correct base mismatches that occur during DNA replication. Besides that, mismatch repair proteins participate in a variety of other DNA transactions that involve heteroduplex intermediates as well as recombination. In higher eukaryotes, complexes of MutS homologues bind to the base mismatch region. The MutL homolog MLH1-hPMS2 is a matchmaker that coordinates the mismatch binding and recruitment of the factors for repair synthesis, like RFC (the replication clamp loader), PCNA (the replication clamp), RPA (the single-stranded-DNA-binding protein), exonuclease I (which hydrolyzes the 5'-ended strand in double-stranded DNA) and DNA polymerase delta (Kunkel et al. 2005). The efficiency of certain genotoxicants such as cisplatin or carboplatin, has been shown to rely on a functional *Mismatch repair* machinery. The DNA lesions produced by these drugs are processed into toxic lesions upon the activation of this repair mechanism, by which newly placed bases are removed instead of the damaged ones, therefore initiating rounds of futile repair that will ultimately lead to cell death (Helleday et al. 2008).

Nucleotide excision repair (NER) is involved in the removal of bulky DNA lesions, like those caused by the reaction with benzopyrene, UV-induced DNA damage like thymine dimers and 6-4-photoproducts, and lesions caused by DNA cross linking agents. NER is sub classified into two different types that share a common core pathway, but differ in the way the damage is sensed. *Global genome repair (GGR)* functions both in

the transcribed and the untranscribed strand and does not require the gene in which the damage occurs to be active. This pathway needs DNA damage sensor proteins XPC-Rad23B that are able to recognize helix distortions, and it is enabled by the DNA binding complex (DDB), formed by DDB1 (p125) and DDB2 (XPE), which binds and bends the DNA making the lesion suitable for GG-NER processing.

Transcription coupled repair (TCR) relies on the sensing of DNA damage by RNA Polymerase, which becomes stalled at bulky lesions. The TCR-NER proteins CSA and CSB facilitate the recruitment of factors of the core pathway. In the core pathway the 10 subunit transcription factor TFIIH is recruited to the region of damage, containing the helicases XPB and XPD which open the DNA; XPA, which is required for positioning the single strand binding protein RPA; and the ERCC1/ XPF endonuclease that cuts the 5' and the 3' end of the lesion. To fill the gap, the intact strand is used as a template for repair and replication requires PCNA, the clamp loader complex RFC and DNA Polymerase δ and ε. Finally, ligation of the nick is performed by DNA Ligase I. Many of the proteins involved in NER have other functions: TFIIH has been associated with general transcription and the endonuclease ERCC1 is important for the removal of DNA crosslinks (Andressoo et al. 2005).

The Fanconi anemia (FA) pathway is named after a rare genetic disease including 15 complementary groups which are associated with the 15 FA proteins (FANCA, B, C, D1, D2, E, F, G, I, J, L, M, N, O & P). A key phenotype of FA is an exceptionally high sensitivity to crosslinking agents. The FA proteins interact with proteins from the HR and the translesion synthesis pathways and have been shown to associate into a supercomplex together with the Bloom syndrome protein (BLM) complex (Blm, Topo IIIα, Blap75, Rpa70, Rpa34, Rpa14 and Blap 250). In the FA core complex, 8 of the FA proteins form a nuclear E3 ubiquitin ligase, which monoubiquitinates FANCI and FANCD2. The FA genes, FANCD1, N and J are identical to the breast cancer susceptibility genes BRCA2, PALB2 and BRIP1, indicating a close relation between the HR pathway in which BRCA2 functions and the FA pathway (W. Wang 2007).

Translesion DNA synthesis (TLS) is a DNA damage tolerance mechanism in which DNA replication is carried on across regions of DNA damage by switching from regular DNA polymerase to special translesion (often low fidelity) polymerases. Some of these polymerases belong to the Y-family, like Polη, Polκ, Polι and REV1. The switch from normal polymerases to translesion polymerases is being mediated by the ubiquitination status of PCNA. In order for the different TLS polymerases to access the stalled replication lesion, PCNA is required given its function as scaffold. PCNA monoubiquitination is mediated by Rad18, which is in turn a target of RPA that is activated upon DNA damage. The monoubiquitination of PCNA allows the removal of replication polymerases and the recruitment of TLS polymerases (Guo et al. 2009).

4. Defects in DNA damage repair pathways – a cause of cancer and premature aging

A deficiency in the repair mechanisms described above can lead to several syndromes that are characterized in general by hyper sensitization to DNA damaging agents or inefficient repair of endogenously caused DNA lesions (Hoeijmakers 2009).

Xeroderma Pigmentosum (XP): defects in either NER or Translesion DNA synthesis (TLS) cause extreme sensitivity to UV radiation. Patients develop skin cancer at a higher rate than the normal population. These patients present high sensitive to DNA damage agents, they have large accumulation of mutations in their genome, and they are prone to develop internal tumors. The accumulation of cancer cells that escape cell death makes this a cancer syndrome rather than an aging one.

Cockayne syndrome (CS): transmitted in an autosomal recessive mode, this disease is caused by a deficient NER mechanism. As a result, cells from these patients present high sensitivity to UV radiation as well as to bulky chemicals that interact with the DNA. Consequently, patients suffer from growth - and developmental arrest, neurological disorders and premature aging. CS has a small effect on generation of mutations, the cells that carry this deficient repair mechanism (Cockayne cells) tend to die prematurely rather than transmit their disturbed genomic information which might lead to cancer; therefore the consequence is premature aging and not cancer.

Additional disorders related to defective responses to DNA strand breaks are: Ataxia Telangiectasia (AT), Nijmegen Breakage Syndrome (NBS), Seckel Syndrome (SS), Severe Combined Immunodeficiency (SCID), which are characterized by DSB repair defects and cause mainly growth and development deficiencies as well as cancer in some cases. Additionally, the Fanconi Anemia (FA) syndrome is caused by a defect in interstrand crosslinks (ICL) repair as well as homologous recombination (HR), and as consequence these patients suffer from different types of cancer and several skin, limbs and renal abnormalities (Ciccia et al. 2010).

Thus deregulated DNA damage response (DDR), depending on the cellular context and genetic background, can induce severe diseases such as cancer or premature aging. A high rate of mutations is generally directly related to cancer in the context of a defective DNA repair mechanism, which can be of hereditary origin. However, certain genetic backgrounds make cells hypersensitive to low doses of damage, which induces cell death to prevent cancer formation, resulting in premature aging.

If DNA damage occurs at an early stage affecting the stem cells, this can also induce cancer and premature aging. Embryonic stem cells are pluripotent cells, meaning that they can give rise to all cellular lineages. Therefore these cells must have a robust DNA damage response in order to avoid transmitting mutations. Accumulation of mutations that can translate into the bypass of cell death activation in stem cells, which are pluripotent, can lead to severe cancer phenotypes. On the other hand, if the enhanced repair mechanisms inherent

to the stem cells are activated upon DNA damage, these cells will undergo cell death to prevent cancer, leading to the loss of the stem cell pool and premature aging.

DNA damage, if not properly repaired, can induce genome instability, which in turn can lead to malignancy as seen in several of the syndromes described above. Anti-cancer drugs often rely on the generation of DNA damage and activate the DNA damage response in order to eliminate the cancer cells (Hoeijmakers 2009). Thus, understanding the mechanisms that underlay the DDR is of crucial importance to improve cancer therapy, as well as to define valid biomarkers to further study diseases derived from DNA damage.

5. Signal transduction regulating the DDR – post-translational modifications

The DDR, which is activated in cells upon receiving an internal or external insult on the DNA, consists of a cascade-like process, which involves sensors that recognize the damage, mediators that recruit additional elements to the damaged site and effectors that slow down the cell cycle, mediate repair or, if the damage is too severe initiate programmed cell death (Fig 1) (Harper et al. 2007; Deribe et al. 2010). In order for the signal to be transmitted ensuring that the process takes place in an orchestrated manner, post-translational modifications (PTMs) are crucial. PTMs modify the properties of proteins by adding chemical groups or other proteins to their amino acids residues. There are several types of PTMs, each of them having a distinct effect on the targeted protein, including control of localization, activity, and stability (Deribe et al. 2010).

Phosphorylation of a protein is the addition of a phosphate group to the residues serine, threonine, tyrosine, histidine or aspartate. This adds a negative charge on the protein, resulting in its conformational change, which in turn can modify its enzymatic activity, as well as its interaction with other proteins or lipids (Johnson et al. 2001; Narayanan et al. 2009). ***Ubiquitination*** consists of the addition of ubiquitin or a small ubiquitin-like modifier (SUMO) by covalent attachment to the protein. This is a three-step process, which involves activation of the ubiquitin by E1 enzymes, conjugation to a ubiquitin carrier protein E2, and, via E3 ligases, ligation (usually to lysine residues) to target proteins (Hershko et al. 1998). ***Acetylation*** of a protein neutralizes its charge and therefore changes its properties. It consists of the transfer of acetyl groups from acetyl coenzyme A onto lysine residues of the target protein. This post-translational modification has been linked to the regulation of gene expression due to its function altering the histone-DNA interaction as well as histone-histone interaction (Strahl et al. 2000). ***Methylation*** is the conjugation of a methyl group to lysine and arginine residues in histones. This process is regulated by methyltransferases and demethylases and its function has been linked to alteration of chromatin structure as well as alteration of the recruitment of nonhistone proteins such as p53 to chromatin, thereby regulating its activity (Kouzarides 2007).

As described above, the DDR signaling network involves several key kinases that phosphorylate a large

number of downstream substrates including those that stay localized at the site of damage (e.g. repair proteins) and others that diffuse away and mediate a more global response (e.g. Chk kinases that act to control the cell cycle) (Falck et al. 2005; J.-H. Lee et al. 2005). In the case of DSBs, dissociated ATM monomers (see above) have become active and cross autophosphorylate each other as well as Chk2, which is then activated and diffuses throughout the nucleus where it acts to inhibit the cell cycle. At lesions induced by UV, activation of ATR leads to phosphorylation and activation of Chk1, which again leads to cell cycle arrest (J.-H. Lee et al. 2005; Ahn et al. 2004).

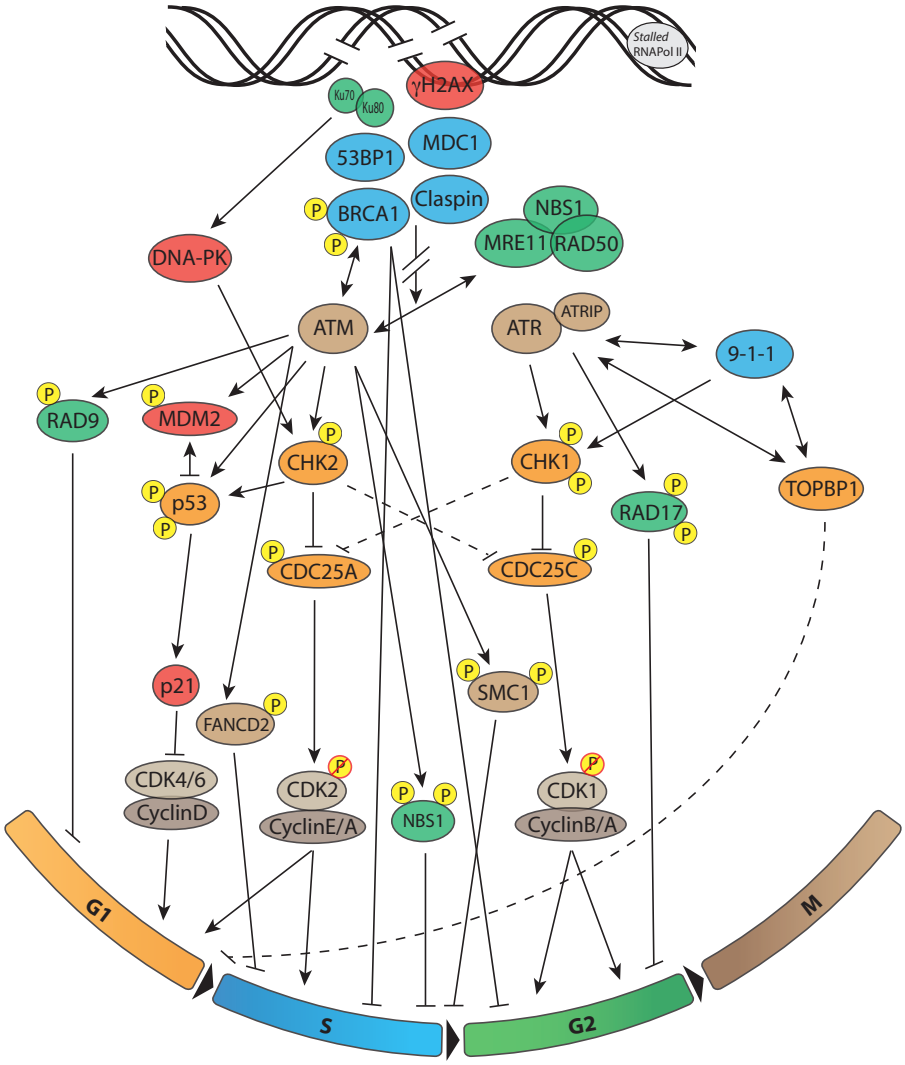

Figure 2. A complex network. Upon DNA damage, there is activation of a complex signaling cascade, which is amplified by the posttranslational modifications. Consequently, the cell cycle is regulated by these signaling events in order to allow repair of the damage. Alternatively, activation of apoptosis is triggered if the damage is too severe. In this way, cells carrying potentially carcinogenic lesions are removed from the organism to prevent cancer.

Once ATM and ATR are activated, they also phosphorylate H2AX, which results in the recruitment of Mdc1. The formation of this complex results in the recruitment of factors that will contribute to the amplification of the signal, such as the E3 ubiquitin ligase Ubc13-Rnf8 that ubiquitinates H2AX in order to recruit 53BP1 (p53 binding protein 1) and BRCA1 (breast cancer type 1 complex). The recruitment of these factors and components of the response leads to the formation of DNA repair foci (Ahn et al. 2004).

It is essential that cells slow down their cell cycle in order to have time to repair the damaged DNA. When Chk1 and Chk2 are activated, they phosphorylate the phosphatases Cdc25A and Cdc25C. When Cdc25C is phosphorylated, it is recruited and inactivated by 14-3-3 proteins. The phosphorylation of Cdc25A leads to the formation of a phosphodegron (proteosomal degradation motif), which is ubiquitinated by the ligase $SCF^{\beta TrCP}$, leading to its degradation.

CDC25 dual specificity phosphatases regulate the activity of cyclin dependent kinases (CDKs) by dephosphorylating their tyrosine/threonine residues, and therefore play a crucial role in the regulation of cell cycle progression. There are three family members of the CDC25 family A, B and C, which cooperate during the regulation of the cell cycle. The CDC25 dual specificity phosphatases are involved in the regulation of the G_1-S transition by dephosphorylating and activating the CDK2-cyclin E and CDK2-cyclin A complexes. On the other hand, they are also involved in the regulation of the G_2-M transitions by activating CDK1-cyclin B complexes (Boutros et al. 2006).

Ubiquitination plays a very important role as a regulatory mechanism during the DNA damage response. Its main function is to lead to protein degradation, which is crucial for attenuating the signal transduction cascades. The repair and checkpoint signaling at DSBs is depending on the E3 ubiquitin ligase BRCA1, which is recruited at the damaged site by multiple ubiquitination steps that are started by the RING finger protein 8 (RNF8) (Ulrich et al. 2010). The feedback loop between p53 and its negative regulator MDM2 is modulated by the ability of MDM2 to ubiquitinate p53 and therefore induce its proteasomal degradation (Ryan et al. 2001).

p53 is an excellent example of a key DDR regulator that undergoes all types of post-translational modifications, phosphorylation, ubiquitination, acetylation, methylation and sumoylation upon DNA damage (Bode et al. 2004; Toledo et al. 2006). In turn, the p53 transcription factor regulates genes involved in all aspects of outcome of DDR signaling, including cell cycle regulation, repair, senescence, and apoptosis (Ryan et al. 2001). Most likely, such a scheme holds through to some degree for all critical proteins in the DDR and we have only scratched the surface. Future studies in this field should further clarify the mechanism underlying the orchestration of a tightly regulated and interrelated network such as the DDR.

6. Different OMICS techniques to unravel signaling pathways

Some 15 years ago, Schena et al made use of complementary DNA (cDNA) chips (microarray) to perform a quantitative

profile of RNA expression in *Arabidopsis thaliana* (Schena et al. 1995). Since then, microarrays have been used extensively to map gene expression profiles of many organisms and cell types in the context of development, disease and toxic response to chemicals (i.e. the search term "microarray" identifies ~42,000 hits in PubMed). Once the sequencing of the human and mouse genomes was accomplished, microarray technology played a very important role in determining which genes were expressed in which cell types, what stages of development, and how gene expression was altered under pathological processes, the latter providing new generation biomarkers for instance for prostate and breast cancer (Shoemaker et al. 2001; Rakha et al. 2010).

Microarray studies have also been used to identify potential biomarkers at the RNA level for the cellular response to various compounds, and have defined signatures for drug responses (Bruheim et al. 2009; Cui et al. 2010; Smalley et al. 2010). As a next layer of information, with the development of mass spec technology, proteomics approaches have matured to a level that allows high throughput determination of protein expression as well as post-translational modifications including phosphorylation and ubiquitination. Combining information from RNA expression and these proteomics studies provides insight into the complex interconnection between signaling networks that are activated in development, disease, and drug responses (Matsuoka et al. 2007; Choudhary et al. 2010).

One major drawback is that these studies do not provide information on the functional involvement of the genes and/or proteins in the process under study: for instance genes whose transcription is not changed may be involved in a response and, vice versa, genes whose expression is altered may not play a role. Although this does not diminish their role as biomarkers, for mechanistic understanding the emergence of a new technology has become important: RNA interference (RNAi) or "functional genomics" screens, in which expression of individual genes is silenced with specific short double stranded RNA molecules (siRNA). Genome-wide RNAi screens have been utilized to unveil components of the DDR in different contexts. Kolas et al, in 2007, identified the ubiquitin ligase RNF8 to be involved in the DNA damage response (DDR) making use of a genome wide screen using ionizing radiation as input and DDR (MDC1 and 53BP1) foci formation as biological endpoint. It was shown that upon DNA damage caused by ionizing radiation, ATM phosphorylates MDC1, which then recruits RNF8 at the damage site. RNF8 then induces the formation of ubiquitination conjugates that mediate the recruitment of the RAP80-BRCA1 complex and 53BP1, thus RNF8 was found to promote G_2/M DNA damage checkpoint, enhancing cell survival (Kolas et al. 2007).

Another genome-wide RNAi screen for the response to ionizing radiation identified components of the Triple T complex (TTI1, TTI2 and TEL2) as crucial mediators of the DDR. These proteins were found to control ATM and ATR stabilization (Hurov et al. 2010). Additional, RNAi screens have identified new players in the maintenance of genomics stability (Paulsen et al. 2009).

A major current challenge is posed by the integration of the various OMICS data, including transcriptomics, proteomics, and functional genomics. This should provide the information to elucidate the full complexity of developmental programs, disease processes, and drug responses (T. Y. Kim et al. 2010; Arrell et al. 2010). Such strategies rely heavily on strong bioinformatics tools needed to combine the technologies into a systems biology approach (Gehlenborg et al. 2010).

7. Scope of this thesis

Cells in the human body have to deal with DNA damage daily, either caused by external or internal sources. The DDR is particularly strong in stem cells. Since these cells have a long life span and are essential for tissue homeostasis, tolerance to damaged DNA would lead to accumulation of mutations and malignant transformation. In addition, accumulation of damaged DNA would lead to loss of the stem cell pool and contribute to aging. In this thesis I investigated various biological the DNA damage response in the context of stem cells as well as cancer cells, from the response to different DNA damaging agents, to the importance of the interaction with the extracellular matrix in combination with the presence of oncogenes. In order to acquire a complete picture of the DNA damage response in mES cells, and therefore elucidate novel pathways involved in this particular response, we combined OMICS techniques such as Functional Genomics, Transcriptomics and Phosphoproteomics, that once overlapped, allowed us to find novel pathways that where not previously described to be involved in the DNA damage response. In ***chapter 2***, DDR and properties of normal stem cells as well as the relation with cancer stem cells is discussed. Additionally the relation of (cancer) stem cells with their microenvironment ("the niche") is also discussed.

In ***chapter 3***, development of a real time live cell imaging technique is described that allows studying the kinetics of apoptosis, one of the potential outcomes of DDR signaling. In healthy cells, phosphatidylserines are localized in the inner leaflet of the plasma membrane and endogenous Annexin V, which is implicated in ion channel formation binds to the phosphatidylserines. During apoptosis, phosphatidylserines translocate to the outer leaflet and our technique makes use of fluorescently labeled recombinant Annexin V to detect this translocation. Automated acquisition of images as well as automated quantification of the accumulation of the fluorescent signal makes this technique suitable for high throughput screening.

During the DNA damage response, post-translational modifications play a crucial role to transmit the signal through this complex signaling cascade. As described in ***chapter 4***, in order to investigate the phosphorylation events and therefore elucidate novel responsive kinases upon the exposure of the cells to cisplatin, a phospho-proteomics approach was performed (Matsuoka et al. 2007; Villén et al. 2007). The serine/threonine protein kinase ATM as well as the tumor suppressor BRCA1, both involved in DNA damage response and expected to be activated, are hyperphosphorylated upon cisplatin exposure. Also several

MAPK family related kinases, which are involved in the response to genotoxic stress (Brozovic et al. 2007), as well as cyclin dependent and PKC related kinases, were hyperphosphorylated. By contrast, factors involved in cytoskeleton reorganization such as Rac1 and Cdc42, were found to be hypophosphorylated. The results indicate that besides the classical DNA damage response, a complex network of survival pathways as well as non-classical DNA damage response pathways are activated upon cisplatin exposure.

In ***chapter 5***, a systems biology approach is described to delineate the DDR in embryonic stem (ES) cells. By combining RNAi screening, transcriptomics and phospho-proteomics, new signaling networks and novel key players have been identified in the response to cisplatin. In addition to the classical p53 pathway, Wnt signaling is identified as a major determinant of DDR in ES cells. Moreover, several of the identified enzymes are validated in cancer cells as potential new drug targets for therapy development.

As described in chapter 2, interactions with the microenvironment can modulate the response to genotoxic compounds and are essential regulators of (cancer) stem cell function. The extra cellular matrix (ECM) forms an essential part of the microenvironment as it provides physical and chemical cues that modulate cell behavior, which is described in ***chapter 6.*** Subsequently, in ***chapter 7***, chemosensitivity in the presence or absence of oncogenes is compared for cells expressing either β1 or β3 integrins. An increased apoptotic response is observed in cells expressing activated c-Src only when these cells also express β1 integrins. Notably, this enhanced response is p53-independent and rather than classical DDR occurs through endoplasmic reticulum (ER) stress and caspase-3 cleavage (activation).

Finally, ***chapter 8*** provides a summary and a general discussion of findings and implications of the work described in this thesis.

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