Changes in chromatin organization of human cells in response to genotoxic stress

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Chapter 1

DNA damage, cellular stress response, and changes in chromatin organization.

DNA damage, cellular stress response and changes in chromatin organization

1.1 Introduction

Stress can be described as a disturbance, which may affect structure, function, growth and survival of cells, tissues and organisms. Organisms are exposed to various endogenous and exogenous stressors such as hyper- or hypothermia, hypoxia, induction of reactive oxygen species (ROS), radiation, starvation, infection, hypo- or hyperosmotic conditions, changes in pH, factors underlying metabolic deficiencies and other pathologic conditions, heavy metals as well as toxic and mutagenic agents (for a review see Tiligada et al., 2002). In order to counteract the deleterious effects of stressors and to restore homeostasis, cells have a variety of protective responses and signal transduction pathways to execute such responses. These responses include activation of protein degradation, antioxidant systems, cell cycle checkpoints, DNA repair and apoptosis.

The cellular targets of stressors are primarily macromolecules. Exposure to stress commonly results in structural deformation or damaging of proteins, nucleic acids or other macromolecules such as lipids. Sensing of damage induced in the macromolecules is the key regulator of the cellular stress response (for a review see Kultz, 2005). It is generally thought that the cellular stress response is mediated by activation of a specific class of genes, i.e. the stress response genes encoding a variety of proteins such as heat shock or stress proteins (HSPs) that act as molecular chaperones (for a review see Jolly and Morimoto, 2000). These genes are conserved in all organisms (for a review see Tiligada, 2006). The heat shock response is the cellular process that is activated by protein damage and mediated by HSPs. The expression of HSPs is generally activated by protein denaturation and might be induced by elevated temperature (heat), but also by other types of stress such as heavy metals, arsenate, alcohol, H_2O_2, UV irradiation, fever and inflammation (Muramatsu et al., 1992; Morimoto, 1993; Garmyn et al., 1995; Trautinger et al., 1995; McDuffee et al., 1997; Richards et al., 1998; Zhou et al., 1998; Trautinger, 2001). The common signal generated by various stress stimuli is likely to be protein damage which occurs mainly as oxidative or structural (unfolding) damage (Pirkkala et al., 2001). Consequently, the protective effects of HSPs, acting as molecular chaperones and

proteases, have been ascribed mainly to their protein stabilizing capacity including recognition and removing of protein damage by refolding of structurally damaged proteins into the native functional state, and proteolysis, i.e. proteolytic degradation of irreversible damaged proteins (for review see Jolly and Morimoto, 2000; Christians et al., 2002; Kultz, 2005).

In case of DNA damaging (genotoxic) agents, the cellular response is termed DNA damage response (DDR) and this response counteracts the adverse effects of genotoxic stress by sensing DNA damage and subsequently activating the downstream effectors, i.e. DNA repair pathways, chromatin remodeling, cell cycle checkpoints, and gene expression. In addition, cells can activate a genetically controlled death program (apoptosis) that removes damaged cells when tolerance limits are exceeded. In this chapter, we focus on DNA damage and DDR with emphasis on the effects of DNA damage on the large-scale chromatin organization.

1.2 Different types of genotoxic stressors

Genotoxic agents may be either of endogenous nature, i.e. the products of normal cellular metabolism, or of exogenous nature, i.e. components in the environment; their critical cellular targets are nucleic acids. DNA damage can be induced by physical agents including ultraviolet and ionizing radiations, as well as chemical moieties such as bulky polycyclic aromatic hydrocarbons (e.g., benzo(a)pyrene), alkylating agents (e.g., MNU and MMS) and crosslinking agents (e.g., psoralens and bifunctional alkylating agents such as mitomycins). In addition, other types of stress such as elevated heat can also induce DNA damage, presumably single- (SSBs) and double- (DSBs) strand breaks as concluded from the induction of histone H2AX phosphorylation by heat in recent studies (Takahashi et al., 2004; Kaneko et al., 2005). Damage to DNA occurs in different forms including SSBs, DSBs, DNA base modifications, DNA- and protein-DNA cross-links and bulky DNA lesions such as benzo(a)pyrene adducts. Unrepaired DNA lesions may interfere with DNA metabolic processes such as replication and transcription leading to cytotoxicity, mutations and genetic instability that can cause various diseases including cancer.

1.2.1 Ionizing radiation

Ionizing radiation (IR) of different linear energy transfer (LET) can induce a wide variety of DNA lesions primarily DNA strand breaks and a broad spectrum of different types of base damage. The principal sources of external exposure are cosmic radiation and radionuclides naturally occurring on the planet. Other sources of IR are artificial such as X-rays used in medical diagnosis and therapy, radiopharmaceuticals used in nuclear medicine and nuclear power plants. IR damages DNA by direct effects resulting from the absorption of the radiation energy by DNA and leading to ionization of bases or sugars (Ward, 1988). The indirect effects result from interaction of DNA with reactive species formed by radiolysis of water or other surrounding molecules (such as the highly reactive hydroxyl radicals) and compromise most of the DNA damage caused by IR (Ward, 1988; Riley, 1994). The direct and indirect radiation effects may result in DNA damage with deleterious biological effects (Ward 1988; Cadet, 1999). Damage to DNA sugars by ionizing radiation is biologically important because of strand breakage. A dose of 1 Gy of low-LET radiation results in the induction of 600-1000 single-strand breaks (SSB), 16-40 double-strand breaks (DSB) and 250 base damages in naked DNA (Ward, 1988; van Loon et al., 1991). The spectrum and frequency of DNA lesions are influenced by the level of oxygen and the packaging of nuclear DNA in chromatin (Ward, 1990; Cadet et al., 2004; Regulus et al., 2007). Although the frequencies of SSBs and base damage clearly exceed the frequency of DSBs, it is obvious that the lethal effects of ionizing radiation can be primarily attributed to DSBs as SSBs and base damage are efficiently repaired (Ward, 1990; Iliakis, 1991). In case of DSBs, both DNA strands are broken and the complementary DNA strand cannot be used for correct repair resulting in higher chance of mis-rejoining of broken ends leading to genomic instability and cytotoxicity.

1.2.2 UV radiation

UV light is electromagnetic radiation emitted from the sun or artificial sources such as sun banks. UV radiation is divided to three areas according to its wavelength, i.e. UVA; 315-380 nm, UVB; 280-315 nm and UVC; 190-280 nm (Setlow, 1974). Although UV radiation has harmful effects on the skin, the most hazardous effect of excess UV in sunlight for human is the increase risk of skin cancer (de Gruijl, 1999; Armstrong and Kricker 2001). DNA absorbs short wavelength UVC very efficiently,

but also absorbs a significant amount of energy from UVB (de Gruijl et al., 2001; Ravanat et al., 2001). The direct absorption of UV by DNA leads to the formation of cross-links between adjacent DNA bases, i.e. cyclobutane pyrimidine dimers (CPDs) and (6-4)-photoproducts (6-4)PPs (Ravanat et al., 2001). The pyrimidone ring of the (6-4)PPs is subjected to further modification by UV irradiation to a photolesion called Dewar valence isomer or Dewar photoproduct (Yamamoto et al., 2006). The (6-4)PP and CPD lesions induce distortions of the DNA helix and halt RNA and DNA polymerases, thus inhibiting gene expression and arresting DNA replication, respectively (Tornaletti and Hanawalt, 1999; Decraene et al., 2001). Oxidative damage to DNA is induced at relatively high frequency following exposure to UVA (Kielbassa et al., 1997). Longer UV wavelengths can also induce DNA breaks and DNA-protein cross-links albeit at low frequency (Tyrrell, 1994).

1.2.3 Cross-linking agents

Cross-linking agents constitute a group of chemicals that has been widely studied and used in chemotherapy regimens for the treatment of various cancers. These agents include bifunctional alkylating agents that can react with two different nucleophilic sites in DNA leading to intrastrand cross-links (if the two sites are situated on the same DNA strand) or interstrand cross-links (if these sites are on the opposite DNA strands). Interstrand cross-links (ICLs) prevent DNA strand separation and hence constitute complete blocks to DNA replication and transcription (Iyer and Szybalski, 1963; Turner et al., 2000). A number of compounds such as mitomycins, nitrogen mustards, cisplatin and photoactivated psoralens can induce ICLs and are extensively used in cancer chemotherapy (Fox and Scott, 1980; Piette et al., 1986; Fram, 1992; Trimmer and Essigmann, 1999). Moreover, ICLs resulting from exposure to cross-linking agents constitute the primary cause of the cytotoxic and antitumor properties of this group of compounds (Iyer and Szybalski, 1963; Palom et al., 2002). Endogenous sources of ICLs also occur such as nitrous acid, which can be formed from dietary nitrites under the acidic conditions of the stomach, aldehydes (e.g., malondialdehyde) formed as products of lipid peroxidation and acetaldehyde resulting from normal cellular glycolysis (Ristow and Obe, 1978; Niedernhofer et al., 2003; Edfeldt et al., 2004).

Mitomycins constitute a group of antibiotics isolated from *Streptomyces caespitosus* and have been discovered to be potent chemotherapeutic cross-linking

agents. Most of the preclinical and clinical investigations have been focused on one member of the group, i.e. mitomycin C (MMC) (for a review see Verweij and Pinedo, 1990). MMC has three functional groups, i.e. quinone, carbamate and aziridine and chemical or enzymatic reduction is required to convert the drug into an alkylating agent capable of mono- or bifunctional covalent interactions with DNA (Reddy and Randerath, 1987; Keyes et al., 1991). Primarily, MMC is regarded as a selective inhibitor of DNA replication and this property is critical for the cytotoxic effect of the drug (Iyer and Szybalski, 1963; Szybalski and Iyer, 1964). Biochemical studies revealed that MMC-induced ICLs occur mainly between the two guanines moieties at CpG sites in the genome (Teng et al., 1989; Borowy-Borowski et al., 1990). The formation of cross-links is further enhanced when the cytosines of CpG sequences are methylated (Millard and Beachy, 1993; Johnson et al., 1995; Li et al., 2000). Many of the CpGs are clustered in GC rich regions, which have been identified in repetitive satellite DNAs (Meneveri et al., 1993). About 60-90% of the CpG sites in human are methylated at the C5 position of cytosine (Bird, 1986). These methylated sequences have been shown to be present in the heterochromatin of human chromosomes, particularly chromosomes 1, 9, 16 and Y as demonstrated by immunocytochemical detection of 5-methylcytosine in the juxtacentromeric regions of these chromosomes (Koch and Stratling, 2004). Indeed, MMC is known to induce chromatid exchanges between the heterochromatic regions of chromosomes 1, 9 and 16 and more specifically between the homologous chromosomes (Shaw and Cohen, 1965; Morad et al., 1973; Abdel-Halim et al., 2005).

1.2.4 Heat shock

Heat shock, i.e. exposure of cells to elevated temperature (above 37°C), affects numerous biological processes including gene expression, DNA replication, DNA repair, cell cycle progression and cell survival (Wong et al., 1989; Laszlo, 1992; Das et al., 1995; Iliakis et al., 2004). All macromolecules are affected by elevated heat; however, proteins are the critical macromolecules for the lethal effects of heat (Laszlo, 1992; Burgman and Konings, 1992; Kampinga, 1993).

The genotoxicity of heat shock appears to be controversial. It has been reported that heat shock does not result in direct damage to DNA (Jorritsma and Konings, 1984; Iliakis et al., 1990; Wong et al., 1995). However, recent studies

suggest that heat shock by itself induces DSBs and other types of DNA damage in mammalian cells. Heat has been reported to induce oxidative base damage in DNA through free radical formation (Bruskov et al., 2002). The study of Nueda et al. (1999) demonstrated that cells deficient in DSB repair exhibited higher levels of heat induced apoptosis than normal cells and the authors suggested the induction of DSBs following heat shock treatment to explain these results. Indeed, recent studies have presented evidence that heat shock induces phosphorylation of histone H2AX in mammalian cells (Takahashi et al., 2004; Kaneko et al., 2005; Hunt et al., 2007). The formation of γH2AX foci is a sensitive marker for the existence of DSBs and also single stranded DNA (ssDNA) regions (Kobayashi et al., 2002; Pilch et al., 2003; Rothkamm and Lobrich, 2003; Marti et al., 2006; Matsumoto et al., 2007). In human cells heat induces DSBs as detected by neutral single cell electrophoresis (Comet) and phosphorylation of H2AX throughout the cell cycle. However, the effect was more pronounced when cells were heated in S phase than in G_1 or G_2 phase. Heat might induce DSBs through the denaturation and dysfunction of heat labile proteins such as DNA polymerases and by blocking of replication (Takahashi et al., 2004). It is also possible that heat induces DSBs via activation of topoisomerase II as shown for low pH (Xiao et al., 2003). Wortmannin, an ATM and DNA-PK inhibitor, strongly inhibits both IR- and heat-induced H2AX phosphorylation suggesting that the same molecular pathway leads to the phosphorylation in heat-treated and irradiated cells (Kaneko et al., 2005). However, in other studies (Zhou et al., 2006; Dong et al., 2007) heat shock did not induce the formation of γH2AX foci and the authors attributed the results contradicting the data reported by Takahashi et al. (2004) and Kaneko et al. (2005) to the different cell lines used.

Heat shock has been used to sensitize tumor cells to ionizing radiation (Overgaard, 1989). The molecular mechanism for this sensitization has been attributed to a heat dependent inhibition of the repair of radiation-induced DSBs (Burgman et al., 1997). Since repair in transcriptionally active genes was also inhibited by heat shock, it was proposed that changes in chromatin structure by increased number of nuclear-matrix attachment sites might underlie the inhibition of DNA repair after heat shock (Sakkers et al., 1999).

Cells respond to heat shock and other stresses by activating the genes that encode heat shock proteins (HSP) and their regulatory heat shock transcription factors

(HSFs) (Morimoto, 1998; Christians et al., 2002). Heat shock proteins are classified according to their molecular weight: HSP25, HSP27, HSP47, HSP60, HSP70/72, HSP90 and HSP110 (for a review see Jolly and Morimoto, 2000). The inducible HSP gene expression is regulated by a family of HSFs, among which HSF1 is responsible for the stress-induced activation of HSP genes in mammalian cells (Morimoto, 1998; Pirkkala et al., 2001). HSF1 is present in an inactive monomeric state in non-stressed cells. Upon exposure to stress such as heat, heavy metals and sodium arsenate, HSF1 undergoes trimerization, post-translational modifications and binding to the heat shock elements of the promoter regions of HSP genes leading to transcription of these genes (Morimoto, 1998; Christians et al., 2002). Increased induction of HSF1, HSP27, HSP70 and HSP90 has been found in cancer tissues (Cen et al., 2004; Khalil, et al., 2006; Wang et al., 2006). In human keratinocytes, the expression of HSP72 is increased by UVB, UVC and psoralen plus UVA light (Muramatsu et al., 1992, 1993; Zhou et al., 1998). Moreover, HSP27 has been shown to protect human cells against UVC-induced DNA damage and was proposed to function in DNA repair (Wano et al., 2004). In addition, HSPs have been proposed to play a role in DNA repair and maintaining genomic stability after exposure of cells to IR (Matsumoto et al., 1995; Calini et al., 2003; Hunt, et al., 2004). Recently, HSP90 has been shown to regulate the Fanconi anemia (FA) pathway, which is a part of the cellular response to crosslinking agents (Oda et al., 2007). In the latter study, HSP90 associated with FANCA which forms with other FA proteins the core complex in response to ICLs. The core complex dependent activation of FANCD2 is crucial for the cellular response to ICLs (for a review see Niedernhofer et al., 2005). Inhibition of HSP90 leads to disruption of the association with FANCA and to impaired activation of FANCD2 suggesting a role of HSPs in regulating the cellular response to genotoxic stress (Oda et al., 2007).

1.3 Cellular responses to genotoxic stress

DNA damage triggers several cellular responses that enable the cell to eliminate the damage, arrest cell cycle progression and activate a programmed cell death process (apoptosis). The DNA damage response (DDR) encompasses complex processes involving numerous proteins, which form intricate and overlapping signal transduction pathways (for a review see Gasser and Raulet, 2006). In case of DSBs, the DDR (Figure 1) is activated via recognition of lesions in the DNA by sensor proteins. After detection of damage, a signal is generated and transmitted through phosphorylation of mediator/transducer proteins to downstream effector molecules involved in activation of DNA repair, cell cycle checkpoints, and apoptosis. Central in the signal transduction pathways are proteins belonging to the family of the phosphatidylinositol 3-kinase related kinases (PIKKs) including ataxia telangiectasia mutated (ATM), ATM- and Rad3-related (ATR) and DNA-dependent protein kinase (DNA-PK) (for reviews see Gasser and Raulet, 2006; Lavin, 2008). These transducers connect sensing and detection of DNA damage to the activation of effector proteins such as p53, which in turn regulate the expression of genes involved in cell cycle checkpoints, DNA repair, chromatin remodeling and apoptosis (for reviews see Cline and Hanawalt, 2003; Sancar et al., 2004; Gasser and Raulet, 2006; Houtgraaf et al., 2006; Bartek and Lukas, 2007). The ability of cells to detect and repair DNA damage is influenced by the chromatin structure. Emerging evidence indicates that various modulations in chromatin structure, such as histone modifications and chromatin remodeling are crucial in many aspects of the DDR (for review see Costelloe et al., 2006). Recent studies have suggested that essential alterations in chromatin structure are induced in the vicinity of DNA lesions such as DSBs. Histone H2AX phosphorylation is one of the best characterized DNA damage-induced histone modifications which occur in regions flanking the sites of DNA DSBs and ssDNA and this phosphorylation is mediated primarily by ATM and ATR and also by DNA-PK (Downs et al., 2004; Shroff et al., 2004; Kinner et al., 2008). In this part of the chapter we focus on DNA repair pathways and transcription response after DNA damage.

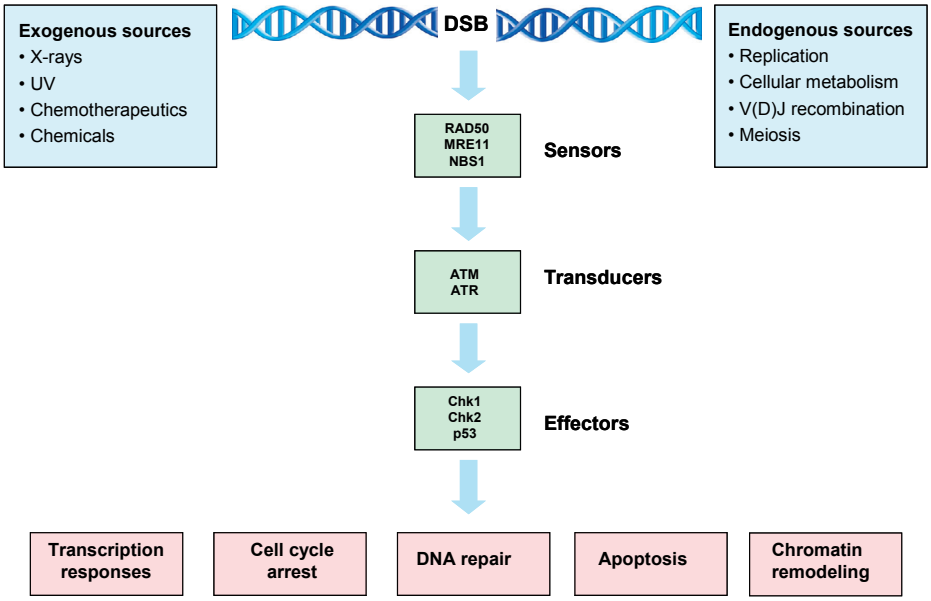


Figure 1: A general scheme for the cellular responses to DSB in DNA (Adapted from Khanna and Jackson, 2001). The presence of DSBs (induced directly by DNA damaging agents or during stalled replication) is recognized by sensor proteins which generate a signal that transmitted to downstream effector molecules. The MRN complex has a crucial role in the sensing of DSBs. ATM and ATR are the central proteins in the signal transduction pathways that transfer the DNA damage signal to the downstream effector kinases such as Chk1, Chk2 and p53. These effector proteins regulate the expression of genes involved in cell cycle checkpoints, chromatin remodeling, DNA repair and apoptosis.

1.3.1 DNA repair

The principal DNA repair pathways include base excision repair (BER), nucleotide excision repair (NER), DSB repair including homologous recombination (HR) and non-homologous end joining (NHEJ), mismatch repair (MMR) and repair of interstrand cross-links (ICL), each of which is dedicated to a particular class of DNA lesions (for reviews see Norbury and Hickson, 2001; Peterson and Cote, 2004; Sancar et al., 2004, Houtgraaf et al., 2006). A brief review of DNA repair pathways relevant for this thesis is given in this section.

Nucleotide excision repair (NER)

The NER pathway is the most important repair system to remove bulky DNA helix distorting lesions that interfere with base pairing and that block transcription and replication. These types of lesions can be induced by UV-irradiation (pyrimidine dimers) and chemical carcinogens such as polyaromatic hydrocarbons (for reviews see Hoeijmakers, 2001; Fousteri and Mullenders, 2008; Shuck et al., 2008). The NER process is initiated by recognition of the DNA damage followed by assembly of a pre-incision complex, excision of the damaged strand, gap-filling DNA synthesis and ligation. NER involves more than 30 different proteins and consists of two sub-pathways, i.e. global genome NER (GG-NER) and transcription-coupled NER (TC-NER). GG-NER and TC-NER differ only in the recognition of the DNA lesion. In GG-NER the entire genome is scanned for helix-distorting lesions while TC-NER is responsible for the repair of damage in the transcribed DNA strand that blocks elongating RNA polymerases.

The individual proteins and protein complexes required for NER have been characterized using in vitro complementation assays (Wood et al., 1988; Aboussekhra et al., 1995; Araujo et al., 2000). The two subpathways of NER are presented in Figure 2. Recognition of DNA damage, i.e. the induced helical distortion, in GG-NER involves XPC-RAD23B and UV-DDB protein complexes. XPC-RAD23B is the principal DNA damage recognition protein and is required for the recruitment of other NER proteins to the site of damage. In TC-NER, damage recognition at stalled RNA polymerase II requires CSA, CSB and XAB2 proteins. After recognition of the DNA damage, GG- and TC-NER follow a common route to complete repair. The DNA duplex is unwound around the damage by the transcription factor TFIIH that contains the two helicases XPB and XPD. During this process, XPA is recruited to the site of the DNA lesion to verify the damage and the single strand DNA-binding protein RPA subsequently stabilizes the open intermediate by binding to the undamaged strand. Finally, the structure specific endonucleases XPG and ERCC1/XPF cleave the damaged strands 3' and 5' of the lesion, respectively, generating a 24-32 base oligonucleotide fragment containing the lesion. The single strand gap is filled in by RFC/PCNA together with DNA polymerases δ and ε that are capable of DNA repair synthesis using the undamaged strand as a template. The remaining nick can be sealed by DNA Ligase I or the XRCC1-Ligase III complex (Moser et al., 2007).

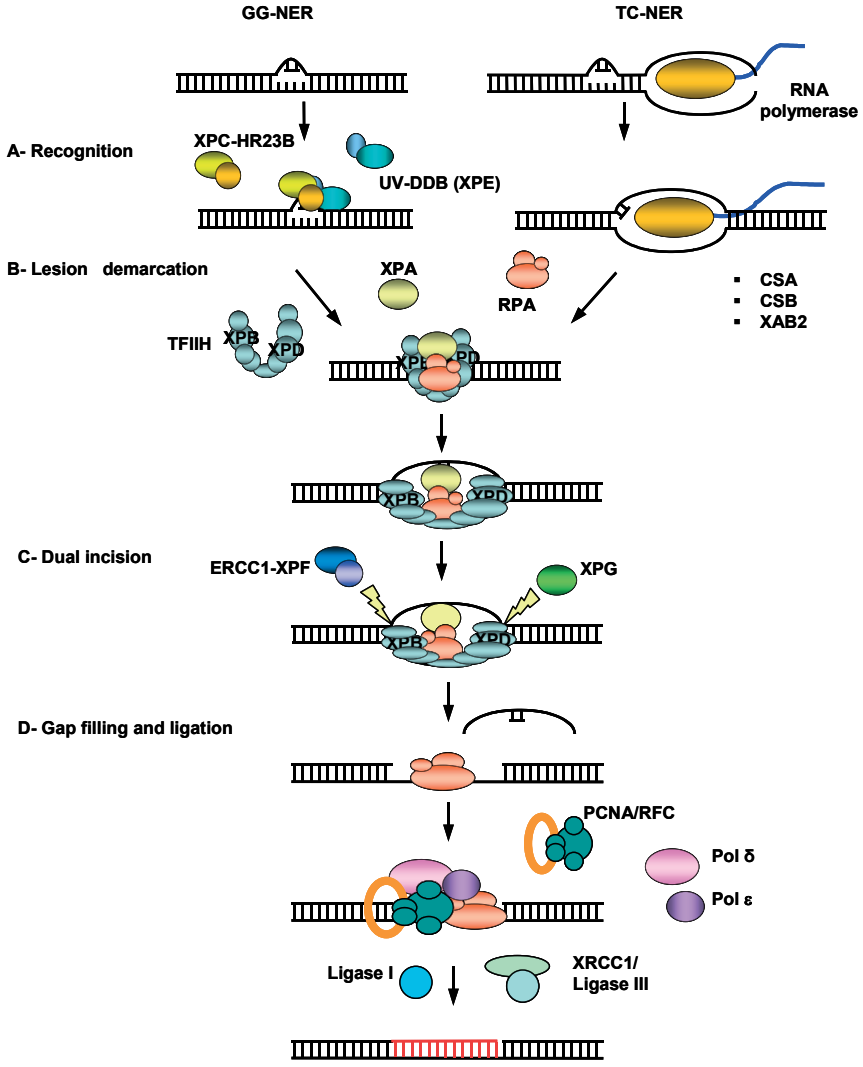


Figure 2: The mammalian NER pathway. (A) Recognition of helix distorting DNA damage during GG-NER or is mediated by two heterodimeric complexex, i.e. XPC-RAD23 and UV-DDB complex during GG-NER. TC-NER is triggered by blockage of RNA polymerase II at the site of DNA damage. In this subpathway damage recognition requires CSA, CSB and XAB2. After damage recognition, the two subpathways converge. (B) Lesion demarcation is mediated by the action of the basal transcription factor THIIF, XPA and the single strand DNA binding protein RPA. (C) 5' and 3' incision flanking the lesion by the structure-specific endonucleases XPF/ERCC1 and XPG respectively. (D) The single stranded gap is filled and sealed by the action of RFC-mediated loading of PCNA, the interaction of DNA polymerases δ and ε and by DNA ligase I and XRCC1-ligase III complex.

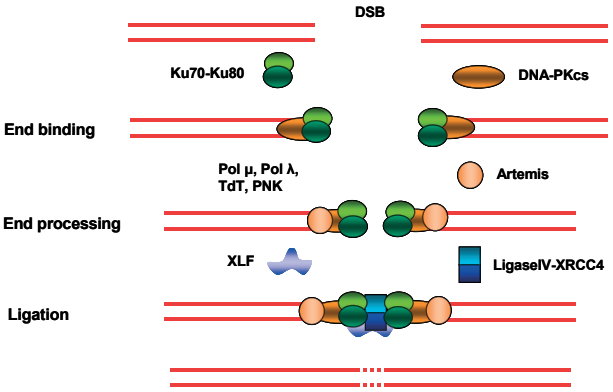
There are at least three rare autosomal recessive diseases in humans associated with defects in NER and characterized by increased sensitivity to sunlight: xeroderma pigmentosum (XP), Cockayne syndrome (CS) and trichothiodystrophy (TTD) (for reviews, see Thoms et al., 2007; Hakem, 2008). XP patients show an extreme skin sensitivity to sun exposure, have more than 1000-fold increased skin cancer risk and often develop neurological abnormalities. XP is caused by mutations in seven genes, *XPA-XPG*, and a single variant gene *XP-V*. CS patients suffer from excessive sun sensitivity but without increased susceptibility to skin cancer. The clinical features of CS comprise growth retardation, progressive cognitive impairment and premature aging. This syndrome is caused by mutations in *CSA* and *CSB* genes and at the cellular level is characterized by TCR deficiency. TTD patients display CS-like symptoms and are also characterized by brittle hair and nails, dwarfism and scaly skin. The most severe cases are associated with mutations in *XPB* and *XPD*, which are subunits of the transcription factor IIH (TFIIH). Proteins encoded by XPB and XPD genes have dual functions, as these proteins are crucial for both NER and transcription (Vermeulen et al., 2001).

DSB repair

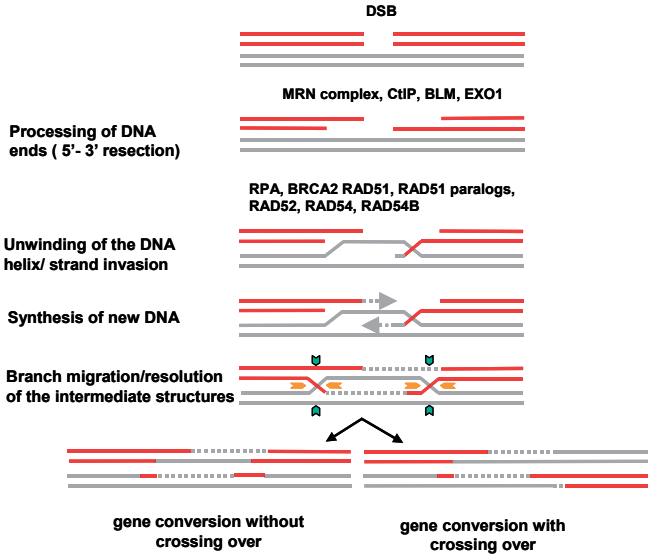
A DNA double strand break (DSB) forms a particular type of DNA damage as both strands of the DNA double helix are affected and hence genetic information on the second strand can not be used for proper repair of the lesion. Non-repair and misrepair of DSBs lead to chromosome fragmentation, translocations and other chromosomal aberrations ultimately leading to cell death or genomic instability and cancer. DSBs are caused by DNA damaging agents such as ionizing radiation, free radicals formed during normal cellular metabolism and certain chemotherapeutic drugs but can also arise as intermediates in normal cellular processes like replication, recombination during meiosis and V(D)J recombination (Richardson et al., 2004; Scott and Pandita, 2006). The detection of DSBs in DNA involves the RAD50/MRE11/NBS1 (RMN) complex and leads to the activation of the ATM protein. ATM is at the apex of a signaling cascade resulting in the modulation of effector molecules involved in DNA repair, cell cycle checkpoints, apoptosis and chromatin remodeling (Iijima et al., 2008).

Figure 3

(A) NHEJ



(B) HR



(C) SSA

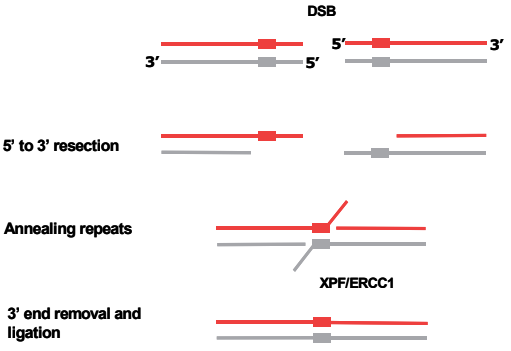


Figure 3: DSB repair pathways in mammalian cells. (A) In NHEJ the KU heterodimer binds to the broken DNA ends and recruits DNA-PKcs. Upon binding to the DNA ends and KU, DNA-PKcs posphorylates itself and mediates the accessibility of other factors to the site of the DNA damage including Artemis, polymerases μ, λ, TdT and PNK. After end processing by the latter enzymes ligation of the DNA ends is mediated by LigaseIV/XRCC1/XLF. (B) HR is initiated by processing of the broken DNA ends leading to the formation of ssDNA overhangs. This resection is dependent on the RMN complex, CtIP, BLM and EXO1. The ssDNA tails are first engaged by the ssDNA binding protein RPA. Subsequently, RPA is replaced by the RAD51 protein. The assembly of RAD51 filaments involves the recombination mediators including BRCA2 and RAD52. RAD51 filaments mediate the search for a homologous undamaged DNA repair template and include other members of the RAD52 group. Strand invasion leads to the formation of a joint molecule between the broken DNA ends and the homologous template. Synthesis of DNA and ligation lead to the formation of a double Holiday junction that can be enlarged by branch migration. Resolution of the Holiday junction followed by ligation of DN ends can lead to gene conversions without or with crossing over. The presence of crossovers in mitotically dividing mammalian cells is very rare and recombination models without the formation of holiday junctions have been proposed (Ref). (C) SSA requires the presence of repeated sequences on both sides of the break. During resection of the 5' ends the homologous repetitive regions become exposed and annealing of these regions may occur. After removal of the non-homologous ends by XPF/ERCC1 nuclease and gap filling the DNA ends are ligated. As a consequence of this mode of repair, a deletion is introduced in the DNA.

In eukaryotes DSBs can be repaired via two processes: non-homologous end joining (NHEJ) and homologous recombination (HR). The relative contribution of both pathways in mammalian cells varies during embryonic development and is also dependent on the cell cycle. During G_0 and G_1, NHEJ is the predominant repair pathway, whereas both HR and NHEJ contribute to DSB repair during S and G_2. Currently, the factors that govern the choice between HR and NHEJ in S/G_2 are largely unknown. HR requires a second homologous sequence present on the sister chromatid or the homologous chromosome that can be used as a template for high fidelity repair. In NHEJ, on the contrary, there is no need for extensive homology and this pathway is less accurate and may give rise to insertions or deletions.

In mammals, the NHEJ pathway (Figure 3A) involves the KU heterodimer (KU70/KU80), the DNA protein kinase catalytic subunit (DNA-PKcs), XRCC4, Ligase IV, Artemis and the recently identified XRCC4-like factor Cernunnos-XLF (for review see Kanaar et al., 2008; Weterings and Chen, 2008). The heterodimers of KU bind to the ends of DNA DSBs and in turn facilitate recruitment of DNA-PKcs to the site of the damage. The association of DNA-PKcs with DNA ends and KU complex is necessary for the activation of its kinase activity. Autophosphorylation of DNA-PKcs is essential for the efficient repair of DSBs. Binding of DNA-PKcs to DNA ends mediates tethering of both ends and results in accessibility for processing enzymes, e.g. Artemis, DNA polymerases μ, λ terminal deoxynucleotidyl transferase

(TdT) and polynucleotide kinase (PNK). Artemis is important for the opening of hairpin structures at the coding ends during V(D)J recombination and for the repair of DSBs presumably in heterochromatin after exposure to X-rays (Goodarzi et al., 2008). The last step in NHEJ is dependent on the Ligase IV/XRCC4 complex. The XLF factor interacts with the Ligase IV/XRCC4 heterodimer and promotes ligation of DNA ends.

HR is a multistep process (for reviews see Pastink et al., 2001; Hakem, 2008; Kanaar et al., 2008) and requires an undamaged sister chromatid or homologous chromosome that can be used as a template for repair synthesis (Figure 3B). The first step in HR is the formation 3' single-stranded ends. The RMN complex, consisting of RAD50, MRE11 and NBS1, has been implicated in this step. Recently it has been shown that the RMN complex initiates 5' resection, while the Bloom's (BLM) protein (Sgs1 in yeast) together with a nuclease such as CtIP and EXO1 are required for more extensive resection of the ends (Sartori et al., 2007; Gravel et al., 2008). Subsequently the single-strand DNA binding protein RPA binds to the 3'ends followed by the assembly of Rad51 nucleoprotein filaments. RAD51 is the eukaryotic homolog of RecA in bacteria and is the central protein in recombination. In mammalian cells the filament formation is dependent on BRCA2 but also involves members of the RAD52 group including RAD52, RAD54, RAD54B and the RAD51 paralogs (RAD51B, RAD51C, RAD51D, XRCC2 and XRCC3). The RAD51 filaments catalyze the search for a homologous double-stranded DNA template and mediates strand invasion to form a joint molecule between the broken DNA ends and the repair template. After DNA synthesis using the homologous sequence as a template, the intermediate structures are resolved by unwinding followed by the annealing of the broken DNA ends and ligation (see Figure 3B). Using the sister chromatid as a template for repair synthesis leads to correct restoration of the original sequence. When the homologous chromosome is used as a template, HR may lead to homozygosity for certain mutations and is one of the mechanisms leading to LOH events which are frequently associated with tumor formation. Single strand annealing (SSA) is a subpathway of HR in mammalian cells and is dependent on the presence of sequence repeats on both sides of DSBs (Figure 3C). Resection of broken DNA ends is required and may lead to annealing of complementary DNA sequences. Subsequently, the ssDNA tails are removed by the endonuclease XPF/ERCC1 before ligation (Al-Minawy et al., 2007).

Impairment of HR and NHEJ pathways is associated with severe genetic disorders characterized by developmental defects, immunodeficiency, neurological degeneration, radiosensitivity, genomic instability, or cancer predisposition (for review see Scott and Pandita, 2006; Sung and Klein, 2006; Hakem, 2008). In humans, ataxia-telangiectasia like disorder (ATLD) and Nijmegen breakage syndrome (NBS) result from mutations in MRE11 and NBS1, respectively. Mutations in ATM and ATR lead to Ataxia-telangiectasia and Seckel syndrome, respectively whereas mutations in Artemis, DNA-PKcs and Ligase IV lead to radiosensitive severe combined immunodeficiency (SCID) disease. Biallelic mutations in BRCA2 give rise to Fanconi anemia (complementation group FA-D1), which at the cellular level is manifested by a defective HR pathway (Howlett et al., 2002).

ICL repair

DNA interstrand crosslinks (ICLs) are very toxic to dividing cells because ICLs prevent proper strand separation during DNA replication. ICLs can lead to mutations, chromosomal translocations, deletions and cell death. As in yeast, mammalian cells repair ICLs through the coordinated action of several DNA repair pathways including NER, HR and postreplication repair/translesion synthesis (TLS). Moreover, additional proteins such as Fanconi anemia (FA) and BRCA proteins have impact on the cellular response to ICL agents and constitute a signal transduction pathway that coordinates cell cycle checkpoint responses and ICL repair (for reviews see Dronkert and Kanaar, 2001; McHugh et al., 2001; Niedernhofer et al., 2005; Mirchandani and D'Andrea, 2006). Current models of ICL repair (Figure 4) propose that mammalian cells use the endonucleases to unhook the crosslinks at any stage of the cell cycle followed by TLS bypass and HR to resolve the generated DSBs during DNA replication (Rothfuss and Grompe 2004; Clingen et al., 2007). In yeast and human cells TLS polymerases were shown to be involved in ICL repair during G_1 (Sarkar et al., 2006; Mogi et al., 2008). After sensing of ICL, a structure is generated allowing the endonucleases XPF/ERCC1 and possibly MUS81/EME1 to perform incisions at both sides of the ICL which generate a monoadduct (Niedernhofer et al., 2005). Based on the studies of Rothfuss and Grompe (2004) and our study (Chapter 5), the unhooking of ICLs may occur in the absence of DNA replication. FANCM helicase (a component of the FA core complex) might be involved in the unwinding of the crosslinked DNA during the unhooking process (Niedernhofer et al., 2005). The activation of the FA/BRCA

pathway was suggested to stabilize stalled replication forks (Lomonosov et al., 2003). After unhooking of the ICL, TLS polymerases continue DNA synthesis to bypass the lesion and often introduce point mutations, whereas regular DNA polymerases continue on the other strand. The generated DSBs can be repaired by the HR machinery including RPA, BRCA2 (FANCD1) and RAD51 and the unhooked ICL adduct is removed by NER to reestablish DNA replication (Figure 4).

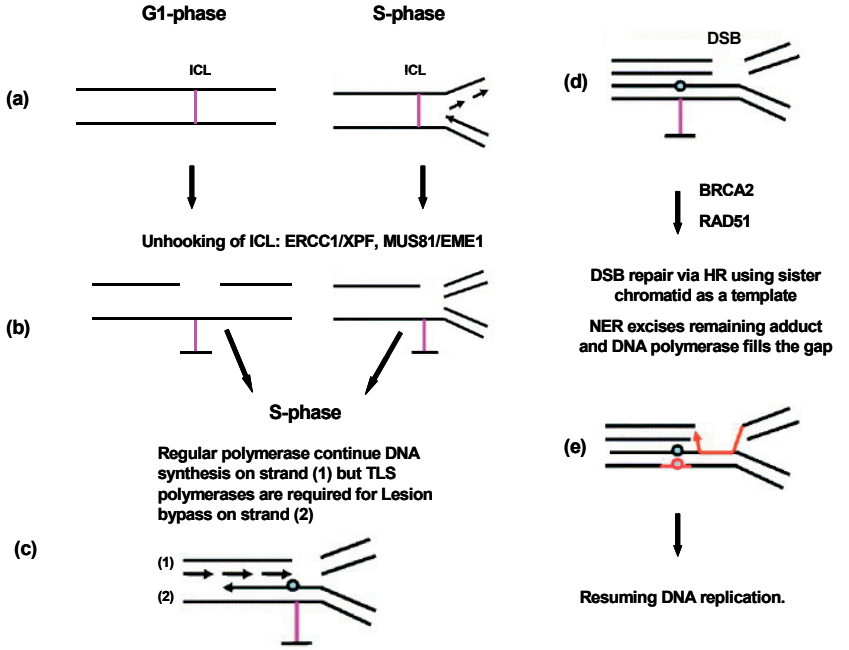


Figure 4: A model for crosslink repair in mammalian cells (adapted from: Rothfuss and Grompe 2004; Niedernhofer et al., 2005; Mirchandani and D'Andrea, 2006). (a) A DNA interstrand crosslink (ICL) is recognized in G_1 phase (left) or in S-phase at stalled replication fork (right). (b) In S-phase cells (right) stalled replication forks provide substrates for incisions by the heterodimeric endonuclease ERCC1/XPF and possibly the MUS81/EME1 endonuclease leading to unhooking of the ICL. This step generates a monoadduct and a gap. These endonucleases might also unhook the ICLs in G_1 (*continued*) (left); during progression through the S-phase replication forks stall at the incised ICL. (c) DNA synthesis by regular polymerases continues on the undamaged strand (1), whereas gap-filling DNA synthesis by a TLS polymerase (such as ζ, η or κ) is required to bypass the lesion and restore the damaged template strand (2), which frequently creates a point mutation at the site of damage. (d-f) The resulting DSB can be repaired by the HR and consequently DNA replication is resumed. The remaining unhooked ICL carrying a short oligonucleotide is removed by NER.

1.3.2 Transcription response

The transcription response of cultured human cells upon (geno)toxic exposure has been assayed by genome wide measurement of mRNA expression using DNA microarrays (Lamb et al., 2006). Arrest of the transcription machinery by DNA damage has been suggested to trigger DNA-damage signaling pathways. In response to DNA damaging agents and other types of cellular stressors, blockage of transcription triggers the activation (stabilization and phosphorylation) of the tumor suppressor protein p53, which accumulates in the nucleus and acts as a transcription factor regulating genes contributing to cell cycle arrest, DNA repair, and apoptosis (for reviews see Ljungman, 2000; Vousden and Lu, 2002; Ljungman and Lane, 2004; Latonen and Laiho, 2005).

Small noncoding RNA molecules called microRNAs (miRNAs) have been found to regulate gene expression by controlling protein concentration at post-transcriptional and translational levels and regulating cellular signaling network components and transcription factors. miRNA acts through the RNAi (RNA interfering) pathways and functions to downregulate the expression of many proteins by base-pairing with target mRNAs leading to degradation or translational repression of mRNAs (for reviews see Stevenson and Jarvis, 2003; Cui et al., 2006; Jackson and Standart, 2007). miRNAs play key roles in diverse cellular processes such as cell proliferation, cell-cycle progression, apoptosis and the response to stress and also have tumor suppressor or oncogenic activities (Chang et al., 2007; Ivanovska et al., 2008; for review see Taylor and Gant, 2007; Kundu and Surh, 2008). Several recent studies have implicated miRNAs (for example the miR-34 family) in the regulation of the p53 tumor suppressor network; the expression of these miRNAs is induced by DNA damage and oncogenic stress in a p53 dependent manner (Chang et al., 2007; for review see He et al., 2007). Moreover, alteration in the expression of miRNAs has been reported in mammalian cells following exposure to genotoxic stress including ionizing radiation, etoposide and H_2O_2 (Ishii and Saito 2006; Simone et al., 2007; Weidhass et al., 2007).

The expression of heat shock proteins (HSPs) is highly induced when cells are exposed to different types of environmental stress including DNA damage (Muramatsu et al., 1992, 1993; Morimoto, 1998; Zhou et al., 1998; Hunt, et al., 2004). The expression of heat shock genes is regulated by the heat shock transcription factor

(HSF1) which forms nuclear stress granules after exposure to elevated temperatures. These granules colocalize primarily to the satellite-III sequences in the pericentromeric heterochromatin of human chromosome 9 and also on the centromeric regions of chromosome 12 and 15 (Denegri et al., 2002; Jolly et al., 2002). RNA processing factors such as hnRNP-A1-associated protein (HAP) have been shown to translocate to the nuclear stress granules upon heat shock, supporting the hypothesis that the stress granules could be related to transcription and splicing activities during stress (Weighardt et al., 1999; Denegri et al., 2001). Moreover, in cells exposed to heat, HSF1- and RNA polymerase-II-dependent transcription of chromosome 9-specific satellite-III repeats generates large and stable RNA molecules present in the same stress-granules with HSF1 (Jolly et al., 2004; Rizzi et al., 2004). The transcripts are likely noncoding RNA molecules, as they remain associated with chromosome 9 during cell cycle progression (Jolly et al., 2004). The function of these transcripts is largely unknown, however, they were suggested to be involved in the negative regulation of gene expression as in case of miRNAs and in maintaining chromatin structure (Jolly et al., 2004; Biamonti, 2004; Sandqvist and Sistonen, 2004). Other studies demonstrated that the organization of silent pericentric heterochromatin and the inactivated X-chromosome is dependent on noncoding RNA molecules (Avner and Heard, 2001; Cohen and Lee, 2002; Maison et al., 2002). Also in yeast, RNAs encoded by centromeric repeats have been proposed to influence chromatin architecture by participating in the formation and maintenance of heterochromatin (for review see Jenuwein, 2002).

1.4 Chromosome Organization in Interphase Nuclei

The organization of chromosomes in interphase nuclei was first proposed by the Rabl model. In this model most chromosomes are polarized within the nucleus with centromeres and telomeres are at opposite poles of the nucleus (Cremer et al., 1993). Conventional microscopic studies of interphase nuclei reveal chromatin regions of different staining intensity, representing heterochromatin fractions of high density, euchromatin of lower density and nucleoli of lowest density. Interphase chromosome organization could be clearly traced when the technique of chromosome painting by fluorescence in situ hybridization (FISH) became available allowing determination of the arrangement of different chromosomes within nuclei by 3-dimentional (3D)

microscopy (for review see Cremer and Cremer, 2001; Parada and Misteli, 2002). The latter studies demonstrated that during interphase each chromosome occupies a distinct spatially well-defined subvolume of the nucleus called chromosome territory (CT). In addition to the ordered organization of chromosomes, protein components of the cell nucleus also exist in subnuclear compartments including the nucleoli, splicing factor compartments and PML bodies (Dundr and Misteli, 2001). In mammalian cells, there is a tendency toward clustering of centromeres inside the nucleus (Haaf and Schmid, 1991; Ferguson and Ward, 1992). In many cell types, heterochromatic regions including centromeres are observed as darkly stained chromocenters that have been suggested to form structure centers for chromatin organization and to influence essential nuclear processes such as gene expression, DNA replication and cell division (Schmid et al., 1975; Manuelidis, 1990; Haaf and Schmid, 1991). Moreover, in a recent study using confocal microscopy, spatial associations of centromeres were observed in human cells leading to cell-type specific heterochromatic compartments or chromocenters (Alcobia et al., 2000). The organization patterns of the genetic material and many of the components involved in gene expression point towards a role for spatial organization in epigenetic control of tissue specific gene expression (Alcobia et al., 2000; for reviews see Marshall, 2002; Parada et al., 2004).

Two models describing the distribution of CTs within the nucleus have been proposed (for reviews see Parada and Misteli, 2002; Misteli, 2004). One model based on the radial arrangement of CTs between the center and the envelope of the nucleus, suggests that in human lymphocytes gene-dense chromosomes (such as chromosome 19) are located preferentially in the center of the nucleus; the opposite holds for gene-poor chromosomes (Croft et al., 1999; Cremer et al., 2001). Similarly, transcriptionally active genes are positioned more closely to the interior of the nucleus than inactive genes (Roix et al., 2003; Zink et al., 2004; Williams et al., 2006). Radial positioning has been observed in human fibroblasts for chromosomes of different size; the smaller chromosomes are positioned more in the center of the interphase nucleus (Sun et al., 2000; Bolzer et al., 2005). In quiescent human fibroblasts the gene-poor chromosome 18 moves from nuclear periphery to a more internal location in the nucleus (Bridger et al., 2000). The other model is based on specific relationships between two or more CTs or chromosomal regions (for reviews see Parada and Misteli, 2002; Meaburn et al., 2007). Evidence for nonrandom positioning of chromosomes relative to each other has been reported for mouse chromosomes 12,

14 and 15. Clusters containing one copy of each of these three chromosomes have been frequently identified in mouse lymphocytes (Parada et al., 2002). Chromosomal regions containing ribosomal genes are known to cluster in most of eukaryotic cells forming two or more nucleoli, which are the sites of ribosomal gene transcription and rRNA processing. A similar clustering of tRNA genes has been reported in *S. cerevisiae* (Thompson et al., 2003). Association of human chromosomes 6 and 7 with specific transcription domains has been reported in G1 cells (Pombo et al., 1998). Recent evidence suggests that the proximal location of chromosomes also has a prominent role in gene expression (Osborne et al., 2004; Spilianakis et al., 2005) and in the formation of chromosome translocations (Kozubek et al., 1999; Elliott and Jacin 2002; Parada et al., 2002; Boei et al., 2006), thus influencing genome stability. In yeast, spatial proximity of genome regions also appears to influence recombination events (Bressan et al., 2004). The spatial vicinity of radiation induced DNA breaks leads to the formation of exchanges between chromosomes containing these lesions (Savage 2000). The preferential induction of exchanges between the homologues of human chromosome 19 by IR was found to correlate with their preferred proximal position in the nuclear center (Boei et al., 2006). Moreover, chromosome rearrangements associated with disease or cancer state were found to originate from closely positioned chromosomes in the corresponding normal tissue (Nikiforova et al., 2000; Parada and Misteli, 2002). In addition to proximity effects, homology-dependent DNA repair might also be responsible for the increased levels of exchanges between homologous chromosomes formed after exposure of human cells to IR (Plan et al., 2005). In the latter study, the observed frequency of dicentrics involving homologous chromosomes was about 3-folds higher than the expected frequency based on the probability of occurrence by chance. The studies focusing on higher-order chromatin arrangements in a variety of human cells including fibroblasts and lymphocytes revealed high variability in the distances between CTs of homologous chromosomes (Ferguson and Ward, 1992; Cremer et al., 2001; Bolzer et al., 2005). Thus homology dependent DNA repair might explain DNA damage-induced association of homologous chromosomes in interphase (Dolling et al., 1997; Abdel-Halim et al., 2004; 2005).

Recent studies revealed that chromatin fibers from one CT intermingle with adjacent CTs (Branco and Pombo, 2006). In the latter study high-resolution fluorescent microscopy and electron micrographs of regions where CTs are in contact,

showed condensed bundles containing chromatin from different chromosomes. Chromatin mobility was proposed as a mechanism to mediate chromosome intermingling (Aten and Kanaar, 2008). Moreover, a significant correlation between the extent of intermingling and the frequencies of radiation-induced chromosome translocations has been found. Interestingly, inhibition of transcription lead to changes in the intermingling patterns of certain chromosomes (Branco and Pombo, 2006) indicating that the degree of intermingling and dynamics of chromatin are closely linked to chromatin function.

1.4.1 Pairing of homologous chromosomes

Pairing of homologous chromosomes is an essential feature of meiosis to promote genetic recombination and to allow correct segregation of homologues in the first meiotic division (for review see McKee, 2004). Moreover, homologous pairing also occurs in somatic and germ-line mitotic cells of Dipteran insects forming polytene chromosomes (Hiraoka et al., 1993; Csink and Henikoff, 1998; Fung et al., 1998). For a long time, conflicting evidence existed as to whether somatic pairing occurred outside of what was seen in Diptera (Haaf and Schmid 1991). Chromosome associations in non-mammalian organisms, i.e. yeast (Burgess et al., 1999) and plants (Bender, 1998; Watanabe et al., 2005), are widely observed for genomic regions that contain stretches of repetitive elements. Simple-repeat elements found at telomeres or centromeres are obvious candidates for long-range pairing, which in turn will contribute to the positioning of interphase chromosomes (for review see Taddei et al., 2004). Accumulating evidence indicates that in mammalian somatic cells pairing or association between homologues does occur in a locus- and cell-type-specific manner in both normal and malignant cells. For example, in human cells nonrandom association of homologues is characteristic of Sertoli cells (Chandley et al., 1996) and quiescent fibroblasts (Nagele et al., 1999). Similarly, in earlier work by Arnoldus et al. (1989), the existence of tissue dependent somatic pairing was observed. In the latter study, homologous association of human chromosome 1 was detected in nuclei taken from cerebellar tissue but not in nuclei from cerebral tissue. Also, pairing of centromeric regions of chromosome 17 was observed in cells obtained from different brains using satellite DNA probes (Arnoldus et al., 1991). Moreover, using 3-D FISH techniques combined with fluorescence-activated cell sorting, LaSalle and Lalande

(1996) found in human lymphocytes that homologous associations occur specifically at the imprinted 15q11-q13 regions during late S-phase of the cell cycle. In mouse fibroblasts preferential pairing of the imprinted regions on chromosome 7 has also been observed in s-phase (Riesselmann and Haaf, 1999). Cells from normal and malignant human prostates showed a significant incidence of somatic pairing of the centromeres of chromosomes 17 (Brown et al., 1994) and chromosomes 9 and 17 (Williams et al., 1995) during interphase. In the latter study, chromosome 9 exhibited somatic pairing in both prostate and lymphoblastoid cells and the pairing appeared to be limited to the satellite-III pericentromeric heterochromatin. Somatic pairing was also observed for the centromeres and p-arms of chromosome 15 in normal and malignant cells of the hematopoietic and lymphoid system (Lewis et al., 1993) and for centromeres of chromosomes 7 and 10 in follicular lymphoma (Atkin and Jackson, 1996). In addition, evidence was obtained for interphase somatic pairing of centromeric heterochromatin of chromosomes 1 and 16 in lymphocytes of patients with ICF syndrome (Maraschio et al., 1992) and for pairing of the subtelomeric regions of chromosome 4 and 10 in facioscapulohumeral muscular dystrophy (FSHD) patients (Stout et al., 1999).

Numerous models have been proposed for the mechanism by which homologous chromosomes become paired. In general, these models suggest either random (driven by diffusion) or active movement of chromatin to bring homologous regions into contact (Fung et al., 1998; for review see Barzel and Kupiec, 2008). The biological relevance of pairing of homologous sequences in somatic cells compared to meiosis is less clear. Various investigators have suggested a role of somatic pairing in promoting DSB repair via HR, or facilitating interactions between homologous transcriptional regulatory sequences (Hiraoka et al., 1993; Csink and Henikoff, 1998; Fung et al., 1998; Vazquez et al., 2002). Pairing of heterochromatin regions in somatic cells was also suggested to be one of the factors responsible for producing some degree of order within the interphase nucleus (Comings, 1980). Moreover, homologous somatic pairing is thought to stimulate the development of the heterochromatin variants of human chromosomes by induction of translocations with breakpoints localized within the heterochromatin (Schmid et al., 1983; Haaf et al., 1986).

1.4.2 Heterochromatin: structure and function

Heterochromatin is generally transcriptionally silent, replicated late during S-phase, localized at the nuclear periphery, and is relatively inaccessible to DNA-modifying enzymes (Jackson, 1997). In most eukaryotes heterochromatin is concentrated in pericentromeric and telomeric regions. There are two types of heterochromatin, i.e. constitutive and facultative, which differ slightly depending on the DNA content. Facultative heterochromatin is C (centromeric specific)-band-negative, characterized by the presence of G-bands (staining AT-rich gene poor chromatin regions) and rich in LINE-type repeated sequences. Its configuration is reversible, i.e. changeable between the heterochromatic and euchromatic state depending on the developmental stage or the cell type, as in the case of one of the X-chromosomes (Barr-body) of females (Lyon, 2000). Constitutive heterochromatin is stable, C-band-positive, and its heterochromatic properties are conserved during all stages of development and in all tissues. Heterochromatin contains a particular type of DNA called satellite DNA, which is highly repetitive and highly condensed forming the compact structure of the constitutive heterochromatin. Satellite DNA consists of large numbers of short tandemly repeated sequences classified into different satellite DNA families (for review, see Lee et al., 1997). Alpha-satellite DNA is AT rich and present in the centromeric regions of all human chromosomes (Manuelidis, 1978). Satellite-I DNA is located more specifically at the pericentromeric regions of chromosomes 3 and 4, and the pericentromeric regions and the short arms of the acrocentric human chromosomes (Tagarro et al., 1994). Satellite-II DNA sequences are predominantly localized at the heterochromatic regions (secondary constrictions) of chromosomes 1 and 16 (Moyzis et al., 1987; Tagarro et al., 1994). Satellite-III DNA is localized mostly at the secondary constriction of chromosome 9, but also to some extent located at the heterochromatin of chromosome 1, short arms of acrocentric chromosomes and the Y chromosome (Frommer et al., 1988; Schwarzacher-Robinson et al., 1988; Tagarro et al., 1994; Lee et al., 1997). The DNA satellite-I is AT rich whereas satellites II and III are composed of both AT and GC rich DNA sequences (Jeanpierre, 1994, Lee et al., 1997). Another GC rich DNA repeat family is the beta satellite DNA, which localizes also at the short arms of acrocentric chromosomes, and the pericentromeric regions of chromosomes 1, 3, 9, and Y (Waye and Willard, 1989; Greig and Willard, 1992; Meneveri et al., 1993).

Currently, heterochromatin is defined on the basis of DNA composition, associated proteins, post-translational histone modifications, and function, i.e. epigenetic gene silencing. However, some of these characteristics are common to euchromatin. For example, heterochromatic regions are enriched with hypoacetylated histone, methylated histone H3 lysine 9 and heterochromatin protein 1 (HP1) but euchromatic regions also contain these heterochromatic modifications and proteins (for a review see Peng and Karpen, 2008). Although heterochromatin is often described as mainly comprising transcriptionally inert junk DNA sequences, it contains the highly active ribosomal RNA and other protein-encoding genes (Hoskins et al., 2007; Smith et al., 2007). It is well known that the positioning of genes close to heterochromatin blocks can strongly affect their transcription. The study of Tsukamoto et al. (1997) revealed that in yeast proteins associated with heterochromatin-mediated transcriptional silencing, i.e. Sir proteins, are also involved in the repair of DNA damage induced by ionizing radiation suggesting a connection between heterochromatin-mediated silencing and DNA repair. In the latter study, the yeast Ku70 was shown to interact with Sir4 and inactivation of Sir2, Sir3 and Sir4 lead to severe defects in Ku-dependent DNA NHEJ suggesting that Sir2, Sir3 and Sir4 are components of the Ku-dependent DNA repair apparatus. Heterochromatin is also essential for chromosome organization and for centromere and telomere functions (Bernard et al., 2001; de Lang 2005) and it is formed and regulated by transcripts and the RNAi pathways (Peng and Karpen, 2008). Some histone modifications including acetylation and methylation are responsible for the compartmentalization of the genome into euchromatin and heterochromatin (Martin and Zhang 2005). It has been shown using the enhanced green fluorescent protein (EGFP)-HP1-β fusion protein that exposure of cells to IR and also the clastogenic drug etoposide induces dynamic alterations of chromatin triggered by mobilization (releasing from chromatin) of HP1-β (a chromatin factor bound to histone H3 methylated on lysine 9). This mobilization promotes a DNA damage response and H2AX phosphorylation in mammalian cells (Ayoub et al., 2008). In the latter study, the alteration of EGFP-HP1-β was induced in both euchromatin and heterochromatin and inhibition of the release of HP1-β diminishes H2AX phosphorylation (Ayoub et al., 2008). Other studies also show that changes in characteristic features of heterochromatin such as CpG methylation, HP1 hypermethylation and changes in histone modifications are correlated with certain

diseases and cancer progression (Ding and Kleer, 2006; Norwood et al., 2006; Scaffidi and Misteli, 2006; Tan et al., 2007).

The heterochromatic region (9q12) of chromosome 9 is a mixture of several GC-rich satellite DNAs specially satellite III enriched in methylcytosine containing DNA (Miller et al., 1974; Gosden et al., 1975; Rocchi et al., 1991; Meneveri et al., 1993; Tagarro et al., 1994; Koch and Stratling, 2004). It is well known that this region is heteromorphic, i.e. characterized by heritable variations at homologous chromosomal sites between different individuals as revealed by standard cytogenetic techniques such as C- and Q-banding (Caspersson et al., 1970; Arrighi and Hsu, 1971; Kim, 1974; for a review see Kowalczyk et al., 2007). The heteromorphisms in the pericentric heterochromatin region of chromosome 9 include mainly variations in the size of this region, which have been suggested to originate from rearrangements and exchanges (including intra- and inter-chromosomal recombination) involving the different classes of satellite DNA repeat sequences (Wang and Miller, 1994; Macera et al., 1995; Ramesh and Verma, 1996; Samonte et al., 1996; Park et al., 1998; Willatt et al., 2007). Heteromorphisms inside repetitive sequences were initially proposed to have no impact on phenotype however, in more recent research a correlation was found between the heteromorphism of chromosome 9 heterochromatin and reproductive and infertility problems, mental retardation and cancer predisposition (Sasagawa et al., 1998; Starke et al., 2002; Madon et al., 2006). There is a tendency for the heterochromatic regions to pair when they have similar repetitive sequences (Haaf et al., 1986). Moreover, the study of Starke et al. (2002) suggested several hotspots for recombination in the pericentromeric heterochromatic region of chromosome 9; moreover, a general role for the GC-rich repetitive heterochromatic sequences in meiotic and somatic pairing has been proposed (Chandley et al., 1989; Vazquez et al., 2002). The presence of highly repetitive satellite sequences and the duplicated nature of the region 9q12 reflect its complexity. This region has been shown extremely difficult to sequence and still contains a large number of sequence gaps (Eichler et al., 2004; Humphray et al., 2004). Nonetheless, 138 gene features were annotated within the pericentromeric heterochromatin of chromosome 9 (Humphray et al., 2004). Moreover, The study of Gilbert et al (2004) using sucrose gradient sedimentation to separate open and compact chromatin fibers revealed that the satellite-III DNA containing region 9q12 hybridizes strongly to both open and compact chromatin. This indicates that this region of the genome is heterogeneous

and contains both heterochromatin and euchromatin. Interestingly, transcriptional activity of satellite-III DNA repeats of 9q12 region has been reported in cells exposed to stress suggesting that this region has properties of euchromatin (Jolly et al., 2004; Rizzi et al., 2004). Moreover, HSF1 containing stress granules formed at the 9q12 region, were shown to be juxtaposed to HP1-β fluorescent signals on the centromeres of chromosome 9 demonstrating that the paracentromeric 9q12 region does not display conventional heterochromatic features (Jolly et al., 2004).

1.4.3 Changes, repositioning and pairing of chromatin after DNA damage

The studies of chromatin using live microscopy methods revealed a high degree of local chromatin dynamics related to gene expression (for a review see Lanctot, et al., 2007; Soutoglou and Misteli, 2007). In general, the mobility of gene loci and chromosomes in interphase is restricted by the nuclear architecture of CTs and occurs by local diffusion within a volume of approximately 1 μm (Marshall et al., 1997; Vasquez et al., 2001; Walter et al., 2003). This can explain chromosome intermingling at borders of CTs (Aten and Kanaar, 2008). However, large-scale movement of chromosome domains over distances of several micrometers can occur by unfolding of chromatin resulting in colocalization of distal genes during their transcription (Osborne et al., 2004; Sproul et al., 2005). Also cell cycle dependent movement of centromeres from the nuclear periphery to the interior of the nucleus has been shown in human cells (Ferguson and Ward, 1992). Transcription activation of certain chromosome regions can also induce the movement of these regions to the nuclear interior and modify their cell cycle dependent positioning (Tumbar and Belmont, 2001).

An important factor that might lead to changes in the positioning of chromosomes or genetic loci is the signaling and/or processing of DNA damage. In order for chromosomes to change position there must be an underlying movement of chromatin per se. Indeed, evidence for the dynamic nature of the interphase nucleus and chromatin movement after infliction of DNA damage has been accumulated in recent years. There is biochemical evidence that chromatin structure is remodeled in the presence of DSBs, i.e. the higher order structure of chromatin is altered to permit the access of proteins involved in cell cycle checkpoints and DNA repair (for reviews see Peterson and Cote, 2004; van Attikum and Gasser 2005). Dynamic changes in

histone acetylation and phosphorylation (such as H2AX phosphorylation) in chromatin flanking DSBs were suggested to mediate the accessibility of damaged DNA to signaling proteins and chromatin remodeling complexes (Pilch et al., 2003; Kusch et al., 2004; Murr et al., 2006). The relaxation of chromatin structure also occurs within the entire nucleus after exposure to UV irradiation (Rubbi and Milner, 2003). Changes in the relative positions of the *ABL* and *BCR* genes in stimulated human lymphocytes were found after exposure to ionizing radiation (Kozubek et al., 1997). The study of Figgitt and Savage (1999) using FISH in G_0/G_1 human fibroblasts revealed chromosomal domain break-up and movement within 1 hour after exposure of cells to IR. Moreover, changes in the spatial distribution leading to clustering of DSB-containing chromosome domains occurred within minutes after exposure to α particles indicating movements of the domains (Aten et al., 2004). In the latter study, movement was noticed in the distance range of several μm. However, using the GFP technology to examine the mobility and structure of chromatin containing DSBs in living cells, Kruhlak et al. (2006) found that chromatin containing DSBs exhibits limited mobility but undergoes an energy-dependent local expansion immediately after DNA damage probably to facilitate DNA damage signaling and repair. This process occurs in both euchromatin and heterochromatin with similar kinetics. Live cell imaging in mammalian cells revealed that chromatin at both sides of the DSB exhibited only small-scale local motion (Soutoglou et al., 2007). In the latter study, significant mobility and separation of broken ends were visible in Ku80 mutant mammalian cells, which are defective in NHEJ repair. The authors suggested that in the absence of NHEJ, repair by HR requires extensive mobility of the broken ends in order to find homologous sequences (Soutoglou et al., 2007).

Damage to DNA has also been reported to influence the position of chromosome domains with respect to their homologues in prokaryotes and eukaryotes. Alignment of homologous chromosomes in the bacterium *D. radiodurans* has been suggested to explain the extreme resistance to ionizing radiation (Daly and Minton, 1996). The authors proposed a model in which pairing along the entire length of homologous chromosomes would permit efficient repair of potentially lethal DSBs induced by radiation. In human interphase cells, Dolling et al., (1997) demonstrated that the homologues of chromosomes (7 and 21) rearrange and become more closely oriented to each other in response to radiation exposure. Also, a reduction in the inter-

homologue distances of different genetic loci including the centromeres of chromosomes 6, 8, 9 and 17 was found in human leukemia cells after γ irradiation (Skalnikova et al., 2000; Jirsova et al., 2001). The authors attributed these results to the decrease in the radial position, i.e. reduction in the distance between the examined loci and the nuclear center. Moreover, treatment of human lymphocytes with L-histidine in the presence of H_2O_2, which is known to induce single- and double-strand breaks, induced an increased association of the homologous pericentric regions of chromosomes 1, 15 and X (Monajembashi et al., 2005). The alterations in nuclear topography after exposure to DNA damage were suggested to be important for DNA repair and the induction of chromosomal exchanges (Kozubek et al., 1997; 1999; Figgitt and Savage, 1999; Spitkovsky et al., 2002; Aten et al., 2004).

In earlier cytogenetic studies, the examination of chromosome damage induced by MMC in human cells revealed an overrepresentation of interchanges between the heterochromatin of chromosomes 1, 9 and 16 particularly homologous exchanges (Shaw and Cohen, 1965; Morad et al., 1973). In these studies the authors suggested that heterochromatic regions are somatically paired, either spontaneously or by the effect of MMC, in the interphase nuclei to explain the preferential formation of homologous exchanges. Measurement of the distances between the paracentromeric heterochromatic regions of human chromosomes 9 homologues revealed random distribution of these regions in interphase confluent fibroblasts (Abdel-Halim et al., 2004; 2005). Exposure of these cells to IR, MMC treatment, UV irradiation and also heat shock treatment revealed repositioning and colocalization (pairing) of the heterochromatic regions in a sub-population of cells indicating large scale movement (Abdel-Halim et al., 2004, 2005, 2006). Most notably, lack of pairing of homologous heterochromatic regions in XPF and FANCD1/BRCA2 deficient cells goes together with the lack of induction of H2AX phosphorylation after MMC treatment (Abdel-Halim et al., 2005; Chapter 5 of this thesis). We propose that the spatial chromosome associations (pairing) and the reduction of distances between homologous chromosomes induced by DNA damage might be a consequence or a prerequisite of homology dependent repair of DNA damage that may lead to the formation of homologous chromosome exchanges. In addition, transcription activation may play a role in bringing homologous regions together.

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