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Chromatin modifiers in DNA repair and human disease

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GENERAL INTRODUCTION

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DNA is the macromolecule that encodes the genetic information of life. It defines the structure, organization and function of each cell and therefore it is crucial to preserve the integrity of the DNA during lifespan. However, the DNA is constantly exposed to various genotoxic threats that lead to around 1.000 to 1.000.000 lesions per cell each day (Lindahl, 1993). If these lesions are repaired incorrectly or left unrepaired, genetic alterations (mutations) occur that can lead to cell death and/or genome instability, and consequently to human diseases such as neurodegeneration and cancer.

DNA organization

In eukaryotes chromosomal DNA is organized into a highly condensed structure called chromatin. The basic unit of chromatin is the nucleosome, which is composed of ~147 base pairs of DNA that is wrapped around histone octamers in two left-handed superhelical turns. Each histone octamer contains two copies of each of the four conserved core histones H2A, H2B, H3 and H4. However, several histone variants can be incorporated that can affect nucleosome or higher-order chromatin structure. In addition, the binding of non-histone proteins can add to the degree of chromatin compaction. Very condensed chromatin is called heterochromatin, whereas very open and transcriptionally active DNA structures are referred to as euchromatin.

DNA damage response

The packaging of DNA into chromatin does not protect DNA from the constant attacks by various exogenous and endogenous DNA damage-inducing agents causing a large variety of structural different DNA lesions. Fortunately, cells have evolved sophisticated mechanisms that can sense DNA damage. Subsequently, a multi-step signaling cascade is triggered to transduce the DNA damage signal and to promote the recruitment and/or activation of effector proteins that can mediate DNA damage repair, change the chromatin composition, adjust the transcriptional program and pause cell cycle progression if necessary. However, if the occurred DNA damage is beyond repair, a cell can also enter programmed cell death called apoptosis. These events are collectively referred to as the DNA damage response (DDR) and take place simultaneously with the ultimate goal to maintain DNA integrity. Thus, although discussed separately below, the signaling and repair of DNA damage operate in chorus and several proteins actually function within both parts of the DDR.

Since the DDR maintains the stability of the genome in cells, it is extremely important for human health. It is therefore not surprising that inactivating mutations in DDR genes cause rare hereditary genetic disorders like Xeroderma Pigmentosum and Ataxia Telangiectasia (De Boer and Hoeijmakers, 2000; McKinnon, 2012). Patients that suffer from such disorders are often not able to effectively respond to DNA damage, and hence display a highly increased risk to develop DNA damage related disease such as cancer. AT patients additionally present with defective brain development and a weakened immune system.

DNA damage response upon DNA double-strand breaks

One of the most toxic forms of DNA damage is the DNA double-strand break (DSB), which is due to the menacing information loss on both DNA strands when a DSB occurs. Replication fork stalling or collapse as well as the covalent attachment of a protein such as SPO11 during meiosis can lead to DSB induction. Additionally, the exposure to ionizing radiation (IR), the

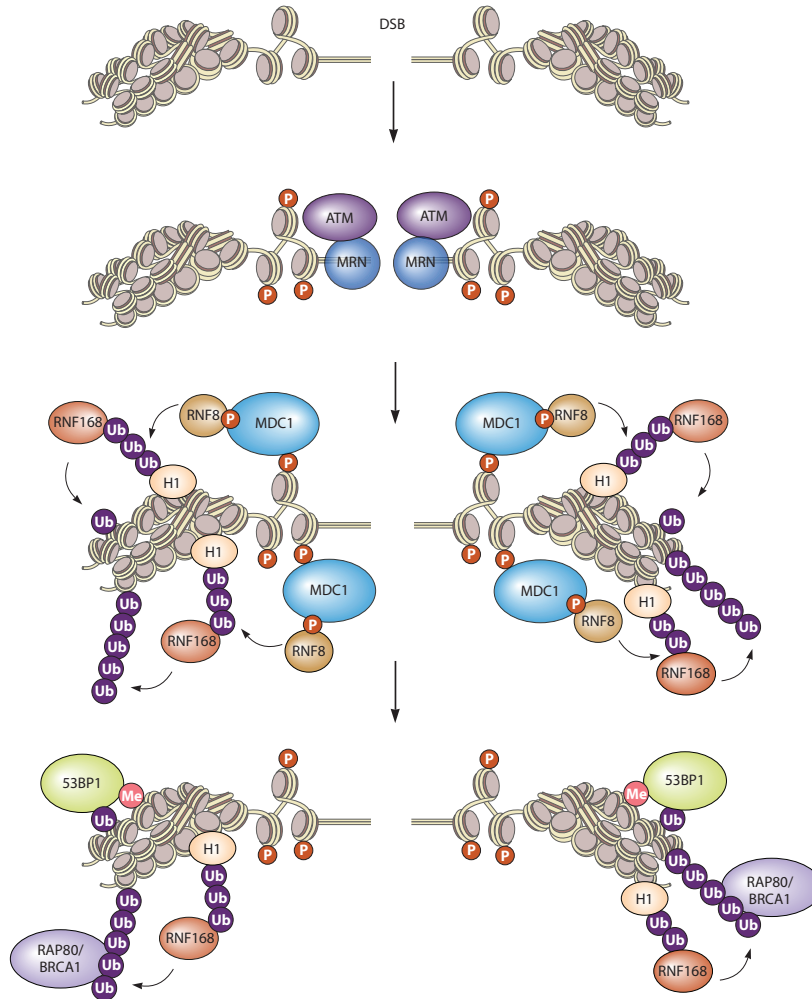


Figure 1. Overview of the signaling response to DSBs. DSBs are sensed by the MRN complex that directly recruits the ATM kinase to the lesion. The subsequent ATM-dependent phosphorylation of histone H2AX (called γ H2AX) in DSB flanking chromatin facilitates the binding of MDC1 nearby the site of DNA damage. MDC1 functions as a binding platform for the RNF8 E3 ubiquitin ligase. RNF8 initiates an ubiquitin-dependent cascade by ubiquitylating histone H1. The formed poly-ubiquitin chains are subsequently bound by the E3 ubiquitin ligase RNF168, which targets H2A(X). These events eventually culminate in monoubiquitin-dependent accrual of 53BP1, that is simultaneously reliant on the availability of methylated histone H4 (H4K20me is a pre-existing methylation mark and thus not DNA-damage induced, however it is not shown in all panels for clarity reasons.), and agglomeration of poly-ubiquitylated H2A(X), that for instance attracts the RAP80-BRCA1 complex.

treatment with chemicals such as camptothecin or the occurrence of several DNA lesions within a relatively small region can also result in DSB formation. When a DSB is inflicted, a fine-tuned DDR is triggered that coordinates cell cycle progression and DNA repair (Ciccia and Elledge, 2010; Jackson and Bartek, 2009; Smeenk and van Attikum, 2013). A key feature of the DDR is the assembly of signaling and repair factors in the vicinity of DSBs (Bekker-Jensen and Mailand, 2010; Huen and Chen, 2010). Initially, DSBs are sensed by the Mre11-Rad50-Nbs1 (MRN) complex (Petrini and Stracker, 2003), which directly attracts the PIKK

kinase ATM at the lesion and assists in phosphorylation dependent ATM activation (p-ATM); subsequently, p-ATM phosphorylates all three members of the MRN complex to initiate downstream signaling. Phosphorylation of histone H2AX (called γ H2AX) by ATM in DSB flanking chromatin culminates in the binding of MDC1 nearby the site of DNA damage. The subsequent binding of the RNF8 E3 ubiquitin ligase to MDC1 in turn triggers a ubiquitin-dependent cascade, involving the recruitment of the E3 ligase RNF168 to poly-ubiquitylated histone H1, the subsequent ubiquitylation of histone H2A/H2AX by RNF168, as well as the ubiquitin-dependent accrual of 53BP1 and the RAP80-BRCA1 complex (Fig. 1) (Doil et al., 2009; Lok et al., 2012; Stewart et al., 2009; Thorslund et al., 2015; Wang and Elledge, 2007).

Double-strand break repair - Homologous recombination

Two major pathways facilitate the repair of DSBs namely homologous recombination (HR) and non-homologous end-joining (NHEJ). HR mediates the error-free repair of DNA breaks during the S or G2 phase of the cell cycle by using the sequence information from an undamaged, homologous template, usually the sister chromatid (San Filippo et al., 2008). In more detail, MRN facilitates short-range degradation of the broken DNA ends together with CtIP to create 3' single stranded DNA (ssDNA) overhangs. This is followed by long range end-resection mediated by either EXO1 alone or the concerted action of the nuclease DNA2 with the BLM helicase (Liu et al., 2014). The ssDNA overhangs are bound and stabilized by RPA to prevent degradation and the formation of secondary structure. Simultaneously, the Partner and localizer of BRCA2 (PALB2) is recruited in a BRCA1-dependent manner and the retention of PALB2 at chromatin is mediated by its Chromatin Association Motif (ChAM) (Bleuyard et al., 2012; Zhang et al., 2009b; Zhang et al., 2009a). PALB2 also comprises a WD40 domain that facilitates its interaction with BRCA2, an event that is crucial for BRCA2 recruitment to DSBs (Sy et al., 2009; Xia et al., 2006). Subsequently, BRCA2 promotes RPA displacement and loading of the RAD51 recombinase, forming an ssDNA-containing nucleoprotein filament. Once bound to ssDNA, RAD51 can search for and invade a homologous duplex DNA template. Subsequently, restoration of the original DNA sequence is achieved by DNA synthesis and ligation (Fig. 2) (Liu et al., 2014).

Double-strand break repair - Non-homologous end-joining

Classical NHEJ (c-NHEJ) is the dominant pathway for DSB repair in mammalian cells. It re-joins the broken DNA ends and is active throughout the whole cell cycle. However, c-NHEJ has no inherent mechanism to ensure the restoration of the original DNA sequence in the vicinity of DSBs and can therefore be either error-free or error-prone. During c-NHEJ repair, the DNA ends are bound and held in close proximity by a single molecule of the heterodimer Ku70/Ku80, which attracts the DNA-dependent kinase DNA-PKcs to form the DNA-PK complex. DNA-PKcs mainly undergoes autophosphorylation, but also displays activity towards other NHEJ factors. A subset of DSBs requires DNA end-processing before re-joining can occur. In that case, the endonuclease Artemis can resect the broken DNA ends upon interaction with DNA-PKcs. On the contrary, the DNA polymerases μ and λ can add nucleotides to fill in remaining gaps. These events are subsequently followed by DNA ligation, a process that is facilitated by the DNA ligase IV, XRCC4 and XLF/Cernunnos complex (Fig. 3) (Kakarougkas and Jeggo, 2014; Lieber, 2010; Liu et al., 2014).

Noteworthy, a second NHEJ repair pathway has been discerned and is referred to as alternative NHEJ (alt-NHEJ). While c-NHEJ, as described above, is the only DSB repair pathway that can operate during all phases of the cell cycle, alt-NHEJ mainly operates during

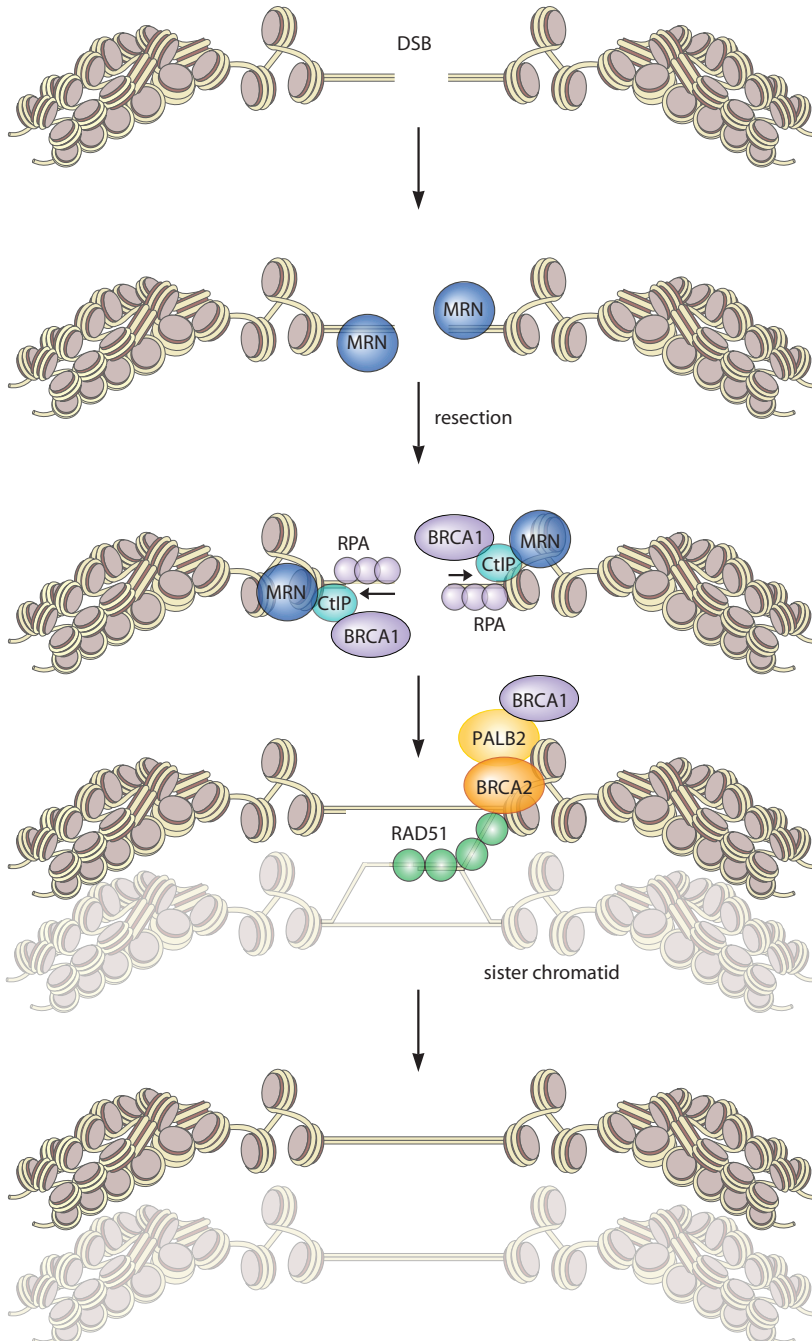


Figure 2. Overview of DSB repair by the homologous recombination (HR) pathway. 5'–3' DNA end resection is initiated by the MRN complex together with CtIP and the 3' ssDNA is coated by RPA. BRCA1 and CtIP physically interact at DSBs, while BRCA1 also recruits and binds PALB2, which in turn facilitates the accrual of BRCA2. Eventually, RPA is exchanged for RAD51 by BRCA2. The RAD51 filaments mediate the search for a homologous sequence and invasion of the homologous strand. Upon DNA synthesis, the formed DNA structures are resolved and the DNA strand is restored in an error-free fashion.

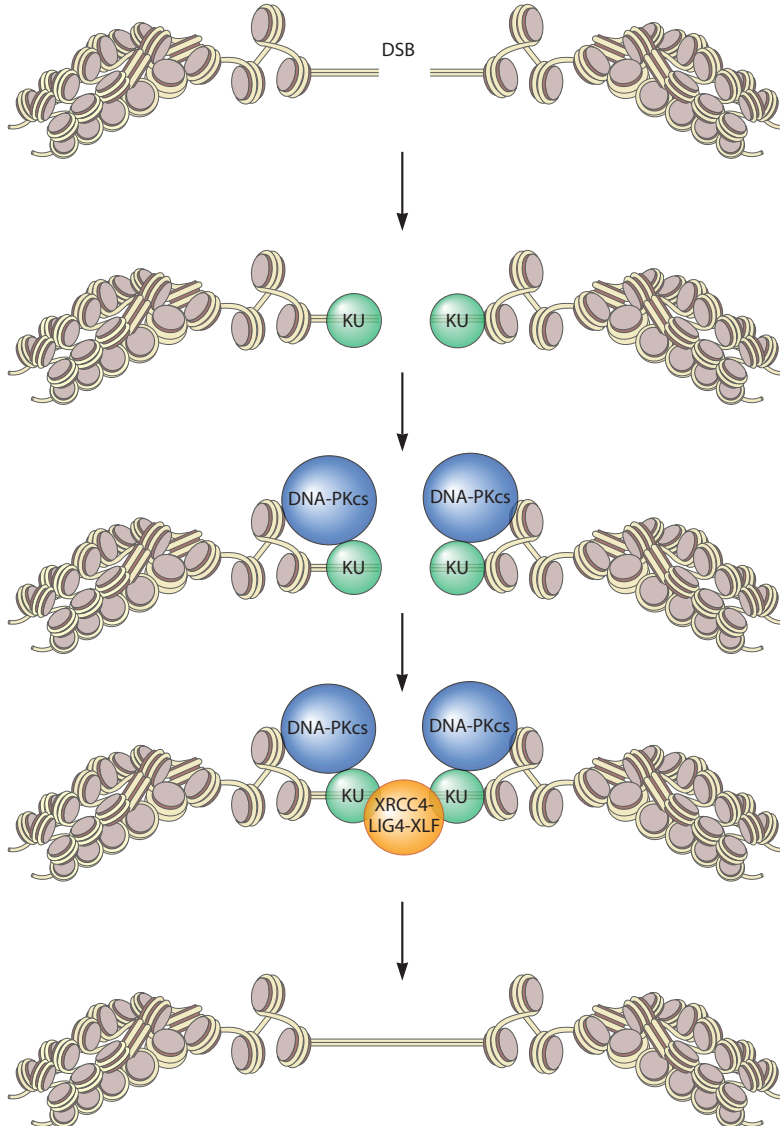


Figure 3. Overview of the Non-homologous end-joining (NHEJ) pathway. The Ku70/80 dimer binds DNA ends and recruits DNA-PKcs that undergoes activation. End-processing enzymes are attracted, which modify the DNA ends. The accumulation of the XRCC4-LIG4-XLF complex results in the ligation of the broken DNA ends.

S-phase and only if classical NHEJ is not functional i.e. when proteins like Ku70/80, DNA-PKcs or XRCC4/LigaseIV are unavailable or inactive (Lieber, 2010). This alternate pathway is initiated through the binding of PARP1 to the DSB, which can be in competition with Ku-binding (Wang et al., 2006). Next, the end-processing enzymes MRN, CtIP and BRCA1 assemble to facilitate DSB end resection. Alt-NHEJ occurs if micro-homologies of 5-25 bp are exposed upon end resection that enable the DNA single strands to anneal. Due to the use of micro-homology to stabilize the DSB ends, alt-NHEJ is also frequently referred to as micro-homology mediated end-joining (MMEJ) (Liu et al., 2014). Finally, the ligation of the broken ends involves either the LigaseIII/XRCC1 complex or DNA LigaseI in mammalian cells (Fig. 4).

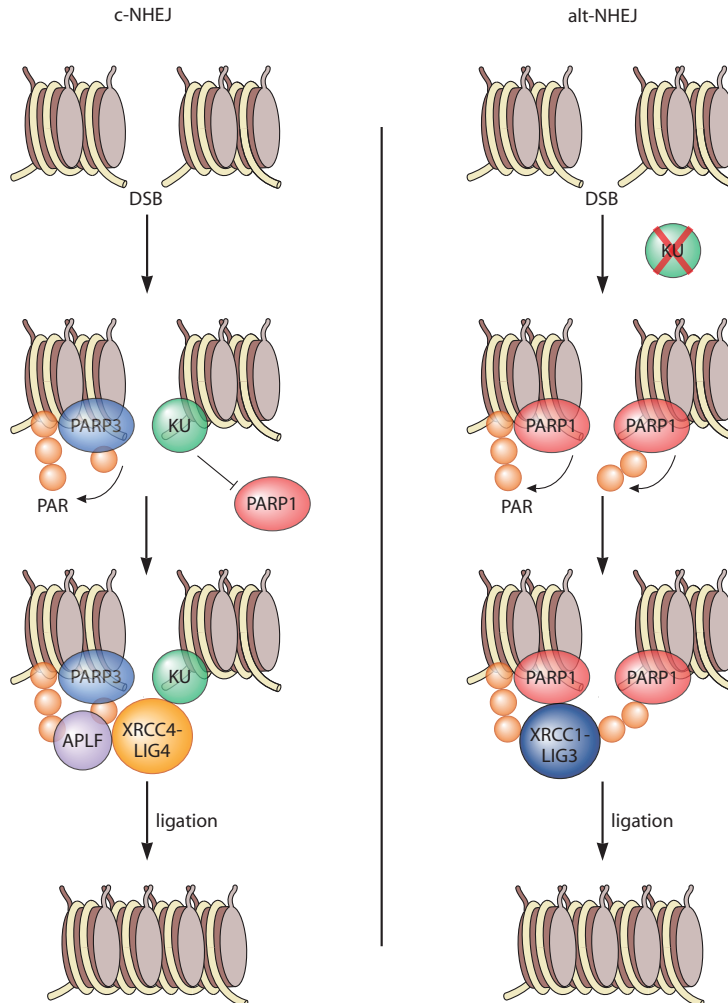


Figure 4. Classical versus alternative NHEJ and the role of PARP1. DSBs are mainly repaired through rapid classical NHEJ. However, in the absence of Ku, PARP1 binds efficiently to DSBs, which leads to its activation, resulting in auto-poly(ADP-ribosyl)ation. The synthesis of poly(ADP-ribosyl) (PAR) chains initiates the recruitment of the XRCC1-LIG3 complex leading to a slow sealing of the DSB in an XRCC4-LIG4 independent manner.

Since deletions regularly occur upon DSB end processing during alt-NHEJ, this pathway is considered to be an error-prone pathway.

NHEJ also has an essential role during the somatic gene rearrangement process V(D)J recombination and throughout the process of immunoglobulin (Ig) gene-diversification called class-switch recombination (CSR). These processes take place at the immunoglobulin heavy chain (IgH) locus that comprises the variable (V), diversity (D) and joining (J) gene segment and the constant region (C) (Fig. 5). During V(D)J recombination the RAG1/2 complex deliberately generates sequence-specific DSBs. One segment of each V, D and J region is subsequently joined through c-NHEJ and together these regions encode for the variable domain of the Ig that defines the antigen specificity (Fig. 5). In maturing B and T lymphocytes, V(D)J occurs in a multistep rearrangement process at the Ig or T cell receptor locus respectively, leading to the generation of a diverse repertoire of Igs and T cell receptors.

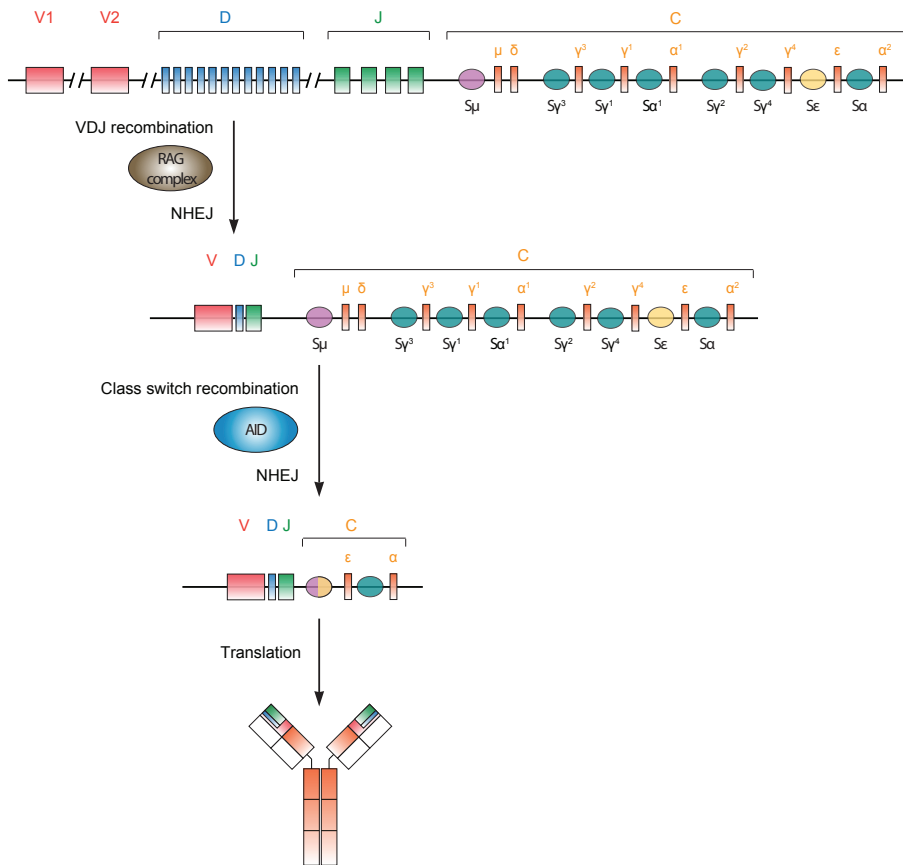


Figure 5. Variable (V), diversity (D) and joining (J) recombination and class switch recombination (CSR) of the IgH locus. Rearrangements of the IgH locus depend on the deliberate induction of either sequence-specific DSBs by the RAG complex during V(D)J recombination or on the induction of base mismatches by the deaminase AID that eventually lead to DSB formation throughout CSR. The formed DSBs are re-joined through classical NHEJ, a possibly error-prone process that can allow functional rearrangements to occur. The switch from IgM to IgE is depicted. Once the final transcript is generated, RNA is produced from the newly arranged IgH locus and translated into a specific immunoglobulin. These processes contribute to the variety of immunoglobulin species within the immune system. Figure adapted from (Mani and Chinnaiyan, 2010).

CSR on the other hand changes the production of Igs in B cells from one type to another when facilitating the exchange of the constant region of the IgH gene locus by a set of constant-region genes located further downstream within the same locus. Here the deaminase AID converts cytidines (C) preceded by W(A/T)R(A/G) dinucleotides to an uracil (U) within the switch regions (S μ - α) located upstream of the different constant region genes (C μ - α) (Fig. 5). This leads to the generation of mismatches, which can subsequently transform into single strand breaks (SSBs) when excision repair pathways attempt to repair these lesions. Due to the high density of AID motifs within the switch regions and the induction of numerous SSBs, DSBs ultimately arise during CSR. Upon DSB repair via c-NHEJ, different constant regions can be ligated together and subsequent transcription will determine the B-cell immunoglobulin isotype to which the cell will switch (Chaudhuri and Alt, 2004). The effector function of the Ig is changed during such a CSR event, but the V(D)J-mediated antigen specificity of the Ig remains unaltered.

Double-strand break repair pathway choice

How DSB pathway choice is determined during the cell cycle has been subject of numerous investigations. A combination of factors seems responsible, such as the availability of DNA repair proteins, cell cycle stage, chromatin environment and DNA damage complexity. Ku70-Ku80 has high affinity for DSB ends and thus accumulates within seconds encircling the DNA at both DSB ends in a sequence-independent manner. Ku thereby forms the scaffold for downstream c-NHEJ repair factors and mediates the fast repair of DSBs through c-NHEJ, while inhibiting other DSB pathways (Wang et al., 2006). This makes c-NHEJ the first choice DSB repair pathway. However, if re-joining of a DSB is delayed due to the absence of crucial c-NHEJ factors or because the DSB ends require major DNA end processing, either alt-NHEJ or HR can take over.

53BP1 is an important regulator of DSB repair pathway choice, which promotes NHEJ. Upon DSB induction, 53BP1 binds to nucleosomes that are both di-methylated at H4K20 and mono-ubiquitylated at H2AK15 (Fradet-Turcotte et al., 2013) (the subsequent modifications will be discussed in more detail below). Its binding affinity proximal to DSBs is mediated through histone acetyltransferase TIP60/TRRAP-induced acetylation of histone H4 on lysine (K) 16 (H4K16ac) upon damage induction that blocks 53BP1 binding to the neighbouring H4K20 methylation mark and inhibits DSB repair via HR. However, the antagonizing deacetylation of H4K16 by histone deacetylase 1 (HDAC1) and HDAC2 is then required for efficient 53BP1 binding to H4K20me2 (Hsiao and Mizzen, 2013; Tang et al., 2013). 53BP1 nucleosome binding is followed by its ATM-dependent phosphorylation, that is required to recruit RIF1 and PTIP to DSBs. RIF1 functions as the effector protein of 53BP1 in the G1 phase of the cell cycle and inhibits DNA end resection. In G2/S phase, RIF1 recruitment is suppressed by BRCA1 and its interacting protein CtIP, providing a switch to DSB repair via HR (Chapman et al., 2013; Escribano-Diaz et al., 2013; Zimmermann et al., 2013). PTIP also counteracts resection upon direct binding to ATM-phosphorylated 53BP1 and Artemis via its BRCT domains. Artemis thereby seems to function as downstream effector and limits DNA end resection at DSBs (Callen et al., 2013; Wang et al., 2014).

If rapid re-joining of the DSB via NHEJ does not ensue, HR can also be the DSB resolving pathway during S or G2 phase of the cell cycle (Shibata et al., 2011). If necessary, a switch from NHEJ to HR is mediated by BRCA1 and the deubiquitylating enzyme POH1, which belongs to the proteasomal machinery. BRCA1 recruits POH1 to DSBs, which promotes RPA-mediated resection through the removal of RAP80 from ubiquitin conjugates. The latter is required, since RAP80 blocks ubiquitin proteolysis and thus has a protective role towards ubiquitin. However, in the absence of RAP80, ubiquitin chains are degraded leading to the loss of 53BP1 in damaged chromatin and initiation of DNA end resection (Butler et al., 2012; Kakarougkas et al., 2013). CtIP is of great importance for this process, because it stimulates DSB repair via HR by promoting end-resection. Activation of CtIP is regulated on the one hand through its cell-cycle dependent expression, being up-regulated during S/G2 phase, and on the other hand by the p-ATM dependent recruitment of CtIP to DNA damage. Also the DSB-induced deacetylation as well as MRE11-CDK2-dependent phosphorylation of CtIP both regulate its action and promote its binding to BRCA1 (Buis et al., 2012; Kaidi et al., 2010; You et al., 2009). Thus, a multitude of interactions and posttranslational modifications (PTMs) mediate the local chromatin environment of DSBs and the key players regulate the cells' choice for a particular DSB repair pathway during the cell cycle.

Chromatin structure changes through histone posttranslational modifications and chromatin remodeling

Various regulatory mechanisms control the folding-state of DNA to provide access to proteins involved in DNA-based metabolic processes including transcription, DNA replication and DNA repair. First, histones can be posttranslationally modified through the action of enzymes that covalently modify residues at their inner core or at their N- and C-terminal tails. In that way not only the physical properties of the chromatin, but also the binding of non-histone proteins to chromatin can be altered. Besides phosphorylation, histones can also be ubiquitylated, SUMOylated, methylated, acetylated and poly(ADP-ribosyl)ated; the combinatorial nature of these modifications forms what is called the 'histone code' (Jenuwein and Allis, 2001).

Alternatively, ATPase-containing multi-subunit chromatin remodeling complexes can change the biophysical properties of chromatin through sliding nucleosomes along the DNA, evicting histone dimers or octamers and exchanging core histones or histone dimers with histone variants (Clapier and Cairns, 2009) such as H2A.Z (Xu et al., 2012) (discussed in more detail below).

Previous studies have shown that histone modifiers (Luijsterburg and van Attikum, 2011) and ATP-dependent chromatin remodelers are involved in the human DDR (Luijsterburg and van Attikum, 2011; Smeenk and van Attikum, 2013). In the following section more information on our current understanding of the role of chromatin modifications and chromatin remodelling in the DSB response is presented.

Posttranslational modifications during the DSB response

Phosphorylation

Upon phosphorylation, a phosphate group is attached to an acceptor protein at a serine (S) or threonine (T) residue. Among the huge number of phosphorylated proteins, hundreds of proteins have been found to contain SQ/TQ motifs, which can undergo DNA damage dependent phosphorylation by kinases from the phosphatidylinositol-3 kinase (PI3K)-family including ATM, ATR and DNA-PKcs (Matsuoka et al., 2007). Phosphorylation can thereby facilitate phospho-specific interactions with one of the many DDR factors that contain phospho-binding motifs, such as the Breast-cancer C-terminal (BRCT) domain or the Forkhead associated (FHA) domain (Mohammad and Yaffe, 2009). Also histones are phosphorylated upon DNA damage induction with the phosphorylation of the histone H2A variant H2AX on serine S139 (γ H2AX) as a key example. H2AX differs from H2A by an additional SQ(EY) motif at the C-terminus and engulfs about 10-15% of the H2A pool in higher organisms (Stucki and Jackson, 2006). ATM is the primary kinase that phosphorylates H2AX at DSBs (Burma et al., 2001) but acts in a redundant fashion with DNA-PKcs (Stiff et al., 2004). Conversely upon UV damage or replication stress, H2AX becomes phosphorylated primarily by ATR (Ward and Chen, 2001).

γ H2AX spreads over more than 20 megabases of chromatin surrounding the DSB (Fig. 1) (Iacovoni et al., 2010) and interacts with MDC1 through the BRCT domain of the latter. γ H2AX maintenance and MDC1-binding is regulated by the Williams syndrome transcription factor (WSTF), also called BAZ1B, which has kinase activity and was found to phosphorylate histone H2AX on tyrosine T142 independently from DNA damage. While WSTF is not directly involved in the DNA damage-induced phosphorylation of H2AX on Ser139, it does help to maintain γ H2AX levels following DNA damage (Barnett and Krebs, 2011; Xiao et al., 2009). Furthermore, the antagonizing activity of the EYA1/3 phosphatases

is required to dephosphorylate H2AX T142 following DNA damage, thereby promoting the chromatin assembly of MDC1 and counteracting an apoptotic response driven by T142 phosphorylation (Cook et al., 2009; Krishnan et al., 2009). MDC1 then provides a binding platform for several downstream DDR factors at DSBs (Stucki and Jackson, 2006). The formation of γ H2AX is further required to arrest cell cycle progression upon exposure to low doses of IR (Fernandez-Capetillo et al., 2002). Another crucial role of γ H2AX in the DDR is the MDC1-mediated recruitment of the E3 ubiquitin ligases RNF8 and RNF168 to DSBs, which facilitate the accumulation of 53BP1 and BRCA1 through the formation of ubiquitin conjugates on several H1 and H2A residues (discussed below) (Doil et al., 2009; Huen et al., 2007; Kolas et al., 2007; Mairand et al., 2007; Stewart et al., 2009; Thorslund et al., 2015; Wang and Elledge, 2007).

A different, but important event during the DSB response is the ATM-mediated phosphorylation of KAP1 on serine S824 in heterochromatic regions (Goodarzi et al., 2008; Lee et al., 2010b; Noon et al., 2010; Ziv et al., 2006). Heterochromatin comprises about 10-25% of total DNA within a cell, dependent on age, cell type as well as species. Importantly, heterochromatin forms a barrier for efficient DSB repair that is overcome by ATM-dependent KAP1 phosphorylation. Phosphorylated KAP1 interferes with the SUMO-dependent interaction between KAP1 and the nucleosome remodeler CHD3, leading to CHD3 dispersal from DSBs in heterochromatic regions (Goodarzi et al., 2011). Additionally, the chromatin remodelers SMARCA5 and ACF1 are recruited by RNF20/40 to heterochromatic DSBs and induce Artemis-dependent chromatin relaxation. This leads to a transient and local increase in the accessibility of the heterochromatin and enables the repair of the damaged DNA (Klement et al., 2014).

Apart from kinases, a number of dephosphorylating enzymes (phosphatases), including PP2A α , PP2A β , PP4C, PP6C and WIP1 have been linked to the DSB response and were shown to be involved in γ H2AX dephosphorylation (Cha et al., 2010; Chowdhury et al., 2008; Douglas et al., 2010; Keogh et al., 2006; Macurek et al., 2010; Moon et al., 2010; Nakada et al., 2008). The absence of either of these phosphatases leads to defective γ H2AX removal from DSBs and impairs the completion of DSB repair rendering cells hypersensitive towards IR. This shows the importance of a tight regulation of the phosphorylation events during the response to DSBs.

Ubiquitylation

Ubiquitin is a small protein of 76 amino acids (8.5 kDa) that is essential and highly conserved throughout evolution. The versatile cellular signals given by various types of ubiquitin modifications control a large variety of biological processes including protein degradation and DNA repair. Ubiquitin is expressed in cells as a precursor protein, which requires cleavage for its activation upon which a carboxyl-terminal di-glycine motif is exposed. Ubiquitin can then be covalently conjugated onto a target protein in a three-step enzymatic process that facilitates the binding of the ubiquitin carboxyl-terminus to a ϵ -amino group of a lysine within a substrate. This process requires an E1- (activating), an E2- (conjugating) and an E3- (ligase) enzyme. The latter type of enzymes thereby belongs to one of the three main families: HECT-domain E3 ligases, RBR E3 ligases and RING E3 ligases. The HECT and RBR E3 ligases contain an active cysteine to which ubiquitin is transferred from the E2 before it is conjugated onto the substrate. In contrast, RING E3 ligases do not bind ubiquitin directly, but rather bind the ubiquitin-charged E2 and the substrate simultaneously (Brown and Jackson, 2015).

Interestingly, no consensus motif exists for ubiquitin conjugation, hence substrate specificity is determined by the E3 ligase, its interacting partners and the substrate itself (Mattioli and Sixma, 2014). Ubiquitin can be conjugated as single molecule on one or more lysine residues of a substrate but also in chains due to the presence of 7 lysine residues (K6, K11, K27, K29, K33, K48 and K63) within the ubiquitin amino acid sequence that can undergo autoubiquitylation. Ubiquitin chains are named after the ubiquitylated lysine linking the ubiquitin molecules. The regulatory role of ubiquitylation differs according to its type of linkage: monoubiquitylation can for instance affect transcription and chromatin remodeling, while polyubiquitylation by means of K48-linked ubiquitin chain formation can target proteins for proteasomal degradation. Moreover, K63-linked ubiquitin chains are required for a proper response to DSBs and provide a binding platform for several DSB signaling proteins when generated in the vicinity of these lesions (Panier and Durocher, 2009).

At the vicinity of DSBs RNF8 binds to phosphorylated MDC1 via its FHA domain and initiates the ubiquitin signaling cascade (Huen et al., 2007; Kolas et al., 2007; Mailand et al., 2007) providing an important link between the two PTMs. Together with the E2 enzyme UBC13, RNF8 creates K63-linked ubiquitin chains on histone H1 within DSB-flanking chromatin (Fig. 1) (Doil et al., 2009; Pinato et al., 2011; Stewart et al., 2009; Thorslund et al., 2015). Furthermore, RNF8 also attracts the polycomb protein BMI1, which has been shown to monoubiquitylate H2A and H2AX at K119 and K120 in cooperation with other components of the polycomb repressive complex 1 (PRC1) like E3 ligase RNF2 (Facchino et al., 2010; Ginjala et al., 2011; Ismail et al., 2010; Pan et al., 2011; Wu et al., 2011). Moreover, the RING E3 ligase RNF168 is recruited through binding of the RNF8-induced K63-linked ubiquitin chains on histone H1 via its tandem ubiquitin interacting motifs (UIMs) (Doil et al., 2009; Stewart et al., 2009; Thorslund et al., 2015). RNF168 then generates more K63-linked ubiquitin chains and monoubiquitylates H2A/H2AX at K13-15 (Mattioli et al., 2012). Interestingly, RNF168 was recently found to also induce K27-linked ubiquitin chain formation on H2A and H2AX (Gatti et al., 2015). These K27- and K63-linked ubiquitin chains form the basis for the recruitment of 53BP1 by means of H2AK15ub, to which 53BP1 binds with an ubiquitylation-dependent recruitment motif (Fradet-Turcotte et al., 2013). Also the assembly of the BRCA1-A complex to DSBs is facilitated by this ubiquitin conjugate formation (Fig. 1) (Gatti et al., 2015; Mattioli et al., 2012).

BRCA1 dimerizes with the BRCA1-associated RING domain protein BARD1, which together function as an E3 ubiquitin ligase (referred to as BRCA1 core complex) (Baer and Ludwig, 2002; Hashizume et al., 2001; Ruffner et al., 2001; Wu et al., 1996). When ABRAXAS, BRCC36, MERIT40 and RAP80, interact with this BRCA1 core complex the so called BRCA1-A complex is formed (Shao et al., 2009; Wang and Elledge, 2007). RAP80 has been shown to directly bind K63-linked ubiquitin chains through its UIMs (Sato et al., 2009) as well as K27-linked ubiquitin chains (Gatti et al., 2015). In that way, RAP80 targets the BRCA1-A complex to the damaged DNA in a manner dependent on K63-linked ubiquitin conjugate formation by RNF8 together with UBC13 and RNF168 (Fig. 1) (Thorslund et al., 2015; Wang and Elledge, 2007). The assembly of BRCA1 within the BRCA1-A complex might simultaneously restrict the amount of BRCA1-CtIP and BRCA1-PALB2 complex formation and consequently DNA end-resection and BRCA2-RAD51 loading at DSBs, respectively (Coleman and Greenberg, 2011; Hu et al., 2011; Typas et al., 2015).

Besides RAP80, also 53BP1, RNF168 and RNF169 interact directly with K27- and K63-linked ubiquitin (Gatti et al., 2015). RNF169 thereby is an RNF168-related ubiquitin

ligase that provides an interesting example for negative regulation of the DDR by simply competing with 53BP1 and the BRCA1-A complex for binding to ubiquitylated chromatin and limiting their recruitment to DSBs (Chen et al., 2012a; Poulsen et al., 2012).

The HECT domain containing protein HERC2 provides an additional regulatory level to the ubiquitin cascade by controlling the ubiquitin-dependent retention of DDR factors (53BP1 and BRCA1) on damaged chromatin. It has been shown that upon exposure to IR, HERC2 interacts with RNF8 in a manner dependent on its phosphorylation at threonine Thr4827 (Bekker-Jensen et al., 2010). Moreover, the RNF8-dependent SUMOylation of HERC2 by the E3 SUMO ligase PIAS4 is also required for the HERC2-RNF8 interaction (Danielsen et al., 2012). Mechanistically, HERC2 is thought to facilitate the assembly of the RNF8-UBC13 complex, which promotes K63-linked polyubiquitylation and simultaneously restricts the interaction of RNF8 with other E2 conjugating enzymes. HERC2 also stabilizes RNF168 and its absence severely affects ubiquitin conjugate-formation and the recruitment of downstream repair factors like 53BP1 and BRCA1 (Bekker-Jensen et al., 2010).

Besides H2A, also H2B has been reported to be a target for monoubiquitylation when DNA damage is induced. H2B ubiquitylation is facilitated by the E3 ubiquitin ligase RNF20-RNF40, which form a heterodimer. This E3 ligase is recruited to DSBs upon ATM-dependent phosphorylation and is important for the timely repair of DSBs. Furthermore, RNF20 has been shown to promote the accumulation of NHEJ as well as HR repair factors and, interestingly, also the accrual of chromatin remodeler SMARCA5/SNF2h which facilitates repair (discussed below) (Moyal et al., 2011; Nakamura et al., 2011).

The tight control of the ubiquitylation cascade by ubiquitin ligases and the indirect contribution of chromatin remodeling enzymes entails yet another important level of regulation that is mediated by the group of deubiquitylating enzymes, shortly termed DUBs. Five distinct families subdivide approximately 90 potential DUBs encoded by the human genome: ovarian tumor proteases (OTUs), ubiquitin-specific proteases (USPs), ubiquitin carboxy-terminal hydrolases (UCHs), Machado-Joseph disease enzymes (MJDs) and JAB1/MPN/MOV34 metalloenzymes (JAMMs). OTUB1 binds directly to and inhibits the E2 enzyme UBC13, preventing the interaction of UBC13 with RNF168. This subsequently suppresses the RNF168-dependent ubiquitylation of DSB-containing chromatin (Nakada et al., 2010). Other DUBs that have roles within the DDR are USP44 and USP33, which both antagonize the RNF8/168-dependent ubiquitin conjugation on H2A and in the latter case also (γ)H2AX (Mosbech et al., 2013; Sharma et al., 2014). Moreover, a recent genetic screen identified hitherto unknown DUBs to be potentially involved in the DDR (Nishi et al., 2014), while a similar screen in our lab identified USP26 and USP37 as DUBs that are critical for the DDR. Both DUBs actively degrade RNF168-induced ubiquitin conjugates at DSBs, which averts BRCA1 sequestration via the BRCA1-A complex and reverses the RAP80-inhibitory effect on DSB repair via HR. Hence, this may subsequently promote the assembly of BRCA1 with PALB2-BRCA2-RAD51 to regulate HR (Typas et al., 2015).

SUMOylation

The small ubiquitin-like modifier (SUMO) has been implicated in the modification of a vast variety of proteins and the regulation of many cellular processes, including transcription, chromatin remodeling and DNA repair (Flotho and Melchior, 2013; Hickey et al., 2012; Jackson and Durocher, 2013). Like ubiquitin, SUMO is synthesized as a precursor protein and requires processing by SUMO-specific proteases (Fig. 6A). The subsequent exposure of the di-glycine motif that is needed for SUMO conjugation functions via a 3-step enzymatic

1

cascade as described for ubiquitin. The dimeric E1 activating enzyme is SUMO-Activating Enzyme Subunit 1 and 2 (SAE1/SAE2), while Ubiquitin Carrier Protein 9 (UBC9) forms the E2 conjugating enzyme (Bernier-Villamor et al., 2002; Desterro et al., 1999; Schulman and Harper, 2009). The combined action of E1 and E2 is only sufficient for a few target proteins to become efficiently SUMOylated, instead, a series of E3 SUMO ligases is required to enhance SUMO conjugation specificity and efficiency (Flotho and Melchior, 2013; Hay, 2005; Johnson, 2004; Nagy and Dikic, 2010) (Fig. 6A). SUMO is mainly conjugated to lysines, which are part of a SUMO consensus motif comprised of a large hydrophobic residue (ψ) that is followed by the SUMO acceptor lysine (K) and a glutamic acid (E) two positions downstream of the SUMOylated lysine [ψ KxE] (Hendriks et al., 2014; Matic et al., 2010).

Three different SUMO modifiers can be distinguished in human cells: SUMO-1, SUMO-2 and SUMO-3. SUMO-2 and SUMO-3 are nearly identical as these two modifiers differ in only three amino acids within the N-terminus and can therefore only be distinguished experimentally with great difficulty. On the contrary, the amino acid sequences of SUMO-2 and SUMO-3 only match for ~45% with that of SUMO-1 (Wang and Dasso, 2009). While SUMO-2/3 comprise an internal SUMOylation site that provides the possibility for polymeric SUMO-chain formation, SUMO-1 lacks this and consequently serves as a SUMO-chain terminator when conjugated (Matic et al., 2008; Tatham et al., 2001; Vertegaal, 2010) (Fig. 6B). Poly-SUMO chains have vital roles during proteasome-mediated protein turnover, the cell cycle regulation, DNA replication and DNA repair (Vertegaal, 2010).

SUMO can be bound by SUMO-interacting motifs (SIMs), which are formed by a stretch of hydrophobic amino acids, or a specific ZZ zinc finger (Danielsen et al., 2012; Song et al., 2004; Vertegaal, 2010). Like all PTMs, SUMOylation is reversible and SUMO conjugates can be removed from target proteins by SUMO-specific proteases (Li et al., 2010b; Mukhopadhyay and Dasso, 2007) thus providing a dynamic response mechanism for cells to react on external and internal conditions and stimuli.

SUMOylation has been implicated in the response to different types of DNA damage (Bergink and Jentsch, 2009). All components of the 3-step SUMO conjugation cascade i.e. SAE1, UBC9, the SUMO E3 ligases PIAS1 and PIAS4 as well as SUMO -1 and SUMO-2/3 have been shown to accumulate at sites of DNA damage (Galanty et al., 2009; Morris et al., 2009). While SUMO-1 requires only PIAS4 for its recruitment, conjugation of SUMO-2/3 is apparently catalysed by both SUMO ligases PIAS1 and PIAS4 in the proximity of DSB induced by laser radiation (Galanty et al., 2009). Moreover, the PIAS4-dependent recruitment of RNF168 and the abrogated ubiquitin conjugate formation in PIAS1- and PIAS4-depleted cells indicate substantial cross-talk between the ubiquitin cascade and the SUMOylation-mediated response to DSBs (Galanty et al., 2009; Morris et al., 2009). The underlying mechanism is thought to involve the PIAS4-mediated SUMOylation of HERC2, which promotes RNF8-UBC13 binding and K63-linked ubiquitin chain formation, of which the latter is required for RNF168 accrual. However, RNF168 itself is also SUMOylated by PIAS4, which might positively regulate its stability (Danielsen et al., 2012). Furthermore, 53BP1 recruitment appeared to be merely dependent on PIAS4, while both PIAS1 and PIAS4 are necessary for the accumulation of the BRCA1-A complex at sites of DNA damage (Galanty et al., 2009; Morris et al., 2009). Besides its UIMs, RAP80 also contains a SUMO-2/3-specific SIM, which is required for its recruitment. Consequently, at DSBs RAP80 probably binds to K63-linked ubiquitin chains and SUMO simultaneously, as was suggested by an *in vitro* binding assay with a Rap80 SIM-UIM-UIM fragment (Hu et al., 2012). The SUMO moiety for RAP80-binding thereby most likely is conjugated onto MDC1 (Hu et al., 2012; Luo et al.,

2012; Strauss and Goldberg, 2011; Yin et al., 2012). Remarkably, while RNF8 and RNF168 are dispensable for PIAS1 and PIAS4 accumulation at DSBs, they still promote the accrual of SUMO-1 and SUMO-2/3, probably by serving as SUMO targets as described above. The recruitment of PIAS1 and PIAS4 is dependent on their SAP domains and while both PIAS1 and PIAS4 are important for the efficient association of BRCA1 with DSBs, the recruitment of RNF168 and 53BP1 only requires PIAS4. Thus it is not surprising, that PIAS1 and PIAS4 have been implemented in the efficient repair of DSBs via NHEJ and HR as well as cell cycle progression (Galanty et al., 2009; Morris et al., 2009).

SUMO has also been implicated in DSB repair by regulating the disassembly of repair complexes at sites of DNA damage. The recruitment of the SUMO-targeted ubiquitin E3 ligase (StUbl) RNF4 relies on its SIM domains, PIAS1 and PIAS4 as well as a number of DDR proteins like MDC1 and BRCA1. When being SUMOylated, these proteins seem to function as binding targets for RNF4 (Galanty et al., 2012; Vyas et al., 2013; Yin et al.,

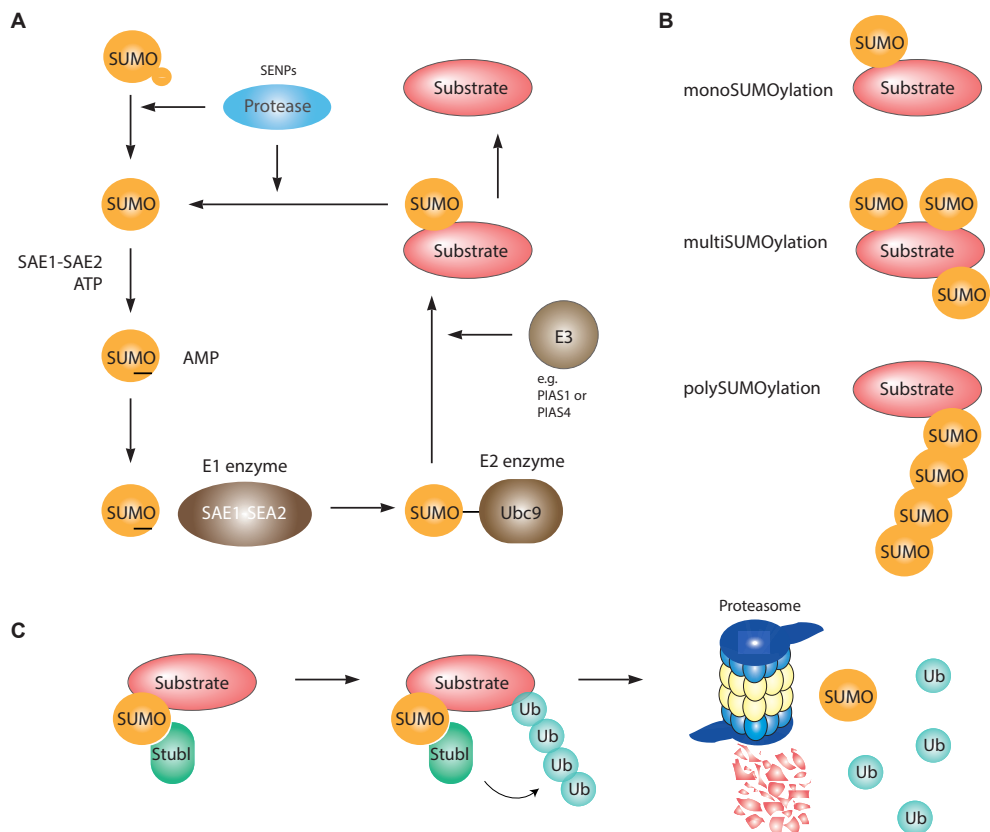


Figure 6. SUMOylation of proteins. (A) The SUMO cycle. Precursor SUMO is cleaved by SUMO specific proteases (SENPs). Via an ATP-dependent cascade involving the activating E1 enzyme SEA1/2, the conjugating E2 enzyme UBC9 and if required a catalytic E3 enzyme, mature SUMO is conjugated onto a lysine of a substrate protein. SUMOylation is a reversible process, because SUMO proteases can deconjugate SUMO from substrate proteins. (B) Substrate proteins can be modified by SUMO by means of monoSUMOylation, multiSUMOylation or polySUMOylation. (C) SUMOylated substrate proteins can be targeted for proteasomal degradation by a SUMO targeted ubiquitin ligase (StUbl). Figure adapted from (Schimmel et al., 2014).

2012). RNF4 promotes the efficient assembly of ubiquitin conjugates at DSBs and that lead to proteasomal degradation of DDR proteins (Fig. 6C), which in turn stimulates DSB repair via both DSB repair pathways, NHEJ and HR. While it is still questionable whether RNF4 mediates NHEJ through the regulation of the rapid turnover of MDC1, RNF4 has been shown to restrict RPA accumulation to DSBs and thus facilitates efficient loading of the HR machinery including RAD51 (Galanty et al., 2012; Luo et al., 2012; Vyas et al., 2013; Yin et al., 2012).

In conclusion, DNA damage triggers a SUMOylation wave that leads to the modification of various DSB repair proteins. It recently has been proposed, that only the simultaneous abrogation of SUMOylation of multiple DSB repair proteins results in a significant defect in DSB repair by HR (Psakhye and Jentsch, 2012). Thus, only the coinciding deSUMOylation of several proteins will lead to detectable phenotypes during the DDR.

Acetylation

Acetylation encompasses the addition of an acetyl group (-COCH₃) to the ε-amino group of a target lysine of a protein. This modification is catalysed onto histones by histone acetyltransferases (HATs) and removed by histone deacetylases (HDACs). Through the neutralization of the positive charge of lysine residues, acetylation can weaken the nucleosomal interactions within chromatin. Acetylated histones are therefore associated with an open chromatin state in which transcription can be active. In general, acetylation is seen as a regulator of higher-order chromatin structure and is important for various cellular processes such as transcription regulation and DNA damage repair.

Upon exposure to IR, both HATs and HDACs accumulate at DNA damage. A well-studied example is the HAT TIP60 that is probably recruited as part of the Nucleosome acetyltransferase of H4 (NuA4) complex. The recruitment of this complex is not sufficient to trigger its activation as only the local transient release of the heterochromatin 1 protein (HP1) upon DSB induction can initiate TIP60 activation. The release of HP1 unmasks the abundant tri-methylated H3K9 mark, to which TIP60 binds with its chromodomain (Sun et al., 2009). ATM activity is subsequently enhanced through TIP60-mediated acetylation and leads to the phosphorylation of numerous downstream targets (Kaidi and Jackson, 2013; Sun et al., 2005). At DSBs TIP60 also acetylates H2AX at K5, which is required for H2AX ubiquitylation at K119 and efficient DSB signaling (Ikura et al., 2007).

Apart from H2AX, also other core histones are targeted for acetylation. Accordingly, TIP60 together with its NuA4 co-factor TRRAP acetylates H4K16 in response to DSBs. The H4K16ac mark mediates the effective accrual of the DSB repair proteins MDC1, 53BP1, BRCA1 and RAD51 and promotes efficient HR (Doyon and Cote, 2004; Murr et al., 2006).

In addition to HR, histone acetylation also plays an important role during NHEJ. The recruitment of CBP and p300 to DSBs induces the acetylation of a number of H3 (K18) and H4 (K5, K8, K12 and K16) residues and promotes the recruitment of the heterodimer Ku70-Ku80 as well as the catalytic subunit of the SWI/SNF chromatin remodeling complex BRM (discussed below) (Ogiwara et al., 2011). Moreover, other labs reported on additional acetylation activity of CBP, p300 and GCN5 on H3K56 and the deacetylation of this mark by the Sirtuin proteins SIRT2 and SIRT3 (Das et al., 2009; Tjeertes et al., 2009; Vempati et al., 2010), showing that the acetylation status of chromatin is dynamically regulated.

Yet another important histone acetylation target is H3K14. Its acetylation has been described to globally increase in cells exposed to IR and to depend on the nucleosome-binding protein HMGN1. Depletion of HMGN1 resulted in decreased ATM-autophosphorylation upon

IR and thus insufficient activation of ATM-targets, while chromatin relaxation induced by HDAC inhibitors bypassed the need for HMGN1-mediated ATM activation (Kim et al., 2009). HMNG1 therefore promotes decompaction of chromatin in the vicinity to DSBs and protects cells from the disastrous effects of IR and UV (Birger et al., 2005).

The acetylation of H4K16A has a central role in the regulation of the DDR, which is linked to releasing higher order chromatin structure (Shogren-Knaak and Peterson, 2006). MOF (or MYST1) is the major HAT that catalyses H4K16 acetylation and its loss causes reduced H4K16ac levels and defects in IR-induced DSB signaling and repair (Li et al., 2010a; Sharma et al., 2010). In more detail, MOF depletion does not affect γ H2AX formation, but is required for the recruitment of MDC1 as well as the downstream factors 53BP1 and BRCA1. This suggests that H4K16ac is crucial for DSB-induced binding of MDC1 to γ H2AX. Interestingly, upon IR-exposure the absence of MOF leads to severe cell cycle arrest at the G2/M border and gives rise to chromosomal aberrations most likely due to severe defects in DSB repair by NHEJ as well as HR (Gupta et al., 2014; Li et al., 2010b; Sharma et al., 2010).

Furthermore, an important role in the DDR has been assigned to HDACs. HDAC1 and HDAC2 are known to rapidly recruit to sites of DSBs, where they deacetylate H3K56. Cells depleted from HDAC1 and HDAC2 show sustained DNA damage signaling, defective DSB repair predominantly by NHEJ and are hypersensitive to IR (Miller et al., 2010). Besides H3K56ac levels, also global H3K9ac decreases upon DNA damage induction in an HDAC-dependent manner (Tjeertes et al., 2009). Furthermore, H4K16ac levels decrease similarly at first through HDAC activity, but increase at later time points during the DSB response in a MOF-dependent manner. Thus H4K16 acetylation has a bi-phasic character during the DDR (Li et al., 2010a; Miller et al., 2010). In addition, HDAC-mediated deacetylation was shown to promote efficient DSB repair via NHEJ (Miller et al., 2010) by the effective disassembly of Ku70 and Artemis from DSBs.

Methylation

Methylation denotes the addition of a methyl group to a lysine or an arginine of a protein. Histone methyltransferases facilitate this reaction on histone proteins through their catalytic SET domain and can either mono-, di- or tri-methylate histones. Proteins harbouring a chromo or a tudor domain are able to bind to these methyl moieties. Similar to other PTMs, histone methylation is a reversible process due to the activity of histone demethylases.

Condensed chromatin displays high levels of H3K9 trimethylation (H3K9me3) rendering the DNA inaccessible for repair proteins and transcriptionally inactive. The histone methyltransferase SUV39H establishes the H3K9me3 mark, to which HP1 directly binds (via its chromodomains) and contributes to the maintenance of heterochromatin (Cheutin et al., 2003). Very recently, scientists found SUV39H to be recruited to DSBs in association with KAP1 and HP1 SUV39H, which thereby locally increases H3K9me3 levels and creates more binding positions for HP1 and subsequently more KAP1-HP1-SUV39H complex. The H3K9me3 mark eventually also becomes available for TIP60 binding mediating acetylation and activation of ATM (Sun et al., 2005; Sun et al., 2009) and rapid phosphorylation of KAP1. These events are followed by the release of the repressive KAP1-HP1-SUV39H complex from damaged chromatin and thus describe a negative feedback loop for the activation of ATM. The transient formation of repressive chromatin might thereby be important for stabilizing the damaged chromatin and might generate a suitable template for DNA repair proteins (Ayrapetov et al., 2014).

Another important methylation mark is H4 dimethylated at lysine 20 (H2K20me2).

This mark, together with RNF168-ubiquitylated H2AK15 is critical for the recruitment of 53BP1 to DNA damage, demonstrating that 53BP1 is a bivalent PTM reader. Hence, only the recognition of nucleosomes comprising both marks leads to actual 53BP1 binding at DSBs involving H4K20me2 with its Tudor domain and H2AK15ub with its ubiquitylation-dependent recruitment (UDR) motif (Botuyan et al., 2006; Fradet-Turcotte et al., 2013; Mattioli et al., 2012). While it is clear, how H2AK15ub is formed during the DDR, quite some debate prevails within the field about H4K20 methylation and the responsible methyltransferase(s). Pei and colleagues have shown that the histone methyltransferase MMSET is recruited to DNA damage in a γ H2AX-MDC1-ATM-dependent manner and that it increases local H4K20me2/3 levels at DSBs to facilitate 53BP1 recruitment in human cells (Pei et al., 2011). Additionally, the activity of the H4K20 monomethyltransferase SET8 (or PR-SET7) was shown to be required for 53BP1 foci formation in human cells (Dulev et al., 2014; Hartlerode et al., 2012). In contrast to these findings, MMSET and the H4K20 dimethyltransferase SUV420H were not requisites for 53BP1 recruitment in mouse embryonic fibroblasts (MEFs) (Hartlerode et al., 2012). This implies that the function of these methyltransferases might not be conserved from mice to humans.

Alternatively, it has been suggested that 53BP1 assembles onto H4K20me2 established in a DNA damage-independent fashion, since H4K20me2 is a very abundant histone mark. This would argue against the involvement of these methyltransferases within the DSB response and proposes that H4K20me2 could rather represent an additional binding interface for 53BP1, which is important for its stable association to damaged chromatin. Interestingly, 53BP1 binding to H4K20me2 can be perturbed by other proteins that have affinity for this histone mark in the absence of DNA damage, such as the Polycomb protein L3MBTL1 and the demethylase JMJD2A (or KDM4A) (Acs et al., 2011; Mallette et al., 2012). Both proteins are ubiquitylated by RNF168 upon DNA damage induction. L3MBTL1 is subsequently removed from chromatin by proteosomal degradation (Butler et al., 2012; Mallette et al., 2012), while JMJD2A gets evicted from the histone mark by the ATPase activity of VCP (or p97) (Acs et al., 2011; Meerang et al., 2011). These processes thus unmask the H4K20me2 marks locally in the vicinity of DSBs and could also enable 53BP1 binding at damaged chromatin.

Another methylation mark that is involved in the response to DSBs is dimethylated H3K36, which is generated upon DSB induction by the methyltransferase Metnase (also named SETMAR). The accrual of NBS1 and Ku70 is stimulated upon Metnase-mediated H3K36me2 formation at damaged chromatin and specifically promotes DSB repair via NHEJ (Fnu et al., 2011).

Poly(ADP-ribosyl)ation

The process by which a linear or multibranched polymer of ADP-ribose units is attached to a target is termed PARylation for Poly(ADP-ribose)ylation. These polymers can be conjugated onto a glutamate, aspartate or lysine residue of an acceptor protein. PAR is catalysed by poly(ADP-ribose) polymerases (PARPs) that belong to a 17 members counting PARP superfamily, which is further divided into four groups dependent on their domain architecture. One subfamily is formed by the DNA-dependent PARPs: PARP1, PARP2 and PARP3 (Schreiber et al., 2006). These PARPs form the most relevant group for the work presented in this thesis as they have been implicated in the DDR (Pines et al., 2013). PAR-chain synthesis is mediated by PARP, but PAR-chains have a short turnover time due to their rapid degradation by poly(ADP-ribose) glycohydrolase (PARG). PARG functions in a

coordinated manner together with PARPs to regulate various cellular processes. Hence, the amount of PAR-chains is kept in a tight equilibrium to fine tune protein function and cellular processes. PARP1 has been shown to be important during the response to SSBs and DSBs (El-Khamisy et al., 2003; Masson et al., 1998). Proteins are not only PARylated, but can also bind to PAR via several PAR-binding modules: the PAR-binding motif, WWE domains containing a Trp-Trp-Glu motif, PAR-binding zinc fingers and macrodomains that bind to the terminal ADP-ribose of PAR (Gibson and Kraus, 2012).

PARP1 is the main catalyst of PAR and gets activated by binding to DNA damage through its zinc finger domains (Langelier et al., 2010). An essential step in this process is the autoPARylation of PARP1 at the PAR-acceptor sites K498, K521 and K524 (Altmeyer et al., 2009). PARP1 can also PARylate many other targets, including histone proteins (Mortusewicz et al., 2007; Poirier et al., 1982). Histone H2AK13, H2BK30, H3K27, H3K37 as well as H4K16 have all been identified as ADP-ribose acceptor sites and their PARylation might contribute to the rapid recruitment of PAR-binding proteins to DNA lesions. Interestingly, H4K16ac inhibits H4K16 PARylation by PARP1 and thus provides another indication for the existence of functional crosstalk between the different histone tail modifications (Messner et al., 2010). Moreover, the PARylation of nucleosomes has been shown to induce chromatin relaxation and PARP1 activity facilitates expansion of damaged chromatin and the spreading of DDR factors within the damaged chromatin compartment (Poirier et al., 1982; Smeenk et al., 2013).

The chromatin remodeler Amplified in Liver Cancer (ALC1) binds PAR-molecules with its macrodomain at sites of laser-induced DNA damage and thus is an example for a PAR-binding protein. This subsequently leads to its activation and is followed by nucleosome remodeling (Ahel et al., 2009; Gottschalk et al., 2009). Worth mentioning is also the histone chaperone APLF, which incorporates the histone variant MacroH2A1.1 at sites of DNA damage (Mehrotra et al., 2011). Both macroH2A1.1 and APLF bind to PAR-chains through a macrodomain and PAR-binding zinc finger domain, respectively (Ahel et al., 2008; Timinszky et al., 2009). MacroH2A1.1 transiently compacts chromatin and negatively regulates the recruitment of the NHEJ factors Ku70-Ku80 (Timinszky et al., 2009), while APLF promotes NHEJ complex assembly and functions as a scaffold for XRCC4-LIG4-XLF recruitment at DSBs (Ahel et al., 2008; Iles et al., 2007; Kanno et al., 2007; Rulten et al., 2008).

The chromodomain helicase DNA-binding protein CHD4 is the ATPase subunit of the NuRD complex and its recruitment to DNA damage has been shown to be partially dependent on PARP. Remarkably, CHD4 can bind to PAR *in vitro*, despite the lack of any known PAR-binding consensus sites or PAR binding domains (Polo et al., 2010). Another example is the chromatin remodeler SMARCA5 (or SNF2h), which is also recruited in a partially PARP-dependent manner. Upon DSB-induction, RNF168 gets PARylated and interacts with SMARCA5 in a PAR-dependent fashion, contributing to its accrual to DSBs. On the other hand, SMARCA5 supports RNF168 recruitment to DSBs, thereby regulating the RNF168-driven ubiquitin cascade (Smeenk et al., 2013). Hence, the distribution of SMARCA5 and factors involved in this ubiquitin cascade within laser-damaged chromatin compartments was mediated by the activity of PARP. It was suggested that PARP spatially organizes the ubiquitin cascade in response to DNA damage at the level of SMARCA5 as well as RNF168 recruitment, and thereby contributes to efficient ubiquitin conjugate formation and subsequent BRCA1 assembly (Smeenk et al., 2013).

PARP1 can also be activated in the absence of DNA by the mono(ADP-ribosyl)ase PARP3. In contrast to PARP1, PARP3 can auto-ADP-ribosylate without DNA-binding and

the reported interaction of PARP1 and PARP3 seems to be unrelated to repair of at least single strand DNA breaks (Loseva et al., 2010). In line with these findings is the observation that PARP1^{-/-}/PARP3^{-/-} mice are more sensitive to IR compared to the single mutant mice. PARP1 and PARP3 might therefore function synergistically within the DDR (Boehler et al., 2011). However, recent reports suggest that PARP3 also accelerates NHEJ through the ADP-ribosylation of the Ku dimer and histone H1. As such they provide a platform for the PAR-binding protein APLF, which promotes the retention of the XRCC4/LIG4 complex at damaged chromatin (Fig. 4) (Beck et al., 2014; Rulten et al., 2011). These findings thus rather suggest an epistatic role for PARP1 and PARP3 within the DDR.

PTM crosstalk shapes epigenetic environment of DSBs

From the previous sections on PTMs one might start wondering, why there are so many different PTMs and how they relate to each other during the response to DNA damage. PTMs alter protein interactions and influence their translocation or degradation and therefore provide opportunities to regulate and control the activities of distinct proteins like those involved in the DDR. Since there are several different PTMs that can influence protein function, processes can be mediated in a very precise way through numerous different modifications. However, we are just beginning to understand the immense crosstalk of PTMs occurring in the vicinity of DNA damage and its complexity. One interesting finding so far is that the recruitment of several proteins to DNA damage depends on a multiple interaction strategy. 53BP1 is a notable example of a bivalent binding factor, since it binds ubiquitylated and methylated histones in order to robustly enrich and remain at DSBs (Fradet-Turcotte et al., 2013). Moreover, the ubiquitin ligases RNF168 (Mattioli and Sixma, 2014) and RNF4 (Grocock et al., 2014) bind to either ubiquitin or SUMO conjugates, respectively, while they additionally need to interact with chromatin for stable association in close vicinity to the lesion.

Thus, the signaling and repair of DSBs is an extremely fine-tuned multi-step process, where the composition of all PTMs shape the epigenetic chromatin environment of DSBs.

ATP-dependent chromatin remodeling during the DSB response

Chromatin remodelers use the energy from ATP hydrolysis to alter chromatin structure. As previously mentioned, they can do so by sliding nucleosomes along the DNA, exchanging or ejecting histone dimers, or disassembling nucleosomes by ejecting octamers. Originally described in yeast, the sucrose nonfermenting (SNF2) family of chromatin remodelers comprises an ATPase catalytic subunit as well as a helicase domain. The SNF2 family members are further categorized into four subgroups, each group containing different additional functional domains (Fig. 7), which will be addressed per group below. These ATPases often form the catalytic subunit of multi-subunit complexes, in which they assemble together with varying subunits that all contribute to the remodeling activity and/or the functionality of the complex.

SWI/SNF

The catalytic subunit of the Switching defective/sucrose nonfermenting (SWI/SNF) family of chromatin remodelers characteristically comprises an additional helicase SANT-associated (HSA) domain and an C-terminal bromodomain (Fig. 6), the latter is capable to bind acetylated histone tails (Clapier and Cairns, 2009). The SWI/SNF family contains two catalytic subunits, BRM (also SMARCA2) and BRG1 (SMARCA4), and each forms a multi-subunit complex with

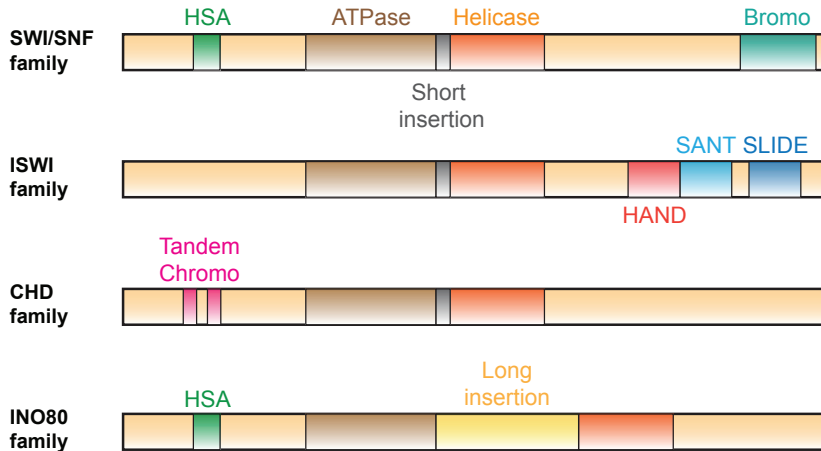


Figure 6. Schematic representation of the SWI2/SNF2 chromatin remodeling superfamily. Next to the catalytic ATPase and helicase domain, all SNF2 family members contain additional domains, by which they are classified into the four subfamilies: SWI/SNF, ISWI, CHD and INO80. The SWI/SNF family comprises a helicase-SANT-associated (HSA) domain, which facilitates the binding to nuclear actin-related proteins, and a bromodomain that is capable of binding acetylated lysines. ISWI chromatin remodelers are equipped with an HAND, SANT and SLIDE domain that can mediate interactions with proteins and DNA. The CHD family members contain an N-terminal tandem chromodomain, which enables these chromatin remodelers to bind methylated lysines. In contrast to the other subfamilies, INO80-related enzymes have a longer insertion between the ATPase and helicase domain as well as an HAS domain within the N-terminal part.

8 - 10 BRM- or BRG1-associated factors (BAFs). Both remodelers can facilitate nucleosome repositioning, dimer or octamer ejection and nucleosome unwrapping (Kasten et al., 2011).

SWI/SNF has been implicated in the DSB response: a positive feedback loop has been described for γ H2AX formation at DSBs, constituting the rapid and transient phosphorylation of BRG1 on Ser-721 by activated ATM. This stimulates the binding of BRG1 through its bromodomain to acetylated H3 in chromatin comprising γ H2AX-containing nucleosomes and the phosphorylation of H2AX by ATM through the remodeling activity of BRG1. Simultaneously, the HAT GCN5 is recruited to DNA damage in a γ H2AX-dependent manner and acetylates H3 within the chromatin surrounding the lesion. This subsequently induces the recruitment of additional BRG1 and facilitates the spreading of the γ H2AX signal, as well as DNA damage signaling and repair (Kwon et al., 2015; Lee et al., 2010a; Park et al., 2006). Furthermore, BRG1 has just recently been suggested to function in the HR pathway, while NHEJ efficiency was normal in BRG1-depleted cells (Qi et al., 2015). This study showed that BRG1 regulates HR through the exchange of RPA with RAD51 at DSBs.

SWI/SNF has also been shown to be regulated by BRIT1 (or MCPH1), which associates with core subunits of the SWI/SNF complex in an ATM-/ATR-dependent manner and promotes the recruitment and binding of SWI/SNF at DSBs (Peng et al., 2009). Probably through the interaction with SWI/SNF and indirectly γ H2AX, BRIT1 attracts DDR response factors like NBS1, p-ATM, MDC1 and 53BP1 (Rai et al., 2006; Wood et al., 2007). Thus, consistent with BRIT1 contributing to early DSB signaling, the absence of BRIT1 resulted in a G2/M checkpoint defect and abrogated DSB repair via HR and NHEJ (Lin et al., 2005; Peng et al., 2009). Interestingly, defects in BRIT1 were also shown to promote tumor development and underlie primary microcephaly, a neural development disorder characterized by reduced brain size (Chaplet et al., 2006).

ISWI

Thus far, seven different complexes of the mammalian Imitation SWItch (ISWI) family have been described (Erdel and Rippe, 2011; Toto et al., 2014). Two ATPase subunits built the core of these complexes: SMARCA5 (or SNF2H) and SMARCA1 (or SNF2L). In addition to their ATPase domain located within the N-terminus, both ATPases possess a HAND, SANT and SLIDE domain within their C-terminus (Fig. 6) (Grune et al., 2003).

SMARCA1 has been found in the CERF (Banting et al., 2005) and NURF (Barak et al., 2003) complexes, which function within the central nervous system during neurulation or neuronal development, respectively. However, to our knowledge a function for these factors in the DDR has not yet been established. In contrast, SMARCA5 has been implicated in the DDR. In fact, we and others have recently shown that upon DSB induction the RNF8/RNF168-induced ubiquitylation response is tightly controlled by the chromatin remodeler SMARCA5 and PARP (Lan et al., 2010; Smeenk et al., 2013). PARP thereby regulates the distribution of SMARCA5 and factors of the RNF168 signaling cascade throughout the damaged chromatin compartment of a cell, which subsequently leads to the efficient formation of ubiquitin conjugates at and the assembly of BRCA1 to the lesion (Smeenk et al., 2013). However, the recruitment of SMARCA5 is also mediated by the histone H3K56 deacetylase SIRT6 (Toiber et al., 2013) and the RNF20-RNF40 ubiquitin ligase, which ubiquitylates H2B to promote the assembly of HR repair factors (Nakamura et al., 2011; Oliveira et al., 2014). The depletion of RNF20 or SMARCA5 renders cells defective in DNA end resection and unable to recruit BRCA1 and RAD51 to DSBs. Interestingly, RNF20 also facilitates DSB-induced chromatin relaxation in heterochromatin downstream of KAP1 phosphorylation and the dispersal of CHD3 in an SMARCA5-dependent fashion, which is favourable for the repair of DSBs via an Artemis-dependent NHEJ pathway (Klement et al., 2014). Thus, SMARCA5 is important for the proper execution of the two DSB repair pathways HR and NHEJ (Lan et al., 2010; Smeenk et al., 2013). Remarkably, SMARCA5 resides in several different complexes, including WICH (SMARCA5 and WSTF), ACF (SMARCA5 and ACF1), CHRAC (SMARCA5, ACF1, CHRAC15 and CHRAC17) and RSF (SMARCA5 and RSF1) (Wang et al., 2007) that contain one or more auxiliary factors in addition to SMARCA5. Thus far, each of these factors has been implicated in the DDR and will be briefly discussed below.

The WSTF kinase and its role in H2AX T142 phosphorylation have been described in the 'Phosphorylation' section. The SMARCA5-complex partner ACF1, also known as BAZ1A, protects cells from various types of DNA damage and facilitates activation of the G2/M checkpoint upon DNA damage induction (Sanchez-Molina et al., 2011). In addition, ACF1 promotes efficient DSB repair by both HR and NHEJ. ACF1 physically associates with the key NHEJ factor Ku70, thereby recruiting the Ku70/Ku80 heterodimer to sites of DNA damage and initiating DSB repair (Lan et al., 2010). The histone fold proteins CHRAC15 and CHRAC17 also facilitate DSB repair by HR and NHEJ although the precise mechanisms are unclear (Lan et al., 2010). The role of the histone chaperone RSF1 during the DDR remains enigmatic and has been investigated in chapters 3 and 4.

CHD

The catalytic subunits of the Chromodomain Helicase DNA-binding (CHD)-type remodelers are characterized by a tandem chromodomain at their N-terminus enabling these remodelers to bind to methylated histones (Fig. 6). Nine different CHD catalytic subunits exist that have various additional DNA- or protein-binding motifs, by which they are discriminated. So far, two CHD proteins, CHD3 and CHD4, were described to mediate the DDR. They are

both mutually exclusive catalytic subunits of the Nucleosome Remodeling and Deacetylase (NuRD) complex. Several of the NuRD subunits were shown to accumulate at sites of DNA damage, including CHD3 and CHD4, HDAC1 and HDAC2, the regulatory subunits MTA1 and MTA2 as well as the methylated DNA-binding protein MBD3 (Chou et al., 2010; Goodarzi et al., 2011; Larsen et al., 2010; Luijsterburg et al., 2012; Polo et al., 2010; Smeenk et al., 2010). Besides chromatin remodeling, the different NuRD complexes also facilitate histone deacetylation through HDAC1 and HDAC2, and have both inhibitory (CHD3) and stimulatory (CHD4) effects on the progression of DSB repair as described below.

The nucleosome remodeler CHD3 possess a small SIM domain, which mediates its binding to SUMOylated KAP1 within undamaged heterochromatin. Upon DSB induction, however, KAP1 becomes phosphorylated by ATM and this modification interferes with the SIM-SUMO interaction between CHD3 and KAP1. This results in the dispersal of CHD3 from heterochromatin surrounding DSBs, local chromatin relaxation and subsequently efficient DSB repair (Goodarzi et al., 2011). Interestingly, since CHD4-depletion did not affect chromatin condensation, only CHD3 seems to collaborate with KAP1 in maintaining heterochromatin compaction (Goodarzi et al., 2011).

A role of CHD4 in DDR has been implicated by several labs. The protein is recruited in a PARP-dependent fashion and physically associates with the FHA domain of RNF8 to promote RNF8-dependent chromatin unfolding and ubiquitin conjugation. These steps are then followed by the assembly of downstream signaling and repair factors, such as RNF168 and BRCA1. Hence, CHD4 was shown to be important for the proper execution of DSB repair (Chou et al., 2010; Goodarzi et al., 2011; Larsen et al., 2010; Luijsterburg et al., 2012; Polo et al., 2010; Smeenk et al., 2010).

The chromatin remodeler ALC1, often referred to as Chromodomain Helicase DNA binding protein 1 like (CHD1L), is related to the CHD family, but contains a C-terminal macrodomain that facilitates PAR-binding (Ahel et al., 2009; Gottschalk et al., 2009). Nonetheless, its exact mode of action during the response to DSBs remains enigmatic.

INO80

ATPases of the INO80 family of chromatin remodelers are divergent from other families by their longer spacer region between the ATPase and helicase domains (Clapier and Cairns, 2009). They also feature an HSA domain which mediates the assembly of actin and actin-related proteins (ARP) to the complex (Fig. 6) (Szerlong et al., 2008). In the human INO80 complex subunits INO80, SRCAP, TIP60/TRRAP with ATPase p400 and SMARCAD1 are unique to the complex, whereas several other subunits are known to be part of different multi-subunit complexes.

Human INO80 accumulates at laser inflicted DNA damage in a manner that is dependent on its subunit ARP8, but independent from γ H2AX (Kashiwaba et al., 2010). In contrast, the recruitment of yeast Ino80 relies on the interaction of the subunit Arp4 with γ H2AX, as well as the on other subunits like Arp8 and Nhp10 (Downs et al., 2004; Kashiwaba et al., 2010; Morrison et al., 2004). Several studies in yeast have described a role for Ino80 in DNA end resection and DSB repair mediated through the removal of H2A.Z/H2B histone dimers from the DNA in the vicinity of DSBs (Chambers and Downs, 2012). This nucleosome remodeling activity contributes to enhanced accessibility of DSBs for repair proteins and ultimately the maintenance of genome stability. Consistently, also mammalian INO80 has recently been suggested to support efficient DSB repair by mediating the 5' to 3' resection of DSB ends (Gospodinov et al., 2011). In more detail, INO80 removes H2A.Z from chromatin

flanking DSBs together with the histone chaperone ANP32E and thereby promotes DNA end resection and DSB repair via HR (Alatwi and Downs, 2015).

The two other human ATPases that belong to the INO80 family are Snf-2-related CREB-binding protein activator protein (SRCAP) and p400. These are part of the SRCAP and TIP60/TRRAP (also NuA4) chromatin remodeling complexes, respectively. Whereas both ATPases have been implemented in the deposition of histone variant H2A.Z in nucleosomes, only p400 additionally incorporates H2A.Z in the vicinity of DSBs (Ruhl et al., 2006; Wong et al., 2007; Xu et al., 2012). Incorporation of H2A.Z promotes an open chromatin configuration through stimulation of H4 acetylation via TIP60 and p400, ubiquitin conjugate formation via RNF8 and subsequent BRCA1 loading. On the other hand, the presence of H2A.Z restricts DNA end resection and loads the Ku70-80 dimer onto DSBs (Xu et al., 2012). Hence, INO80-dependent removal of H2A.Z from damaged chromatin rather promotes end resection and DSB repair via HR (Alatwi and Downs, 2015), as mentioned above. Interestingly, p400 itself has also been described to promote HR through the recruitment of RAD51 (Courilleau et al., 2012), while SRCAP facilitates efficient DSB repair via HR through its DNA damage-induced interaction with CtIP that promotes its recruitment and that of RPA and RAD51 (Dong et al., 2014).

SMARCAD1

The yeast Snf2-related chromatin remodeler FUN30 forms a homodimer in cells and its ATPase activity is stimulated by the presence of DNA (Awad et al., 2010). Fun30 has been implicated in the maintenance of the chromatin structure through the inhibition of euchromatin assembly at heterochromatic regions (Stralfors et al., 2011). Three separate studies showed the recruitment of Fun30 to DNA damage and implicated a role for FUN30 in long-range DNA end resection (Chen et al., 2012b; Costelloe et al., 2012; Eapen et al., 2012). The closest human homolog of Fun30 is SMARCAD1, which similarly promotes the 5' to 3' degradation of DSB ends and facilitates RPA/RAD51 loading onto chromatin (Costelloe et al., 2012). SMARCAD1 thus has an evolutionary conserved role in DSB repair and in the maintenance of genome stability in the context of chromatin.

DDR AND DISEASE

As described in the previous paragraphs, the DNA damage-mediated posttranslational modifications of chromatin and chromatin-associated proteins are crucial for the efficient and timely recruitment of DDR proteins involved in chromatin remodeling, DNA damage signaling, DNA repair (pathway choice), cell cycle progression or transcription, at DNA lesions. The attracted histone modifiers and chromatin remodelers dynamically shape the chromatin environment around these lesions by controlling chromatin organization and the binding of DDR factors to the lesion. In this manner, these chromatin-modifying enzymes regulate the crosstalk between DNA damage signaling and repair as well as other nuclear processes such as replication, transcription and cell cycle regulation (Kruhlak et al., 2007; Shanbhag et al., 2010; Solovjeva et al., 2007; Ui et al., 2015). Consequently, loss of such enzymes can have detrimental effects on genome stability, one of the major hallmarks of cancer. In addition, on the organismal level their loss can cause embryonic lethality, neurodegeneration and premature aging. Thus, it is of great importance to gain further insight in the exact roles of chromatin modifying enzymes during the spatiotemporal organization in diverse cellular processes, including the DDR, in order to understand how and which diseases are caused by their functional loss. Even though our knowledge of the DDR has tremendously increased

over the last decades, novel factors, which often include chromatin modifiers, are still being identified. It is therefore challenging to implement the large number of identified DDR factors in one general model describing their roles during the cellular response to DNA damage. Moreover, parameters like the cell type, the differentiation state of cells and the compaction status of the chromatin surrounding the DNA damage, as well as the type of DNA damage have to be taken into account when trying to get an integrated view on all aspects of the DDR. Future work should therefore focus on mechanistic analysis of each of the identified players in the context of the DDR network in a defined cellular model upon the induction of defined types of DNA damage in order to obtain a more detailed understanding of the complexity of the DDR in the context of chromatin.

Several monogenic diseases are caused by defects in DDR factors and often display pleiotropic clinical phenotypes. For example, mutations in the gene encoding NBS, which together with MRE11 and RAD50 keeps the broken DNA ends in close proximity and activates the ATM kinase (Paull, 2015), lead to Nijmegen Breakage Syndrome. NBS patients display a typical facial appearance, growth retardation, microcephaly, immunodeficiency, IR-sensitivity and predisposition to (lymphoid) malignancies (Chrzanowska et al., 2012; Gladkowska-Dura et al., 2008; Weemaes et al., 1981). Another example represents mutations in ATM, which cause the disorder Ataxia Telangiectasia (AT). AT patients are radiosensitive, display a high incidence of cancer (leukemia, lymphoma) and suffer from immunodeficiency (Staples et al., 2008). The phenotypic manifestation varies in severity with the type of mutation and accordingly with the residual amount of functional ATM kinase present in cells (Verhagen et al., 2012). Finally, mutations in the E3 ubiquitin ligase RNF168 involved in the ubiquitin-dependent signaling of DSBs can give rise to a human disease. Patients with either homozygous or compound heterozygous mutations in RNF168 suffer from RIDDLE syndrome and display immunodeficiency, radiosensitivity, learning difficulties, as well as dysmorphic features (Devgan et al., 2011; Stewart et al., 2009).

Primary immunodeficiencies in patients have been described to originate from mutations in NHEJ genes, which are important for the repair of DSBs. Interestingly, these mutations also hamper the development of B- and T-cells via V(D)J recombination and CSR within the bone marrow, since NHEJ is required for these processes to repair the deliberately induced DNA breaks. The first patient with classical severe combined immunodeficiency (SCID), comprising defective precursor B-cell development and IR sensitivity, was described in 2009 (van der Burg et al., 2009). Mutations in Artemis and members of the LIG4-XRCC4-XLF NHEJ ligation complex have also been linked with such clinical phenotypes. LIG4-deficient patients are sensitive to IR, but dependent on the mutation patients show slightly different additional clinical features such as growth anomalies or immunodeficiency with varying severity (Woodbine et al., 2014). Furthermore, Lig4 is essential in mice, since Lig4 knockout mice are embryonic lethal. In line with this observation, LIG4 deficient patients suffer from tolerable hypomorphic mutations (Barnes et al., 1998; Frank et al., 1998; Gao et al., 1998). In contrast to the LIG4 deficient patients, the immunological phenotype of XLF deficient patients is very severe and these patients additionally display microcephaly, growth retardation as well as sensitivity towards IR (Ahnesorg et al., 2006; Buck et al., 2006; Dai et al., 2003; Dutrannoy et al., 2010). Also a few patients carrying XRCC4 mutations have been described. While they phenotypically showed developmental alterations, no immunological defect was documented for this group of patients, despite the fact that patient-derived cells actually did display defects in NHEJ (de Bruin et al., 2015; Guo et al., 2015; Rosin et al., 2015; Shaheen et al., 2014).

As mentioned above, the aberrant expression of chromatin-modifying enzymes can also lead to various diseases. For example, mutations in several components of the SWI/SNF chromatin remodeling complexes have recently been found in patients with intellectual disability syndromes i.e. Coffin-Siris and Nicolaides-Baraitser (Santen et al., 2012a; Schrier et al., 2012; Van Houdt et al., 2012). On the other hand, expression of the SWI/SNF ATPases BRG1 and BRM is frequently lost in several human tumors, with one or both genes being silenced (Reisman et al., 2009). Moreover, the ISWI chromatin remodeler SMARCA5 (Table 1), which associates with RNF168 and helps to execute the RNF168-mediated DSB response (Smeenk et al., 2013), was found to be either overexpressed or mutated in several different tumors (Cetin et al., 2008; Gigek et al., 2011; Stopka et al., 2000; Sumegi et al., 2011). For an overview of these and other chromatin remodelers that have been causally linked to various diseases due to aberrant expression or mutations, I refer to Table 1.

DDR AND THERAPY

In the last few decades scientists tried to gain more detailed knowledge on the events taking place during the DDR with the purpose to better diagnose and subsequently develop treatments for DDR-associated diseases. To improve the current treatment opportunities of for instance cancer, mechanistic insights in the organization of the DDR are exploited by means of developing small molecule inhibitors for targeted cancer therapies. This form of patient treatment is tailored according to the genetic alterations in their tumor cells, which often have defects in one or more DDR pathway(s). Consequently, the tumor cells are increasingly reliant on the remaining DDR pathways to restore damaged DNA. The concept of synthetic lethality takes advantage of this fact: the targeted deactivation of one DNA repair pathway in combination with a cancer-specific defect in at least one other DNA repair pathway leads to cell death, whereas the deficiency in only one of these repair pathways does not. This approach is very promising since it specifically targets a defect in cancer cells. The PARP1 enzyme has been described to be involved in a number of DNA repair pathways such as SSB repair, base excision repair, nucleotide excision repair and DSB repair (Pines et al., 2013). Its inhibition suppresses DNA repair and sensitizes cells to the cytotoxic effects of DNA damaging agents (Durrant and Boyle, 1982; Nduka et al., 1980). Recently, inhibitors of PARP appeared to have clinical impact on the treatment of cancers lacking functional HR. The best studied example represents BRCA1/2-deficient (breast and ovarian) cancer cells, which proved highly sensitive to PARP inhibitors. The current view is that inhibitor-inactivated PARP becomes trapped at single-strand DNA breaks, which are converted into deleterious DSBs upon DNA replication. Repair of these DSBs normally requires HR (Helleday, 2011; Murai et al., 2012). However, BRCA1/2-deficient cancer cells, when treated with PARP inhibitor, fail to repair these DSBs. This leads to the accumulation of unrepaired DNA breaks till over multiple rounds of replication the level of genomic instability becomes non-viable and eventually causes tumor cells to die (Fong et al., 2010). Recent clinical trials with the PARP inhibitor Olaparib have established the therapeutic potential of PARP inhibitors for BRCA1/2-deficient cancer patients (Feng et al., 2015) and this inhibitor was recently approved for clinical use.

Promising candidates for this PARP-dependent synthetic lethality approach are chromatin remodelers that have recently been linked to DSB repair by HR and to tumorigenesis. For instance, cells depleted of the chromatin remodeling ATPase SMARCA5 are defective in HR and highly sensitive to PARP inhibitor treatment (Smeenk et al., 2013). Furthermore, loss of functional SMARCA5 was found in various cancer cell types (Cetin et

al., 2008; Sumegi et al., 2011), raising the opportunity to treat these cancer cells with PARP inhibitor to induce their cell death. Additionally, loss of the chromatin remodeler CHD4 has also been shown to give rise to significant sensitivity to PARP inhibition as a consequence of defective HR repair (Pan et al., 2012). Remarkably, the expression of CHD4 is lost in about 50% of investigated gastric cancers (Kim et al., 2011), which may sensitize these cancer cells towards PARP inhibitor treatment.

Furthermore, also histone modifiers have recently been identified as promising candidates for PARPi treatment. USP26 and USP37 are two DUBs that regulate RAP80-dependent BRCA1 assembly by reversing RNF168-induced histone H2A(X) ubiquitylation at sites of DNA damage (Typas et al., 2015). Decreased and increased expression of these DUBs lead to defective HR (Typas et al., 2015), and accordingly knockdown of either DUB renders cells sensitive to PARP inhibition. Interestingly, numerous cancer cell lines with decreased or excessive expression of one of these DUBs exist as published in the COSMIC database. As such, these tumors may display defects in HR and sensitivity towards PARP inhibitors. However, since USP37 depletion only results in moderate PARP sensitivity (Typas et al., 2015) patient benefits could be modest with regard to tumor cell death. In this respect it is interesting to note that USP26-deficient cells are almost as sensitive to PARP inhibitor treatment as BRCA1/2-deficient cells (Typas et al., 2015), suggesting that targeting USP26-defects rather than USP37-defects by using PARP inhibitor treatment may be a more promising strategy.

Remarkably, also aberrant levels of epigenetic chromatin modifications have been linked to the development and maintenance of cancer. These (primarily histone) modifications can determine phenotypic characteristics of diseases in a manner that is independent of the patient's genotype. The reversible nature of such epigenetic alterations can provide opportunities for pharmacologically targeted cancer therapies that employ small-molecule inhibitors. Reversing the enzymatic activity of such histone-modifiers can for instance (re-)direct transcriptional processes and (re)activate epigenetically silenced genes in cancer cells. Accordingly, research has focussed on the application of inhibitors of HATs, HDACs, histone methyltransferases and demethylases in cancer therapy (Biancotto et al., 2010). Promising compounds have been found of which some received approval for patient treatment by the US Food and Drug Administration. However, in spite of this achievement it is important to note that clinical responses appeared to be pleiotropic when inhibiting a whole class of enzymes (such as HDACs) (Biancotto et al., 2010) causing unwanted side-effects. Consequently, the development of more specific inhibitors of epigenetic modifiers is a high priority in research. This has been fruitful for instance in the case of HDAC inhibitors, from which an effective anticancer drug (Romidepsin) to cutaneous T-cell lymphomas was generated. Upon Romidepsin-mediated inhibition of HDACs in these cells, acetylation levels of histones and non-histone proteins are maintained, which promote transcriptionally active DNA. The latter can lead to the restoration of gene expression of silenced genes and subsequently inhibited cancer progression (Barbarotta and Hurley, 2015). Furthermore, HDAC inhibitors can induce cell cycle arrest as well as apoptosis and suppress DNA repair through the acetylation or down regulation of DDR genes. The latter effect can be further exploited to increase the lethal effect of HDAC inhibitors on cancer cells when combining the HDAC inhibitor treatment with chemotherapy or radiation (Lakshmaiah et al., 2014).

Specific inhibitors targeting chromatin modifiers can nowadays be used for a cancer treatment approach referred to as personalized medicine, which thrives to identify the genetic background of a patient and unravel the altered biology of their tumor. This information will help clinicians to customize the treatment to each patient's needs and although this

approach sounds very promising, a few drawbacks are still to be overcome. Deciphering the genetic variation(s) in patient tumor cells via genotyping and the subsequent treatment are for example still rather expensive. In addition and more importantly, despite the amount of available genetic information gathered in the last decades, we still lack knowledge of the consequences of (the identified) mutations in cancer cells. Since a significant portion of such mutations have been found in histone modifiers and chromatin remodelers involved in the DDR, mechanistic apprehension of their function can help to define the effect of mutations. We and others are investigating the role of chromatin modifiers in the DDR upon suppression of gene expression, which is a well-defined experimental setting and the easiest to imitate in cell culture. However, whether a mutation in a histone modifier or chromatin remodeler causes complete loss of gene activity in tumor tissue remains questionable and requires further investigation. Eventually, this knowledge will contribute to our understanding of the DDR and could explain the cause of diseases arising from aberrant activity of chromatin modifiers due to (epi-)genetic defects. This fundamental research should eventually contribute to the identification of appropriate targets for future therapies and the development of novel treatment approaches for various human diseases such as cancer.

AIMS AND OUTLINE OF THIS THESIS

Upon the induction of DNA damage, cells initiate a protective response, referred to as the DNA damage response (DDR), to repair DNA damage and maintain genome integrity. This response is driven and regulated by posttranslational protein modifications and chromatin remodeling events. Mutations or aberrant expression of chromatin modifying proteins not only impacts on the DDR, but also causes human diseases with severe clinical phenotypes, illustrating the importance of these proteins for genome stability maintenance and human health. Largely unclear is, however, which and how chromatin modifying enzymes control the complex DDR pathways and in this manner prevent the onset of disease. To this end, we employed cross-disciplinary approaches that combined cell biological, biochemical and microscopic methods to identify histone modifying enzymes, chromatin remodelers as well as other DDR proteins and elucidate their mechanistic role in the response to DNA double-strand breaks (DSBs) and disease prevention.

Chapter 1 comprises a general introduction and reviews the current knowledge on DDR pathways, in particular pathways that respond to DSBs and the role of chromatin modifying enzymes therein. In **Chapter 2**, I introduce the set-up of a siRNA-based screening approach that I used to identify novel chromatin regulators involved in the DSB response. This screen identified the histone methyltransferase EHMT1 as a negative regulator of 53BP1 recruitment to sites of DNA breaks and presents the first evidence for a role in DSB repair by HR and NHEJ. **Chapter 3** addresses the role of the Remodeling and spacing factor 1 (RSF1) during DSB repair via NHEJ. RSF1 deposits the centromeric proteins CENP-S and CENP-X at DSBs. These factors subsequently promote the recruitment of XRCC4 and consequently efficient NHEJ. Additionally, the DNA damage-dependent SUMOylation of RSF1 is presented in **Chapter 4**. The so far obtained data suggests that SUMOylated RSF1 regulates XRCC4 recruitment and possibly NHEJ. In **Chapter 5** I show that ZBTB24, which is mutated in Immunodeficiency, facial anomalies and centromeric instability 2 (ICF2), interacts with key factors of the NHEJ pathway, namely PARP1 and DNA-PKcs. Moreover, I demonstrate that ZBTB24 promotes XRCC4/LIG4 binding, most likely to PARP1 by binding and protecting PARP1-associated PAR chains, facilitating DSB repair via NHEJ. Importantly, ZBTB24's role in NHEJ is required for NHEJ-mediated immunoglobulin class switch recombination (CSR) in B cells, which provides a molecular basis for the immunodeficiency in ICF2 syndrome. Finally, in **Chapter 6**, I generally discuss the implications of the presented studies described in this thesis.

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