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Posttranslační modifikace E3 ubiquitin ligázy RNF168

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I hereby declare that this thesis has been written solely by myself and that the sources used in this thesis are cited and included in the references part.

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SUMMARY

DNA double-strand breaks (DSBs) are caused by many different agents of endogenous or exogenous origin. DSBs are by far the most cytotoxic lesions that may occur in the DNA molecule carrying the essential genetic information. Even a single unrepaired break can lead to large-scale deletions or translocations and eventually to apoptosis or oncogenic transformation. To evade the life threatening damage, eukaryotic cells have evolved a complex network of mechanisms ensuring the cellular response to introduced lesions and their repair. The system includes many different proteins with distinct and specific enzymatic activities organized into an intricate network of signalling cascades. In order to ensure proper detection and repair of the DNA lesion, all steps of the signalization cascades leading to repair and cell cycle checkpoint activation must be tightly regulated. This is mostly achieved by post-translational modifications of the involved proteins. Regulations mediated via the post-translational modifications provide the cells with the required complexity, but at the same time are flexible enough to react to the varying needs of the DNA damage response. The essential role of ubiquitin signalization in DSB repair has been revealed several years ago. Aim of the theoretical part of this thesis is to summarize the role of key factors in the cellular response to double-strand breaks with emphasis on the RNF168 E3 ubiquitin ligase. RNF168 is one of the key proteins involved in signalization of double-strand breaks and facilitates the early stages of their repair by amplifying the ubiquitination signals generated by upstream RNF8 to the threshold required for the recruitment of downstream repair factors such as 53BP1 or BRCA1.

The experimental part of this work was focused on identification of post-translational modifications of RNF168 and characterization of their biological role in the modulation of RNF168 stability and activity, with emphasis on phosphorylations.

SOUHRN

DNA dvouvláknové zlomy mohou být způsobeny celou řadou faktorů endogenního i exogenního původu a představují nejnebezpečnější typ poškození DNA molekuly, která je esenciální pro přenos genetické informace. Jediný neopravený zlom může vést k rozsáhlým delecím a translokacím, a v konečném důsledku k apoptóze, nebo onkogenní transformaci. V průběhu evoluce se u eukaryotických buněk vyvinula komplexní síť mechanismů chránících buňky před touto život ohrožující hrozbou a zajišťujících buněčnou odpověď na vznik tohoto poškození a jeho opravu. Do této členité sítě signálních kaskád je zapojeno mnoho proteinů s různými specifickými enzymatickými aktivitami. Aby bylo možné zachovat funkčnost celého systému zajišťujícího detekci a opravu poškození DNA, všechny kroky signalizačních kaskád vedoucí k opravě a aktivaci buněčných kontrolních bodů buněčného cyklu musí podléhat přísné regulaci. Ta je nejčastěji zajišťována na úrovni posttranslačních modifikací zapojených proteinů. Regulace prostřednictvím posttranslačních modifikací poskytují buňkám potřebnou komplexitu, ale zároveň také flexibilitu, která je esenciální pro vyvolání adekvátní buněčné odpovědi na specifické typy poškození DNA. Před několika lety byla odhalena esenciální úloha signalizace přes ubiquitin při opravě dvouvláknových zlomů. Cílem teoretické části této práce je shrnout úlohu klíčových faktorů buněčné odpovědi na dvouvláknové zlomy, se zaměřením na E3 ubiquitin ligázu RNF168, která představuje jeden z esenciálních proteinů zapojených do signalizace dvouvláknových zlomů. Amplifikací ubiquitinace vytvořené ligázou RNF8 umožňuje rozpoznání místa poškození dalšími proteiny jako 53BP1 nebo BRCA1 potřebnými pro opravu poškození, a zajišťuje tak počáteční fázi opravy.

Experimentální část této práce je zaměřena na identifikaci posttranslačních modifikací RNF168 a charakterizaci jejich biologické úlohy v modulaci stability a aktivity RNF168, se zaměřením na fosforylace.

First and foremost, I would like to thank my supervisor Dr. Juraj Kramara for his advice and patient guidance in the completion of this thesis. His creativity, friendship and passion for science have been an inspiration to me throughout my studies and motivated me for the further education. He has also opened me doors to the promising future scientific career, which I will never forget.

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1. INTRODUCTION

Integrity of the genetic material is essential for its faithful transmission to offspring. Nevertheless, the integrity is constantly threatened by many factors of environmental and endogenous origin. Most living organisms have evolved a complex signal transduction pathway generally termed DNA Damage Response (DDR), which is capable of detecting perturbations in the integrity of the genetic material and transducing the information to relevant cellular processes that mount adequate cell reaction, most importantly, repair of the damage itself through variety of enzymatic tools. On the other hand, activity of the repair proteins must be tightly regulated to prevent occurrence of unwanted alterations in the genetic material such as deletions, amplifications or even large-scale rearrangements (Ciccia and Elledge, 2010).

DDR plays a crucial role in many physiological processes in multicellular organisms. Generation of highly variable T-cell receptors and immunoglobulins during B and T lymphocytes development would not be possible without DDR, as it is dependent on the V(D)J class switch recombination, the only known case of programmed genome alterations in vertebrates (Bassing and Alt, 2004). DDR is also a key mediator in generation of genetic diversity via meiosis and sexual reproduction. In meiosis, the duplicated homologous sister chromatids align and a homologous recombination (HR) driven process termed crossover takes place. Crossover is a controlled shuffling mechanism for generation of new genetic combinations and provides potentially unlimited source of different individual genotypes in progeny (Jackson and Bartek, 2009; Richardson et al., 2004).

DDR defects caused by mutations or dysregulation of the gene encoding the repair, signalling and other DDR related proteins result in diverse pathophysiological states such as cancer (Jackson and Bartek, 2009; Stratton et al., 2009), neurodegenerative disorders (Rass et al., 2007), immune deficiencies (Jackson and Bartek, 2009), infertility (Bartkova et al., 2007), premature ageing syndromes (Schumacher et al., 2008), cardiovascular diseases (Mercer et al., 2007) and metabolic syndromes (Kastan, 2008; Schneider et al., 2006).

Surprisingly, cancer cells often display significant genomic instability resulting from defects in the DDR. Genomic instability may be then characterized by a combination of DNA damage, defects in repair cascades and defects in cell cycle checkpoints. Such a combination of defects in DNA damage repair and cell cycle checkpoint signalization enables extensive genotype variability in tumour cells and is a key mediator of microevolution in tumours. Although these processes represent an advantage for tumours from the microevolutionary perspective, they also provide therapeutic opportunities due to overall lowered repair capacity resulting from the defects in repair cascades and rapid outgrowth (Jackson and Bartek, 2009; Komarova et al., 2008; Pearl et al., 2015).

DNA damage inducing chemotherapy drugs has been mainstay in cancer therapy for decades. Mechanisms of action of these drugs are more or less specific, but all of them eventually lead to induction of specific types of DNA damage, such as covalent crosslinks, DNA alkylation, single-strand breaks (SSBs), or DSBs. Other types of agents induce DNA damage via inhibition of DNA synthesis proteins, e.g. DNA polymerases or topoisomerases (Lord and Ashworth, 2012).

In contrast to these types of DNA damaging agents, selective targeting of the specific players in DDR has arisen as a promising alternative. Usually individual proteins regulating certain steps of DDR cascade are targeted in this strategy, for example DNA-dependent protein kinase (DNA-PK) involved in non-homologous end joining (NHEJ) repair pathway, or cell cycle checkpoints, for example checkpoint kinase 1 (CHK1) or WEE1 (Wee1-like protein kinase). Initial purpose of DDR proteins targeted drugs development has been to use them in combination with radiotherapy and enhance its cytotoxic effect on cancer cells by inhibition of repair processes activated by radiation-induced DNA damage. However the drugs inhibiting DNA damage repair can also be used as a stand-alone strategy as most of the damage occurs in rapidly proliferating cells during replication (Pearl et al., 2015). Very promising is a therapeutical use of poly-(ADP-ribose)-polymerase inhibitors (PARPi), which have been approved for clinical practice recently (e.g. olaparib) for targeting the BRCA1(Breast cancer type 1 susceptibility protein)/BRCA2 (Breast cancer type 2 susceptibility protein) deficient breast and ovarian tumours (Parkes and Kennedy, 2016).

2. THEORETICAL PART

2.1 DNA damage

DNA damage is a constantly occurring event in our cells and it has been shown that it plays a major role in the onset of cancer and aging-related diseases. There are many types of DNA damage arising from different sources of exogenous and endogenous origin. It has been estimated that up to 10^5 spontaneous DNA lesions may occur in each cell every day. For efficient elimination of these genome integrity threats, eukaryotic cells have evolved a complex network of repair processes specialized to each type of lesion (Hoeijmakers, 2009). While environmentally induced damage can be caused by physical or chemical agents, endogenously induced damage is mostly represented by spontaneous hydrolysis of DNA, by attacks of reactive metabolic products or errors produced by DNA replication (Dexheimer, 2013; Lindahl, 1993).

The most common environmental agents represent ionizing radiation (IR) and ultraviolet light (UV) from sunlight. UV radiation is one of the major causes of pyrimidine dimers formation which if left unrepaired, often leads to deamination of cytosines and eventually results in single nucleotide substitutions where cytosines are being replaced by thymines (Lord and Ashworth, 2012; Ward, 1988). Another exogenous physical agent is ionizing radiation (IR). IR originates from natural (cosmic radiation) as well as artificial sources (radiotherapy) and induces a variety of DNA lesions directly e.g. by causing single-strand breaks (SSBs) which can potentially lead to formation of double-strand breaks (DSBs), or indirectly e.g. by enhanced generation of reactive oxygen species (ROS) that in turn damage the DNA molecule (Dexheimer, 2013; Ward, 1988).

DNA damage arising from physiological cellular processes mostly results from two major sources. The first of these sources is the action of the reactive metabolic products such as ROS or reactive nitrogen species (RNS), generally recognized as oxidative/nitrosative DNA stress. The second source is represented by errors of DNA processing mechanisms, in this case, deletions or insertions might

occasionally occur as the consequences of base misincorporations introduced by DNA polymerase (Dexheimer, 2013).

As mentioned earlier, different types of reparation processes have evolved for each subset of DNA lesions. Five main types of repair mechanisms have been defined: mismatch repair (MMR), base excision repair (BER), nucleotide excision repair (NER) and DSB repair which includes non-homologous end joining (NHEJ) and homologous recombination (HR) pathways (Dexheimer, 2013; Jackson and Bartek, 2009). MMR is an essential post-replication repair mechanism dealing with misincorporated bases that escaped the polymerase proofreading activity and deletion/insertion loops generated by polymerase slippage(Dexheimer, 2013; Fukui, 2010). NER and BER are the repair pathways required in cases when bases (BER) or the whole nucleotides (NER) become damaged. NER is a highly versatile pathway capable of sensing the wide range of lesions with helix-distorting properties. Despite the similarities between NER and BER repair, NER requires more complex protein network due to the mechanistical difficulties associated with sensing, opening the chromatin and resynthesizing the fragment of DNA (Dexheimer, 2013; Nouspikel, 2008). In contrast to damage repaired by NER, damaged DNA bases repaired by BER do not distort the overall structure of DNA molecule (Dexheimer, 2013). BER deals with the most forms of spontaneous hydrolytic products in DNA as well as oxidative and alkylating damage of bases and involves various enzymatic tools such as glycosylases, apurinic/apyrimidinic endonucleases, phosphodiesterases, polymerases and ligases (Kim and Wilson, 2012). Since this work is mainly focused on DSB repair and its signalization cascades, other types of DNA repair mechanisms briefly mentioned above will not be further described.

2.2 DNA double-strand break repair

Among all of the different types of DNA damage, double-strand breaks (DSBs) are the most cytotoxic type of lesions and are considered to be the most serious threat to the genome integrity. As already mentioned above, a single unrepaired DSB is sufficient for permanent growth arrest or cell death induction (Bennett et al., 1993; Panier and Boulton, 2014) and can serve as the initiating point for large-scale

chromosomal rearrangements resulting in deletions, amplifications and translocations. Generally, these changes may then result in oncogene activation and/or tumour suppressors inactivations which may promote malignant transformation. Furthermore, defects in DSB repair may also lead to immunodeficiencies or neurodegenerative disorders (Jackson and Bartek, 2009).

DSBs are generated by multiple mechanisms – they can be formed upon IR, or by therapeutical inhibition of topoisomerase II. DSBs may also arise from other types of DNA lesions, mostly SSBs, which give rise to DSBs after replication machinery reaches this kind of DNA lesion (Polo and Jackson, 2011). They also occur during replicative senescence after telomeres become critically shortened and deprotected (d'Adda di Fagagna et al., 2003).

Eukaryotic cells repair DSBs by two major pathways - NHEJ and HR. The pathways are complementary to each other and each of them requires different conditions for their optimal function. Since HR requires homologous template, its functionality is restricted to the S and G2 phases in normal cells, when chromosomes are duplicated and potential homologous templates are available. Use of template during repair by HR also allows for high accuracy of the repair process, which is important for preserving the genetic information prior its transfer to daughter cell by mitosis. In contrast, NHEJ does not require any template and hence it represents a major repair pathway within the cell cycle. Nevertheless, NHEJ is error-prone as it lacks a repair template (Hartlerode and Scully, 2009).

2.2.1 Non-homologous end joining (NHEJ)

The simplest mechanism of restoring chromosomal integrity disrupted by DSB is represented by the NHEJ. In this pathway, the two ends of a DSB undergo processing mediated by various proteins that ensure their compatibility for subsequent ligation of these ends (Figure 1). However, small deletions or insertions may be introduced as a consequence of a missing template and the processing steps of the repair (Weterings and Chen, 2008).

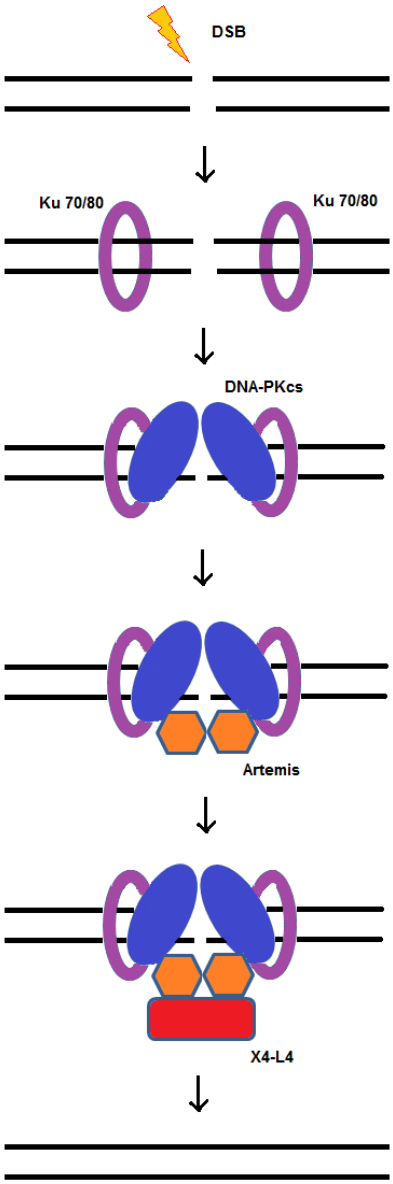


Figure 1. Repair of DSB by the NHEJ repair pathway. Ends of DSBs are recognized by Ku heterodimers. Ku binding prevents extensive resection, while allowing limited processing by downstream factors. DNA-dependent protein kinase catalytic subunit (DNA-PK_{cs}) is recruited to the site and bridges the ends. Subsequently ends are processed by Artemis and damage site is sealed by DNA ligase IV containing X4-L4 complex. Adapted from Hartlerode and Scully (2009).

The NHEJ pathway is initiated by recognition of broken ends of DNA by the Ku heterodimeric complex composed of Ku70 and Ku80 subunits, which serves

as a scaffold for recruitment of downstream proteins and NHEJ synapse assembly, but also as a protection from ends degradation (Hartlerode and Scully, 2009; Symington and Gautier, 2011). It has been shown that Ku complex localizes to laser generated DSBs within seconds (Mari et al., 2006), probably due to its extraordinary binding affinity for DSBs in a sequence independent manner as well as its abundance in the nucleus (Blier et al., 1993).

Ku binding to DNA ends initiates recruitment of other NHEJ factors, including the DNA-dependent protein kinase catalytic subunit (DNA-PK_{cs}), a member of phosphatidylinositol-3 kinase-like kinase family (PIKK) (Davis and Chen, 2013; Hartlerode et Scully, 2009). Interaction of DNA-PK_{cs} molecules with Ku and DNA initiates inward translocation of Ku along the strands and the two molecules of DNA-PK_{cs} subsequently form a synapse between the two ends of the DSB (DeFazio et al., 2002). Furthermore, the interaction of DNA-PK_{cs} with both Ku and DNA ends is also important for activation of the serine/threonine kinase activity of DNA-PK_{cs} (Hartlerode and Scully, 2009; Yaneva et al., 1997). Several phosphorylation targets have been identified in *in vitro* conditions, however it is not clear to what extent are modifications of these targets required for the NHEJ in *in vivo* conditions (Hartlerode and Scully, 2009; Yu et al., 2008). One of the most important substrates for DNA damage induced phosphorylation is DNA-PK_{cs} itself. A number of autophosphorylation sites have been described as well as its role in regulation of conformation and dynamics of the protein. Presumably these automodifications allow further processing of the ends generated by DSBs (Douglas et al., 2002; Goodarzi et al., 2006).

Another important end processing enzyme involved in NHEJ is Artemis. Artemis possesses an endonuclease activity and is recruited to damage sites through its interactions with DNA-PK_{cs}. While its 5'-3'-exonuclease activity is DNA-PK_{cs} independent, its endonuclease activity towards ds-ssDNA transitions and hairpin structures is presumably dependent on DNA-PK_{cs} autophosphorylation. The primary role of this protein is to transform the ends of a DSB to a 5'-phosphorylated and thus ligatable form (Goodarzi et al., 2006; Hartlerode and Scully, 2009; Ma et al., 2002). Inactivation of Artemis in humans leads to severe combined immunodeficiencies (SCIDs), radiosensitivity and defects in class switch recombination; however, DSB repair efficiency in cells lacking functional Artemis do not reflect major defects, which suggests existence of other, Artemis-independent repair mechanism (Wang et al.,

2005). Despite it seems that Artemis is involved in several repair mechanisms, its roles in DDR have still not been completely understood yet (Hartlerode and Scully, 2009; Moscariello et al., 2015). Artemis-processed ends serve as a substrate for a ligation step carried out by the X4-L4 complex containing XRCC4, DNA ligase IV and XLF. It has been shown, that mutation of XRCC4 renders mammalian cells highly susceptible to ionizing radiation. Although it has no known enzymatic activity, stimulatory effect on Ligase IV has been described (Grawunder et al., 1997). However, many other proteins have been implicated to be involved in DNA end processing and ligation steps, but further extensive research is required for the clear understanding of their interplay (Davis and Chen, 2013).

2.2.2 Homologous recombination (HR)

The key steps required for initiation of repair by HR are resection of the ends of DSB and invasion of the 3' single-stranded DNA into a homologous duplex mediated by RAD51 nucleofilament. The duplex containing the invading 3' overhang then serves as a primer and the intact strand of a homologous duplex provides a template for the repair synthesis (Figure 2). Intermediates generated during the synthesis are known as the D-loops (Jasin and Rothstein, 2013)

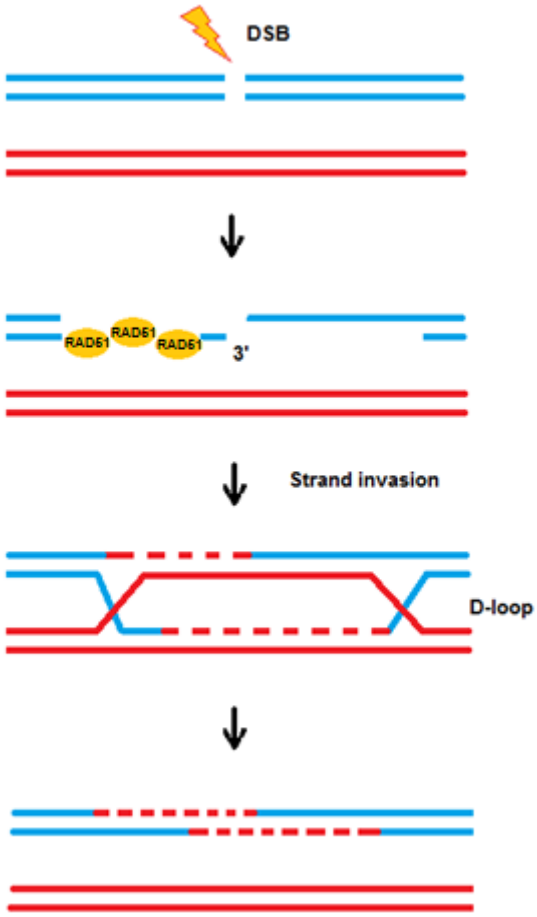


Figure 2. Repair of DNA double-strand break by HR., 3' overhangs are generated by end resection upon DSB induction and ssDNA (single-stranded DNA) flanks are coated by RAD51, which initiates the strand invasion. Sister chromatid serves as a template for resynthesis of the damaged or lost region. Modified from Sung and Klein (2006).

The need of a template for homology directed repair limits its occurrence mainly to the S phase and G2 phase, when DNA is duplicated and the template is available. However, the transitions between HR-mediated and NHEJ-mediated repair are not strict and one repair mechanism is fluently overlaid by the other at the certain cell phase transition points. Thus, even during the S and G2 phases, a small fraction of DSBs might be repaired by NHEJ mechanism and vice versa (Mao et al., 2008).

Besides DSB repair, HR is also involved in other processes, which are crucial for genome maintenance and generally for survival of the organism, such as DNA

replication fork restart, meiotic chromosome segregation and telomere maintenance (Sung and Klein, 2006).

Initiating step of HR is generation of a 3' single-stranded DNA by nucleolytic resection of the 5' strand. Extent of the resection may usually vary from one to several kilobases (Jasin and Rothstein, 2013; Zhou et al., 2014), and requires different enzymatic tools depending on the size of the resected region. While the MRN (MRE11-RAD50-NBS1) complex and CtIP (CtBP-interacting protein) are sufficient for catalyzing the resection several bases long, which promotes the special subtype of NHEJ (known as microhomology-mediated end joinig - MMEJ), more extensive resection involves also the EXO1 (Exonuclease 1) 5'-3' exonuclease and BLM (Bloom syndrome protein) helicase and promotes the classical HR (Ottaviani et al., 2014; Truong et al., 2013). Generally, the extensive resection leading to repair by HR can be carried out by one of the two main machineries, BLM-DNA2-RPA-MRN or EXO1-BLM-RPA-MRN, but many other proteins are implicated in this event. In the first, ATP-dependent pathway, BLM helicase together with the DNA2 bipolar exonuclease mediate unwinding and degradation of the 5' strand, while RPA (Replication protein A) helps unwind the strands by binding the ssDNA thus enhancing the 5'-3' exonuclease activity of DNA2. The MRN exo- and endo-nuclease complex promotes processing by recruiting BLM to the ends. In the second resection pathway, MRN recruits EXO1 and increases its processivity, while BLM is required for enhancing the affinity of EXO1 towards DNA ends (Nimonkar et al., 2011).

CtIP has been identified as an important functionally and physically interacting partner of MRE11 (Sartori et al., 2007). It plays a role in regulation of a pathway choice by promoting end resection, which is the critical decision point leading to repair mediated by HR. Furthermore, both CtIP and MRN are important DNA damage checkpoint triggering sensors (Lavin, 2004; Sartori et al., 2007). CtIP through its interaction with BRCA1 stimulates CHK1 (Checkpoint kinase 1) phosphorylation and controls G2/M DNA damage transition checkpoint (Yu and Chen, 2004), but also promotes ATR (Ataxia telangiectasia mutated and Rad3 related) kinase activation via promoting end resection by MRN interaction (Sartori et al., 2007). MRN-CtIP dependent resection results in a formation of a 3'-tailed DNA, which subsequently serves as a platform for binding of proteins responsible for strand invasion (Cejka, 2015; Sartori et al., 2007).

Based on cytology and chromatin immunoprecipitation data, RPA has been shown to be binding to nascent ssDNA strands with the higher affinity than the RAD51 recombinase (Sugiyama et al., 1997; Sung, 1997). Primary role of RPA is to promote HR by preventing ssDNA flanks from the formation of secondary structures (Sung et al., 2003), but it is also engaged in a negative regulation of HR through the binding competition with a RAD51 the main strand-exchange protein. Importantly, RPA can be subsequently replaced by RAD51 molecules with the help of other mediator proteins. In mammalian cells, the BRCA2 protein is a key mediator protein that stimulates the loading of RAD51 to ssDNA (Jasin and Rothstein, 2013; San Filippo et al., 2008).

Rad51 drives strand invasion by facilitating the physical connection between the invading and template DNA strands. HR intermediates - D-loops are generated during this process (Krejci et al., 2012).

2.2.3 Chromatin response

Since the nuclear DNA of eukaryotic organisms is wrapped around histone proteins and forms tightly packed structure called chromatin, every time that damage occurs, this structure has to be remodelled to allow access to factors of repair machinery to interact with lesion and to promote the adequate cellular response. Hence after generation of DSBs, rapid and extensive chromatin associated response is initiated (Smeenk and van Attikum, 2013; van Attikum and Gasser, 2009).

Two main classes of enzymes facilitate chromatin modifications: chromatin remodelers and histone modifiers. The remodelers catalyse ATP-dependent destabilization, removal or restructuring of nucleosomes to make a discrete region of DNA accessible not only to the DNA repair, but also for other cellular processes like replication and transcription (Clapier and Cairns, 2009). The second class includes proteins modifying chromatin by adding post-translational modification (PTM) marks to histone tail residues. Various modifications can be associated with histones, including acetylations, methylations, phosphorylations, ubiquitinations, SUMO(Small ubiquitin-like modifier)ylations and poly(ADP-ribosyl)ation (also known as PARylation). Proteins involved in attachment of modification marks are known

as writers while proteins with the ability to recognize these marks and interact with them are called readers. Histone code represented by PTMs is very complex, since each of these modifications has a different function depending on several factors, such as its position within the histone. Furthermore, different combinations of modification marks may promote distinct responses. However, the best studied function of these histone modifications is their involvement in recruiting other proteins to the chromatin (Smeenk and van Attikum, 2013).

Since the proper chromatin-based response induced by exposure to damage-inducing stimuli is an essential part of the whole repair process and any defects in this response may have fatal consequences, it is of paramount importance for a cell to tightly guard the histone writers and readers equilibrium. As a consequence of this tight regulation, very prominent and rapid changes in levels of involved proteins and accentuated changes in their subnuclear localization have been observed upon inflicting DNA damage (Smeenk and van Attikum, 2013; van Attikum and Gasser, 2009).

Exposure to ionizing radiation leads to accumulation of specific DDR proteins in discrete foci called ionizing-radiation-induced foci (IRIF) within the nucleus (Fernandez-Capetillo et al., 2003). It was initially unclear how proteins are recruited to IRIFs and what are the exact mechanisms of their retention. Later on, genetic studies in mice have identified H2AX histone variant as a core component for IRIFs formation. This variant represents approximately 10 % of all H2A histone molecules. *H2AX*$^{-/-}$ mice have been shown to be radiation sensitive, growth retarded, immunodeficient and mutant males were infertile. This pleiotropic effect of H2AX deletion was connected to increased chromosomal instability and repair defects caused by overall impairment in recruitment of the basal DDR component factors such as Nbs1, 53bp1 (Tumour suppressor p53-binding protein) and Brca1 (Celeste et al., 2002). In response to DNA damage, the C-terminal Ser139 of H2AX undergoes rapid phosphorylation by ATM (Ataxia telangiectasia mutated) or the other two DDR related PIKK kinases (ATR or DNA-PK) (Figure 3). Although the exact molecular mechanism of ATM activation remains elusive, at least two events have been shown to be required for the activation - relocalization of the Tip60-ATM complex to the DSB site, which has been shown to by critically dependent on the direct interaction with MRN (Sun et al., 2009, Lee and Paull, 2004) and ATM modification by acetylation (Sun et al., 2005).

The phosphorylated form of H2AX is called γH2AX and is recognized as one of the universal markers of induced DNA damage (Sharma et al., 2012). In mammalian cells, H2AX phosphorylation may spread over a 2 Mb long region of DNA surrounding the DSB site. It has been shown, that only 1 min after exposure to ionizing radiation half-maximal amounts of γH2AX are reached, which indicates how quickly cells recognize DSBs and how fast they can react to it (Rogakou et al., 1998). Phosphorylated H2AX then serves as a high-affinity binding site for recruiting downstream DDR factors (Figure 3), which binds the phosphorylation mark through their forkhead-associated (FHA) domain or tandem repeat BRCA1 C-terminal (BRCT) domains (Stucki and Jackson, 2004).

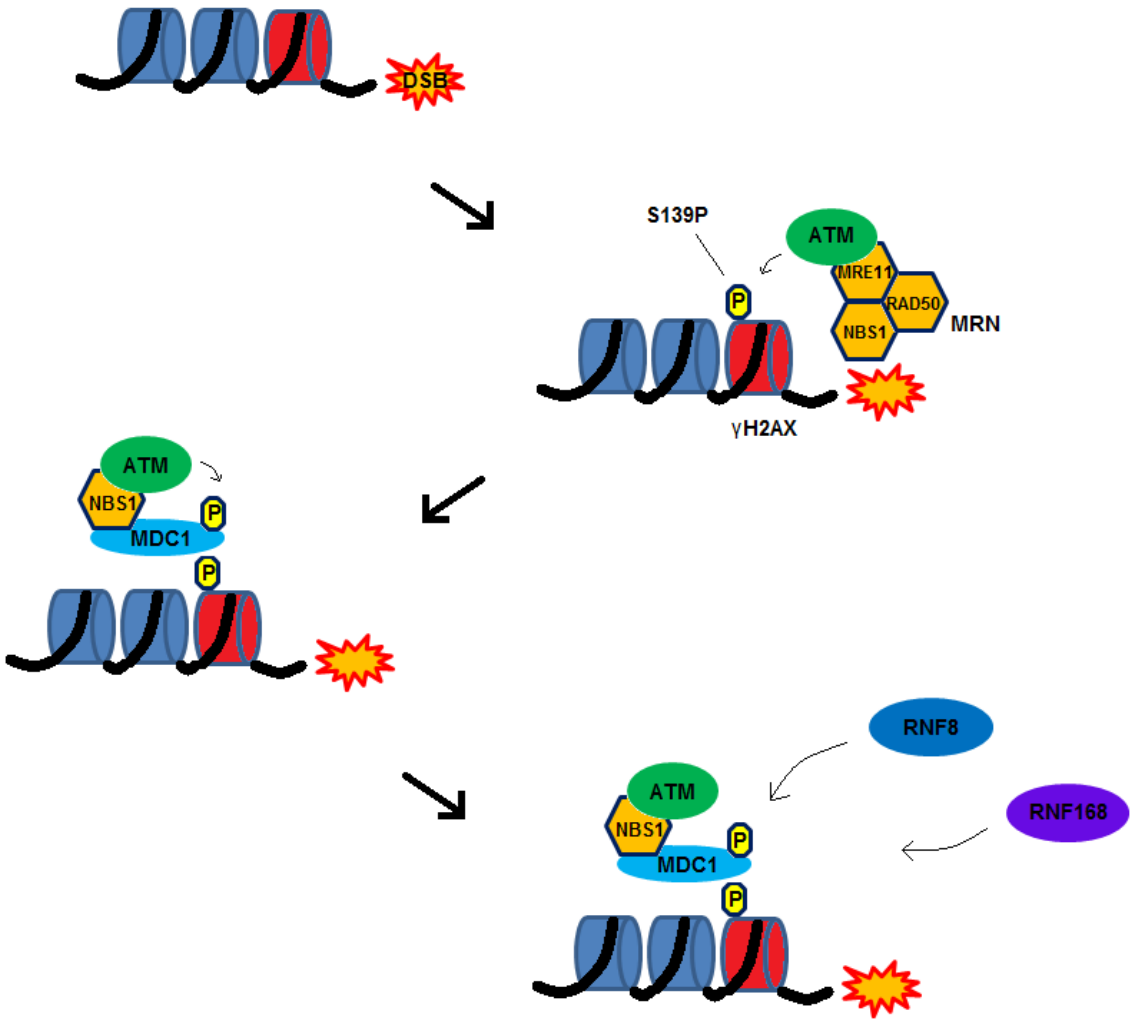


Figure 3. Early stages of DSB repair. Phosphorylation-mediated signalization plays a major role in sensing the damage site and initiation of the DSB repair. Generation of the γH2AX recruits the downstream factors involved in sensing the DNA damage. The second wave of protein recruitment starts with relocalization of RNF8 (RING finger protein 8) and RNF168 (RING finger protein 168) E3 ubiquitin ligases to the damaged chromatin. P - phosphate. Modified from Bekker-Jensen and Mailand (2011) and Citterio (2015).

Several studies identified the protein MDC1 (Mediator of DNA damage checkpoint 1) as a major γH2AX recognizing factor. Formation of the MDC1-γH2AX complex has been shown to be required for an efficient accumulation of downstream DDR factors and physiological radioresistant phenotype. Mutational studies of both MDC1 BRCT domain and H2AX showed that MDC1 and the phospho-acceptor site

of H2AX are vital for the recruitment and retention of other factors in the initial stage of DDR (Stucki et al., 2005). MDC1 is also an important interaction mediator in the γH2AX - MDC1 - ATM positive feedback loop, since interaction of MDC1 with activated ATM stabilizes ATM at the damage site and allows further amplification of H2AX phosphorylation and expansion of an IRIF (Lou et al., 2006). ATM autophosphorylation on Ser1981 has emerged as another essential factor for its sustained retention at DSBs (So et al., 2009).

NBS1, a component of the MRN complex is another key MDC1 interactor. In response to damage, it undergoes activation by the ATM-mediated phosphorylation and transiently interacts with the DSB-flanking chromatin. However, for a sustained accumulation and retention, interaction with MDC1, already associated with chromatin, is essential. This interaction significantly changes dynamics of NBS1 towards relocalization to the repair complex at the damage-associated compartments (Lukas et al., 2004).

Several studies demonstrated that recruitment of DDR factors to the chromatin surrounding DNA lesions is a highly organized process. While accumulation of γH2AX, MDC1 and MRN complex, the most proximal, exceedingly rapid, events of a response to the DSB induction, represent the first wave of protein accumulation, the second wave occurs with a significant delay, but still within minutes (Bekker-Jensen et al., 2005; van Attikum and Gasser, 2009). The second wave of proteins arriving to the chromatin includes mediators of DSB signalling 53BP1 and BRCA1 (van Attikum and Gasser, 2009). It has been shown that their recruitment is highly dependent on the additional histone modifications, especially ubiquitinations carried out by two E3 ubiquitin ligases RNF8 and RNF168 (Doil et al., 2009; Mailand et al., 2007; Stewart et al., 2009). Similarly to H2AX phosphorylation, histone ubiquitinations also spread along the chromatin away from the damage site and provide a docking platform for the proteins of the second wave (Doil et al., 2009). Recently, chromatin SUMOylation mediated by PIAS1 and PIAS4 E3 ligases has been identified as another modification essential for a proper DSB repair, since its required for effective ubiquitin-adduct formation at the chromatin (Galanty et al., 2009).

2.2.4 RNF8

The nuclear factor RNF8 (RING (Really interesting new gene) finger protein 8) is an FHA and RING domain-containing E3 ubiquitin ligase facilitating the initial steps of ubiquitin signalization cascade after DSBs occur (Huen et al., 2007; Ito et al., 2001). Generally, function of E3 ubiquitin ligases is to bring an E2 conjugating enzyme into a sufficient proximity to a substrate and promote the substrate's ubiquitination. This mechanism usually ensures spatio-temporal targeting and degradation of the target proteins by the proteasome. In addition, ubiquitination mediates regulatory functions (Ardley and Robinson, 2005), such as in the case of RNF8 and RNF168. RNF8 plays an essential role in linking the phosphorylation-mediated early stages of DDR involved mainly in recognition of a damage site and ubiquitination-mediated recruitment of checkpoint mediator proteins 53BP1 and BRCA1 (Huen et al., 2007; Ito et al., 2001).

After irradiation, RNF8 rapidly assembles at the damage site via interaction of its N-terminal FHA phosphothreonyl-binding domain with the ATM-generated phosphorylated site on the adaptor protein MDC1, and subsequently catalyzes the ubiquitination of its targets via the catalytically active RING domain localized at the C-terminal end of the protein (Mailand et al., 2007).

In response to DNA damage, RNF8 engages in an interaction with a large (almost 5000 amino acids) E3 ubiquitin ligase HERC2 (HECT domain and RCC1-like domain-containing protein 2). Rather than interfering with RNF8's ubiquitination activity, HERC2 seems to modulate its preference for an E2 ubiquitin conjugating enzyme. Thus, whereas RNF8 has been shown to possess the ability to form functional complexes with various E2 enzymes in order to carry out different processes in cell, interaction with HERC2 shifts its binding choice priority selectively towards UBC13 (Ubiquitin-conjugating enzyme E2 N) to promote the formation of Lys63 (K63) non-proteolytic ubiquitin chains at the damage site (Bekker-Jensen et al., 2010). Interaction of RNF8 with HERC2 is mediated by recognition of ATM-dependent phosphorylation located at the C-terminus of HERC2 by FHA domain of RNF8. Therefore, inherent ability of RNF8 to di- or oligomerize is required for formation of the whole complex MDC1-RNF8-HERC2 at the damage site (Bekker-Jensen et al., 2010).

Although the H2A-type nucleosomal histones have been considered for many years to be the main targets for the RNF8-UBC13 ubiquitination activity, in 2015 Thorslund et al. published their data identifying the H1.2 and H1x, the two isoforms of H1 linker histone, as a major RNF8-UBC13 target (Thorslund et al., 2015).

Using fluorescence recovery after photobleaching (FRAP) experiments, Mok et al. studied differences in kinetics between RNF8 and the other major DDR E3 ubiquitin ligase RNF168 after irradiation *in vivo.* Interestingly, they found that RNF8 displays much higher association/dissociation rate (six-fold) compared to RNF168. This was also confirmed by *in situ* nuclear retention assay which showed much more stable tethering of RNF168 to chromatin-associated structures in the vicinity of DSBs than in the case of RNF8. Furthermore, RNF168 was shown to be more than two-fold abundant at the damage foci (Mok et al., 2014). Generally, both these early stage DDR proteins display faster exchange rates than the downstream repair factors such as BRCA1, 53BP1 and RAD51, which is consistent with their regulatory roles in recruitment of later-stage repair factors to the damage site. Collectively, these findings point to RNF8 as a rate-limiting determinant for focal repair complex assembly (Mok et al., 2014).

Interestingly, a novel non-catalytic role of RNF8 has been described few years ago. RNF8 has been identified as an important player in the CHD4 (Chromodomain helicase DNA-binding protein 4)-mediated chromatin remodelling, which is essential for creating a local chromatin environment permissive to the assembly of checkpoint and repair machinery complexes at the sites of DNA lesions (Luijsterburg et al., 2012).

2.2.5 RNF168

RNF168 is a 571 amino acids long protein encoded by the *RNF168* gene located at the short arm of the chromosome 3. Similarly to RNF8, it is a RING finger domain containing E3 ubiquitin ligase (Figure 4) participating in the early stages of DSB repair pathway signalling. Depletion in the level of this protein has been shown to result in delayed dynamics of repair mechanisms and perturbation of the repair itself owing to impaired recruitment of the downstream repair factors,

such as 53BP1 and BRCA1. Delayed dynamics was monitored at several levels. Irradiated cells lacking endogenous RNF168 displayed significantly increased persistence of MDC1 foci at the damage sites, prolonged phosphorylation of ATM-targeted genome caretakers NBS1 and SMC1 and extended damage-induced G2 arrest (Doil et al., 2009).

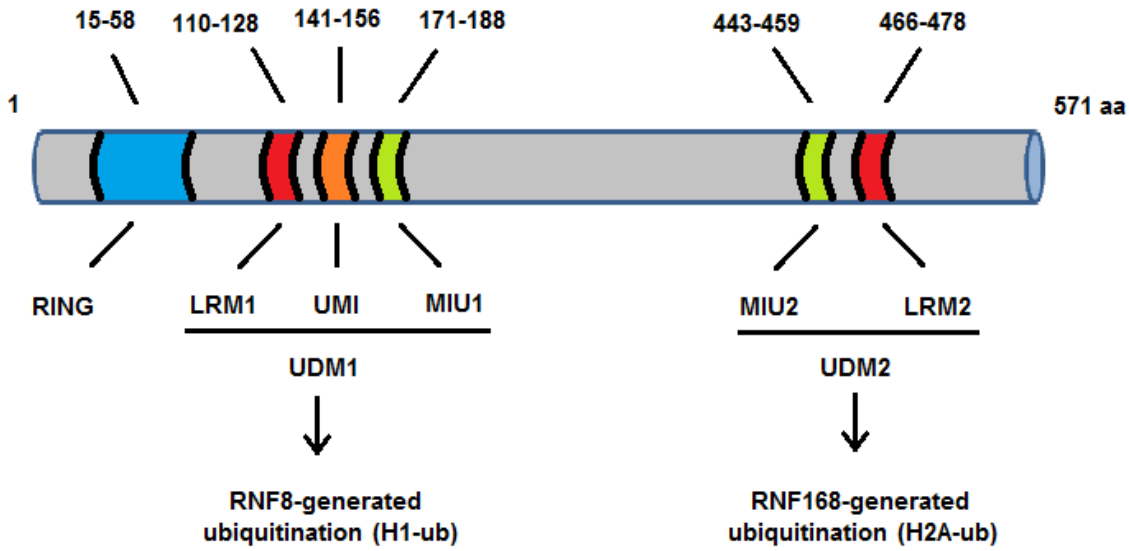


Figure 4. Schematic structure of RNF168. Domains of RNF168 with the indicated amino acid positions within the protein are shown in different colors. Catalytically active RING domain is located at the N-terminus of the protein and is followed by a cluster of protein-interacting motif 1 (UDM1) containing: leucine-rich motif (LRM), UIM- and MIU-related ubiquitin binding domain (UMI) and motif interacting with ubiquitin 1 (MIU1). At the C-terminus the protein-interacting motif 2 containing MIU2 and LRM2 domains is located. Adapted from Huen and Chen (2016).

A specific mutation in the RNF168 gene ablating the catalytic activity of the ligase leads to a rare genetic disease - RIDDLE syndrome. Patients suffering from this disease show increased sensitivity to ionizing radiation, immunodeficiency and also suffer from learning difficulties and dysmorphic features. All these symptoms result from cellular defects in DSB repair, since these patients fail to recruit 53BP1 and BRCA1 factors to irradiation-induced damage sites. It has been shown,

that the abrogated DSB repair can be restored by complementation with exogenous RNF168 expression (Stewart et al., 2009).

Recruitment of RNF168 to the sites of damage is mediated through the recognition of RNF8-generated K63-linked ubiquitination marks mainly at H1 linker histones. Binding to these marks at chromatin is ensured by the N-terminal region containing cluster of protein-interacting motif 1 (UDM1), which possess high-affinity reading activity towards K63 ubiquitinated H1 at chromatin. UDM1 cluster consists of LRM1 (Leucine-rich motif 1), UMI (UIM- and MIU-related ubiquitin binding domain) and MIU1 (motif interacting with ubiquitin 1) motifs (Figure 4). Moreover, the UDM2 domain which is localized at the C-terminal region contains MIU2 and LRM2 motifs (see Figure 1) providing high-affinity reading activity towards the H2A-type histone RNF168-dependent ubiquitination (Huen and Chen, 2016; Thorslund et al., 2015). The mechanisms of this highly ligand-specific recognition of the different ubiquitin binding domains-containing proteins towards different ubiquitinated protein targets were identified recently. The important role of the different LRM motifs was described by Durocher's laboratory. Juxtaposition of ubiquitin binding domain with a short targeting sequence known as LR motif provides the required specificity of ubiquitin binding domains towards different ubiquitinated targets, which is essential for a sequential recruitment of ubiquitin recognizing proteins in DSB ubiquitination cascade (Panier et al., 2012).

Initially, a widely accepted model for sequential recruitment of RNF8 and RNF168 prevailed, postulating that H2A nucleosomal histone ubiquitination is mediated by RNF8 and that this signal is then amplified by RNF168 after its recruitment to the damage site. However, in 2012 Mattiroli and colleagues showed, that initial K13-15 monoubiquitinations of H2A core histone is RNF168-dependent. This observation challenged the accepted model, but still, it confirmed that RNF168 localization to the site of damage requires the catalytic activity of RNF8 at the chromatin (H1 linker histone ubiquitination, Figure 5), but H2A and H2AX are not the targets for these initial steps in ubiquitination cascade and that the amplification of the initial RNF168-generated ubiquitin mark on H2A type histones is orchestrated by both of these E3 ligases (Bartocci and Denchi, 2013; Mattiroli et al., 2012; Thorslund et al., 2015).

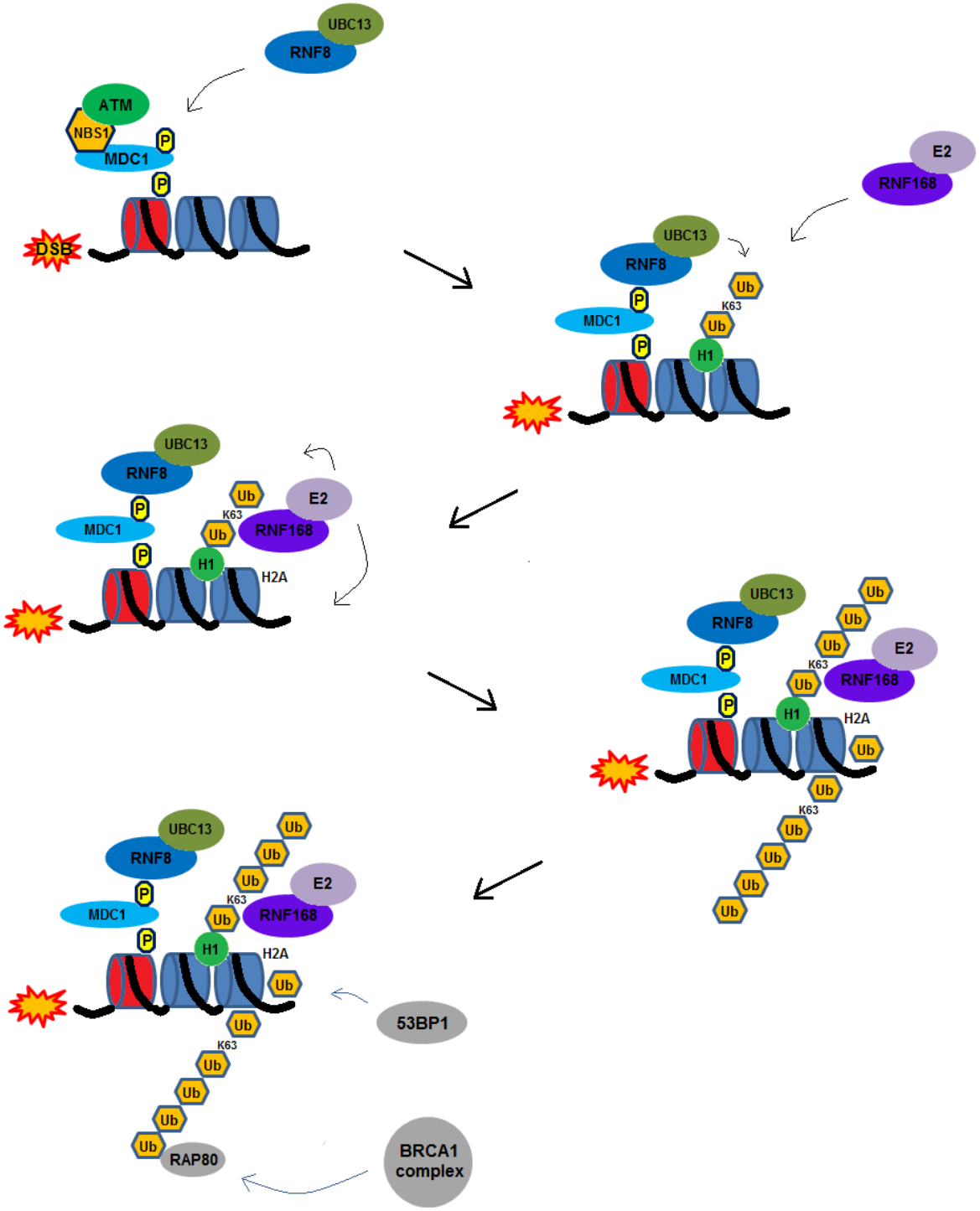


Figure 5. Model of the ubiquitination-mediated DSB signalization cascade. P - phosphate, Ub - ubiquitin. Adapted from Thorslund et al. (2015).

For some time, UBC13 was the only known E2 ubiquitin-conjugating enzyme capable of generation of K63-linked ubiquitin chains. Therefore, it was considered

to be a major interacting partner in the RNF168-mediated ubiquitination. This hypothesis was confirmed by Doil and colleagues in 2009, who showed by pull-down experiments that RNF168 and UBC13 interact (Doil et al., 2009). However, recent structural and biochemical analysis of RNF168 indicated that the protein may also interact with other E2 conjugating enzymes in response to DNA damage (Campbell et al., 2012).

RAD6 E2 conjugating enzyme has recently emerged as a novel interactor involved in target-selective ubiquitination after induction of DSBs. It seems that while RNF168-UBC13 complex is required for amplification of ubiquitination signal at H2A-type core histones, the RNF168-RAD6 complex is required for H1 linker histone ubiquitination and that both of these activities are important for a proper recruitment of downstream factors and fully functional DSB repair (Liu et al., 2013). Involvement of RAD6A and RAD6B subunits (together forming a functional complex) in RNF168-mediated activity has been identified during the screening of tens of E2 enzymes for novel RNF168 partners, when colocalization of different E2s with RNF168 at IRIFs was detected. Interestingly, it has been shown that RAD6 does not form a complex with RNF8, but this is probably caused by differences in the sequence of RING domains of RNF8 and RNF168, which is crucial in this interaction. As expected, *Rad6A*$^{-/-}$ and *Rad6B*$^{-/-}$ cells displayed intact γH2AX, MDC1, RNF8 and RNF168 IRIFs formation, while recruitment of BRCA1 and 53BP1 was significantly impaired. RAD6A and –B knockdown also resulted in suppression of CHK1-dependent G2/M checkpoint activation, which suggests their importance for regulation of G2/M transition after DSBs occur (Liu et al., 2013).

Intriguingly, it has been shown that despite the fact that both RNF8 and RNF168 function together with UBC13 E2 enzyme, UBC13 exhibits much higher activity towards chromatin bound targets when paired with RNF168 compared to RNF8. The molecular mechanism of this phenomenon is still not completely understood, but it seems that the answer could be hidden in the differences in RING domains of these two proteins. (Zhang et al., 2013).

In 2013, Zhang et al. solved the crystal structure of the RNF168 RING domain. The core RING domain represented by residues 16 - 15 comprises of one central α-helix, two antiparallel β strands and two long loops (L1 and L2), which are stabilized by two zinc ions. Although the overall structure of RNF168 RING domain is similar to RING domains of other E3 ligases (e.g. RNF8, TRAF6, CHIP), it also contains

several unique structure features which could be probably involved in specific binding properties of RNF168. Zhang with his colleagues also showed that RNF168 does not form a stable complex with UBC13 *in vivo* and suggested that unique structure features identified in the RING domain may be responsible for the transiency of RNF168-UBC13 interaction, or some other protein could be involved in stabilization of the interaction *in vivo* (Zhang et al., 2013).

2.2.5.1 Regulation of RNF168 activity

Tight regulation of DNA repair processes is of same importance for a cell like the DNA repair itself. Disruption of regulating mechanisms could lead to severe problems, e.g. chromosomal fusions during mitosis which could eventually result in apoptosis or oncogenic transformation. Several levels of DDR regulating mechanisms have evolved in eukaryotes.

Activity of RNF168 is regulated on several levels *in vivo.* Various different proteins and mechanisms are involved in this process, including post-translational modifications, specific degradation of proteins upstream of RNF168 in the DSB signalization cascade or degradation of RNF168 itself, or counteracting the RNF168 activity by deubiquitination of chromatin-associated targets (Bartocci and Denchi, 2013; Gudjonsson et al., 2012).

Recruitment of RNF168 to the damage site requires recognition of the specific ubiquitination marks generated by RNF8 mainly on the H1 linker histone. One of the mechanisms ensuring the dynamic regulation of RNF168 recruitment involves deubiquitinating enzymes. To date, four enzymes counteracting the action of RNF8/RNF168 have been identified: USP3 (ubiquitin-specific protease 3), USP16 (ubiquitin-specific protease 16), BRCC36 (BRCA1/BRCA2-containing complex subunit 36) and OTUB1 (OUT domain ubiquitin aldehyde-binding 1). While USP3, USP16 and BRCC36 are directly involved in negative regulation of RNF8/RNF168 pathway via mediating the deubiquitination of chromatin-associated targets, mechanism of action of OTUB1 is catalytic activity-independent, since it is based on binding competition and inhibition of E2 enzyme UBC13, which is essential

in RNF8/RNF168 pathway (Bartocci and Denchi, 2013; Nicassio et al., 2007; Shanbhag et al., 2010; Wu et al., 2012).

Another major mechanism ensuring the tight regulation of RNF168-mediated signalization operates at the level of regulating the RNF168 protein stability. Two E3 ligases are known to target RNF168 for degradation by ubiquitin proteasome system (UPS) so far: TRIP12 (Thyroid receptor-interacting protein 12) and UBR5 (Ubiquitin protein ligase E3 component n-recognin 5). This mechanism actively reduce the pool of available nuclear RNF168, which is a critical process for keeping RNF168 from the excessive recruitment to the damage sites and spreading the ubiquitination signal across the chromosome in an uncontrolled manner. This level of control is absolutely essential for cells given the processive nature of RNF168 and its damage site self-recruitment capabilities (Gudjonsson et al., 2012).

2.2.6 53BP1

53BP1 is a large protein comprising 1972 amino acids. Despite the fact that it has no apparent enzymatic activity, it plays a key role in DDR because of its ability to interact with various DSB-responsive mediators and effectors. Generally, 53BP1 is an important factor of DSB response since it promotes repair by non-homologous end joining by antagonizing BRCA1 and blocking the repair via homologous recombination. It is also essential for functional class switch recombination and is involved in fusion of deprotected telomeres. 53BP1 deficient mammalian cells display several defects such as deficiencies in checkpoint signalization and sensitivity to ionizing radiation (Nakamura et al., 2006).

In order to function properly, 53BP1 needs to associate with nucleoprotein complexes assembled at the lesion site. A couple of structural elements are required for recognition and binding to the chromatin and for subsequent interactions with downstream effectors (Figure 6) (Fradet-Turcotte et al., 2013; Panier and Boulton, 2014). 53BP1 recruitment to the chromatin in the vicinity of a lesion site is mediated by two major interactions. While the tandem Tudor domains recognize dimethylated H4 histone (H4K20me2) (Botuyan et al., 2006), the ubiquitination-dependent recruitment (UDR) motif interacts with K15 ubiquitination of H2A histone (H2AK15ub)

generated by RNF168. The C-terminal BRCT (BRCA1 carboxy-terminal) domain is responsible for binding to p53 and EXPAND1 (also known as MUM1), the protein involved in chromatin relaxation. The N-terminal part of the protein (residues 1-1220) then contains 28 Ser/Thr-Gln (S/T-Q) consensus motifs that are known ATM-dependent phosphorylation targets and they have been shown to play a key role in interaction of 53BP1 with its effectors such as RIF1 and PTIP (Adams and Carpenter, 2006; Fradet-Turcotte et al., 2013; Panier et al., 2012).

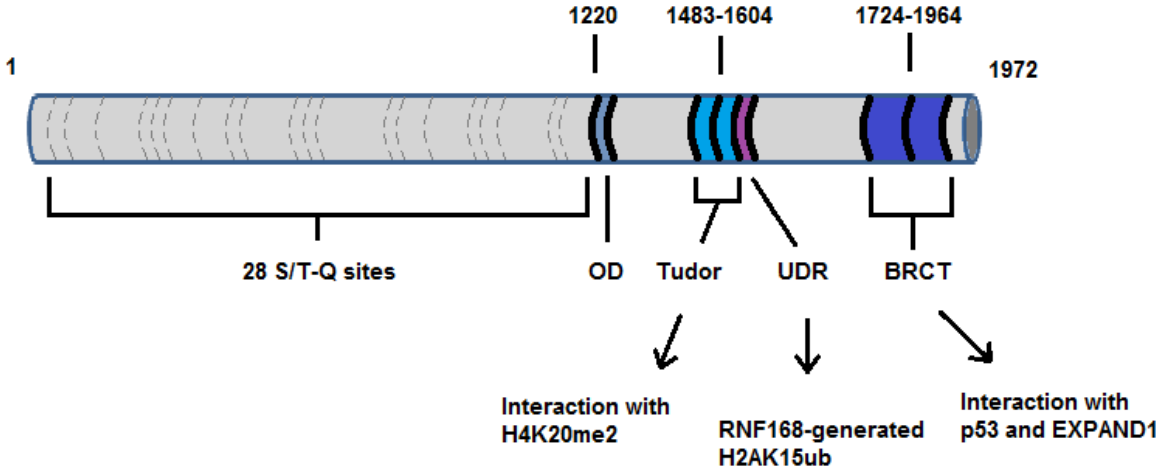


Figure 6. Schematic structure of 53BP1. Individual functional domains of the protein are distinguished by different colors and their positions within the protein are indicated. While the N-terminal half carries 28 ATM-phosphorylatable S/T-Q motifs, C-terminal part contains various functional domains facilitating the protein-protein interactions. Adopted from Panier and Boulton (2014).

For maintenance of a proper balance in DNA repair pathway choice, 53BP1 have to be tightly regulated throughout the cell cycle. UbcH7 (Ubiquitin conjugating enzyme H7) has recently arisen as a novel key player in DSB repair. This E2 enzyme regulates 53BP1 stability by driving its proteasome degradation via ubiquitination. Loss of UbcH7 leads to elevation in 53BP1 levels which forces cells to choose NHEJ repair pathway and leaves them sensitive to genotoxic agents such as camptothecin and other topoisomerase I inhibitors (Han et al., 2014). Also mechanisms regulating 53BP1 at the level of recruitment and retention at the damage sites upon irradiation have been described, among them also regulation mediated by RNF168-dependent

K63-linked polyubiquitination at the K1268 residue. These observations indicate the multistep involvement of RNF168 in DSB repair at different levels (Bohgaki et al., 2013).

53BP1 plays an essential role in repair pathway decision making upon DSB induction. Through its effectors like RIF1, REV7 or PTIP it antagonizes BRCA1 and promotes repair by the error-prone NHEJ in G1 phase (Bunting et al., 2010; Di Virgilio et al., 2013; Escribano-Diaz et al., 2013; Xu et al., 2015). This functional antagonism of 53BP1 and BRCA1 has been studied for several years now and recently has been associated with clinical outcome in patients with homologous recombination-deficient tumours. The error-prone NHEJ promoted by 53BP1 remains the major pathway to repair the damage induced by chemotherapy or radiotherapy treatments in patients with BRCA1 mutation (Bouwman et al., 2010). However, acquired resistance of tumours is commonly observed in these patients. Numerous mechanisms have been identified to be involved in therapy escape in BRCA1-deficient tumours. Significant part of these mechanisms can be explained by loss of different NHEJ-promoting factors or their decreased levels, which results in partial restoration of HR-directed repair. In the last few years, couple of mechanisms leading to poor clinical outcome due to HR restoration have been identified. One of the mechanisms is associated with loss-of-function mutations in 53BP1 gene (*TP53BP1*). Cells with simultaneous deficiency in BRCA1 and 53BP1 have been shown to evolve resistance to anticancer DNA damage inducing treatments, e.g. PARPi (Bouwman et al., 2010). Not only direct loss of 53BP1, but also loss of its effectors and some other proteins involved in blocking the end resection has been recently connected to therapy escape in HR-deficient cancers, e.g. REV7 or HELB (DNA helicase B) (Tkac et al., 2016; Xu et al., 2015). Moreover, also BRCA1 gain-of-function mutations may arise as a consequence of the increased mutagenic potential in cancers (Linger and Kruk, 2010)

2.2.7 BRCA1

The E3 ubiquitin ligase BRCA1 is a well-established tumour suppressor protein involved in number of cellular pathways required for maintenance of genome stability, including DNA damage checkpoint activation, protein ubiquitination, chromatin remodeling, transcriptional regulation and apoptosis (Wu et al., 2010). *BRCA1* gene was the first identified gene predisposing to hereditary breast and ovarian cancer (Hall et al., 1990).

Despite its very limited homology with other proteins, BRCA1 protein contains two functional domains observed also in some other DSB response factors. Its N-terminal catalytic RING domain present also in e.g. RNF8 or RNF168 facilitates formation of K6 polyubiquitin chains and plays a key role in cellular response to DSBs by mediating its repair (Panier and Durocher, 2009; Wu et al., 2010). The second, C-terminal BRCT domain mediates phosphopeptide binding. Many cancer-associated mutations have been identified within these two functional domains, which indicates their high significance for breast and ovarian cancer formation suppression activity (Brzovic et al., 2001; Wu et al., 2010).

As described earlier, upon DSB induction the chromatin surrounding the damage site undergoes a series of phosphorylation and ubiquitination steps. H2A histone conjugated K63-linked polyubiquitin chains generated by RNF168 recruit RAP80 via its UIM. Chromatin bound RAP80 is then recognized by the large complex containing BRCA1, Abraxas (also known as CCDC98), NBA1 (also known as MERIT40), BRE (also known as BRCC45) and BRCC36 proteins (Wu et al., 2010). BRCA1 in this complex then physically interacts with CtIP and MRN proteins to promote the end resection of damage-surrounded DNA and repair by HR (Sartori et al., 2007). However, BRCA1 participation in formation of at least 4 different complexes (A, B, C, D) has been described to be involved in BRCA1-mediated HR repair (Savage and Harkin, 2015).

2.3 DDR in mitosis

Growing evidence has been accumulating on the truncation of the DSB signalization cascade at the apical level upon M-phase entry. While initial steps

of the DSB response consisting of phosphorylation-dependent signalization (γH2AX, NBS1, MDC1) do not display any changes compared to interphase cells, recruitment of the second-wave factors (RNF8, RNF168) facilitating ubiquitinations of chromatin structures have been shown to be abolished. Also other downstream mediators like 53BP1 and BRCA1 do not form IRIFs in mitotic cells. Accumulation of these factors has been shown to be restored in late M phase, approximately at the time of anaphase/telophase transition (Giunta et al., 2010).

Several works have recently shown that functional DDR during mitosis may lead to severe problems potentially resulting in apoptosis or oncogenic transformation (Giunta et al., 2010; Orthwein et al., 2014). Among these problems, induction of structural chromosomal instability (s-CIN) represented by chromosomal rearrangements and consequentially whole chromosomal (numerical) instability (w-CIN) may occur after restoration of RNF8 and 53BP1 accumulation at the site of mitotic damage (Bakhoum et al., 2014). Based on the latest research it seems that different molecular mechanisms can contribute to chromosomal instabilities and aneuploidy in cells with restored DDR. Work of Orthwein and his colleagues showed that aberrantly controlled DSB repair during mitosis results in Aurora B kinase-dependent telomere fusions of sister chromosomes and that this event usually produces dicentric chromosomes and aneuploidy (Orthwein et al., 2014). Another mechanism was described by Backhoum and colleagues who showed that components of sustained mitotic DDR selectively stabilize kinetochore-microtubule (k-MT) attachments via Aurora-A and PLK1 (Polo-like kinase 1) thereby contributing to increase in frequency of lagging chromosomes during anaphase (Bakhoum et al., 2014).

Collectively, there is strong evidence that sustained DSB repair in mitosis is a serious threat to genome integrity; therefore tight regulation is required in order to prevent apoptosis or oncogenic transformation. Molecular mechanisms underlying these M-phase DDR regulations have been uncovered recently (Orthwein et al., 2014). Most of these regulations have been shown to be ongoing at the level of post-translational modifications without any significant fluctuations in RNF8, RNF168 and 53BP1 protein levels throughout the cell cycle (Giunta et al., 2010). Since the first truncated point of DSB response is relocalization of RNF8 to the chromatin and its retention at the damage site, Orthwein et al. investigated whether the phosphorylation-mediated interaction of MDC1 with RNF8 is involved and they

identified T198 residue of RNF8 as a novel target for CDK1 (cyclin-dependent kinase 1)-dependent phosphorylation. Interestingly, while phosphoinhibiting mutation (T198A) of this residue fully restored accumulation of BRCA1, it failed to restore the IR-induced focus formation of 53BP1, which suggests existence of a second, inhibitory mechanism. Using mass spectrometry (MS) analyses they mapped mitotic phosphorylation sites of 53BP1 and identified two residues (T1609, S1618) located in the UDR domain, which is essential for recruitment of 53BP1 to the ubiquitin marks at the chromatin upon damage (Lee et al., 2014; Orthwein et al., 2014). While the T1609 residue is a target of the proline-directed p38 MAP (mitogen-activated protein) kinase (Lee et al., 2014), S1618 is targeted by PLK1 target (Grosstessner-Hain et al., 2011).

3. AIMS

The theoretical part of this thesis aimed to provide an insight into a complexity of the DNA double-strand break signalization and reparation system and to characterize the key factors involved in these essential processes, with emphasis on RNF168 E3 ubiquitin ligase and its involvement in repair pathways.

Goal of the experimental part was to identify post-translational modifications of RNF168 and to characterize their role in activity and stability modulation of the protein, with emphasis on phosphorylations.

4. MATERIALS AND METHODS

4.1 Materials

Table 1. List of used chemicals and materials

Chemicals

Manufacturer	Chemical(s)
Advansta (USA)	Western Bright Sirius HRP substrate
Amresco (USA)	Kanamycin sulphate
Bio-Rad (USA)	Polyacrylamide/Bis Solution 30% 29:1, Coomassie Brilliant Blue G-250
Biotium (USA)	GelRed DNA stain
Lachner (Czech republic)	Boric acid, disodium hydrogen phosphate dodecahydrate, isopropyl alcohol, potassium dihydrogen phosphate, sodium hydroxide
Laktino (Czech republic)	Skim milk (non-fat)
MP Biomedicals (USA)	Tween-20
Penta (Czech republic)	Acetic acid, ethanol, methanol, glycerol anhydrous, aceton
Roth (Germany)	Triton X-100
Serva (Germany)	Agarose, glycine
Sigma-Aldrich (USA)	Bovine serum albumin, ammonium persulphate, EDTA, G418 disulphate salt, HEPES, magnesium chloride, MG132, penicilin/streptomycin solution, ponceau S, potasium chloride, puromycin, S-(+)-glucose, sodium azide, sodium deoxycholate monohydrate, sodium dodecyl sulphate, sucrose, formaline solution 10%, trichloric acid, crystal violet
Thermo Fisher Scientific (USA)	Dimethylsulfoxide, Hoechst 33342
Vector (USA)	Vectashield mounting medium

Enzymes

Manufacturer	Enzymes
New England Biolabs (USA)	*Bam*HI, Calf intestinal phosphatase, Phusion high-fidelity DNA polymerase

Table 1. continued

Antibodies

Manufacturer	Antibody(-ies)
Cell Signaling (USA)	Ubiquitin rabbit polyclonal
GE Healthcare (UK)	Amersham ECL anti-mouse IgG - HRP linked whole Ab Amesham ECL anti-rabbit IgG - HRP-linked whole Ab
GeneTex (USA)	Anti-GAPDH mouse monoclonal
Millipore (USA)	Anti-phospho-histone H2A.X (Ser139) mouse monoclonal, Anti-phosphoserin rabbit polyclonal, Anti-phospho-threonine rabbit polyclonal, Anti-RNF168 rabbit polyclonal
Santa Cruz Biotechnology (USA)	53BP1 (H300) rabbit polyclonal
Sigma-Aldrich (USA)	Anti-SUMO-1 rabbit polyclonal
Thermo Fisher Scientific (USA)	Goat anti-mouse IgG (H+L) - Alexa Fluor 568 conjugate Goat anti-rabbit IgG (H+L) - Alexa Fluor 568 conjugate

Cell cultivation material

Manufacturer	Material
Biosera (UK)	DMEM high glucose medium
Duchefa Biochemie (Netherlands)	Luria Broth (LB) high salt, Luria Broth (LB) Agar, Miller
PAA (Austria)	Fetal bovine serum
Thermo Fisher Scientific (US)	Gibco RPMI medium 1640 GlutaMax

Miscellaneous

Manufacturer	Material
Bio-Rad (USA)	Precision plus dual color protein standard
New England Biolabs (USA)	Gel loading dye - purple (6x), 3.1 buffer, CutSmart buffer, Phusion 5x GC buffer
Promega (USA)	1 kb DNA ladder
Roche (Switzerland)	Complete protease inhibitor cocktail, Phospho stop
Sigma-Aldrich (USA)	ANTI-FLAG M2 affinity gel

Table 1. continued

Kits

Manufacturer	Kit(s)
Macherey-Nagel (Germany)	NucleoBond Xtra Midi/Maxi, NucleoSpin Plasmid
New England Biolabs (USA)	NEBuilder HiFi DNA Assembly Cloning Kit
Qiagen (Germany)	QIAquick Gel Extraction Kit

Solutions

Table 2. List of used solutions

Solution	Composition
10x TBS buffer	150 mM NaCl, 50 mM Tris-Cl pH 7.6
RIPA buffer	150 mM NaCl, 1 % (v/v) Triton X-100, 0.5 % (w/v) sodium deoxycholate, 0.1 % SDS, 50 mM Tris pH 8.0
1x SDS-PAGE running buffer	192 mM glycine, 25 mM Tris, 0.1 % SDS
1x Transfer buffer	192 mM glycine, 20 % (v/v) methanol, 25 mM Tris
2x LSB (Laemmli sample buffer)	0.5 M Tris-HCl pH 6.8, 4.4 % (w/v) SDS, 20 % (v/v) glycerol, 400 mM DTT
5x TBE	1.1 M Tris, 900 mM Boric acid, 25 mM EDTA-NaOH (pH 8.0)
Antibody dilution buffer (ADB)	DMEM, 10 % FBS, 0.02 % sodium azide
Blocking buffer 1	5 % (w/v) non-fat skin milk in TBST
Blocking buffer 2	5 % (w/v) BSA in TBST
Buffer A	10 mM Hepes-KOH, 10 mM KCl, 1.5 mM $MgCl_2$, 0.34 M sucrose, 10 % (v/v) glycerol, 0.1 % (v/)v Triton X-100
Coomassie staining solution	50 % (v/v) methanol, 10 % (v/v) glacial acetic acid, 0.04 % (w/v) Coomassie Briliant Blue G-250
Destain solution	50 % (v/v) methanol, 10 % (v/v) glacial acetic acid
K – acetate	3 M potassium acetate, 5 M acetic acid
PBS	3.2 mM Na_2HPO_4, 0.5 mM KH_2PO_4, 1.3 mM KCl, 135 mM NaCl (pH 7.2)
SDS	10 % (w/v) SDS
TBST	TBS supplemented with 0.1 % (v/v) Tween-20

Cell lines and bacterial strains

HEK293T, adherent cell line derived from human embryonic kidney – ATCC
LNCaP, adherent cell line derived from human prostate cancer – ATCC
U2OS, adherent cell line derived from human osteosarcoma – ATCC
Stable Competent E. coli, New England Biolabs

Genotype: F' *proA+B+ lacI*^{q} *Δ(lacZ)M15 zzf::Tn10* (Tet^{R}) *Δ(ara-leu) 7697 araD139 fhuA ΔlacX74 galK16 galE15 e14- Φ80dlacZΔM15 recA1 relA1 endA1 nupG rpsL* (Str^{R}) *rph spoT1 Δ(mrr-hsdRMS-mcrBC)*

Plasmids

pAcGFP1-N1-RNF168 (Doil et al. 2009)
pAcGFP1-N1-RNF168-S16+19C (catalytically inactive mutant, Doil et al. 2009)
pCAGGS-FLAG-RNF168 (a kind gift from Dr. J. Stark (Munoz et al., 2012))
pSUPERIOR-Puro-RNF168shRNA (Doil et al. 2009)

Oligonucleotides (Generi-Biotech, Czech Republic)

RNF168 C-terminus F (5‘-3‘):	CGCACCCACTTTCTGTTCTG
RNF168 C-terminus R (5‘-3‘):	ACTGATAGATTTGGAGCATCTACTG
RNF168 N-terminus F (5‘-3‘):	ATTTCTTCGGGTATGGTACCGAGTC
RNF168 N-terminus R (5‘-3‘):	CCCTTGAATTCCAGAAAATCTGATC

Synthesized fragments of RNF168 (Life Technologies, 5‘-3‘)

RNF168 C-terminus T+S → A
CAGAACAGAAAGTGGGTGCGCCCCCACATCAGGGGTGACACAGGCAAATGGAAACAAC
ACAGGTGAGACAGAAAATGAAGAGTCGTGCCTACTGATCAGTAAGGAGATTTCCAAAAG
AAAAAACCAAGAATCTTCCTTTGAAGCAGTCAAGGATCCATGCTTTTCTGCAAAAAGAAG

AAAAGTGGCCCCCGAAGCTGCCCCAGATCAAGAGGAAACAGAAATAAACTTTACCCAAA
AACTGATAGATTTGGAGCATCTACTG

RNF168 C-terminus T+S → E

CAGAACAGAAAGTGGGTGCGCCCCCACATCAGGGGTGACACAGGAGAATGGAAACAAC
ACAGGTGAGACAGAAAATGAAGAGTCGTGCCTACTGATCAGTAAGGAGATTTCCAAAAG
AAAAAACCAAGAATCTTCCTTTGAAGCAGTCAAGGATCCATGCTTTTCTGCAAAAAGAAG
AAAAGTGGAACCCGAAGAGGAACCAGATCAAGAGGAAACAGAAATAAACTTTACCCAAA
AACTGATAGATTTGGAGCATCTACTG

RNF168 N-terminus S → A

GACTCGGTACCATACCCGAAGAAATGCTCTCGTCAACGTGGAACTGTGGACGATAATTC
AAAAACACTATCCCAGGGAGTGCAAGCTTAGAGCGTCTGGCCAAGAATCAGAGGAAGT
GGCTGATGACTATCAGCCAGTTCGTCTGCTCAGTAAACCTGGGGAACTGAGAAGAGAAT
ATGAAGAGGAAATAAGCAAGGTGGCGGCAGAGCGACGGGCCAGCGAGGAAGAAGAAA
ACAAAGCCAGTGAAGAATACATACAGAGGTTGTTGGCAGAGGAGGAAGAAGAGGAAAA
AAGACAGGCAGAAAAAAGGCGAAGAGCGATGGAAGAACAACTGAAAAGTGATGAGGAA
CTGGCAAGAAAGCTAAGCATTGATATTAACAATTTCTGTGAGGGAAGTATCTCGGCTGC
TCCCTTGAATTCCAGAAAATCTGATC

RNF168 N-terminus S → E

GACTCGGTACCATACCCGAAGAAATGAACTCGTCAACGTGGAACTGTGGACGATAATTC
AAAAACACTATCCCAGGGAGTGCAAGCTTAGAGCGTCTGGCCAAGAATCAGAGGAAGT
GGCTGATGACTATCAGCCAGTTCGTCTGCTCAGTAAACCTGGGGAACTGAGAAGAGAAT
ATGAAGAGGAAATAAGCAAGGTGGCGGCAGAGCGACGGGCCAGCGAGGAAGAAGAAA
ACAAAGCCAGTGAAGAATACATACAGAGGTTGTTGGCAGAGGAGGAAGAAGAGGAAAA
AAGACAGGCAGAAAAAAGGCGAAGAGCGATGGAAGAACAACTGAAAAGTGATGAGGAA
CTGGCAAGAAAGCTAAGCATTGATATTAACAATTTCTGTGAGGGAAGTATCTCGGCTGA
ACCCTTGAATTCCAGAAAATCTGATC

4.2 Methods

4.2.1 Agarose gel electrophoresis

Gels were prepared using 0.8 - 1.5 % (w/v) agarose in 0,5x TBE. GelRed was added to the solution according to manufacturer's instructions. Prior to loading DNA samples were supplemented with loading dye to a final 1x concentration. 1 kb DNA ladder (Promega) was used as a molecular weight marker. Gels were run in 0,5x TBE buffer at a voltage gradient 5 - 10 V/cm. Separated DNA was visualized using the BioRad ChemiDoc MP system.

4.2.2 Cell culture

U2OS and HEK293T cell lines were cultured in standard Dulbecco's modified Eagle's minimal essential medium and LNCaP cells were cultured in Gibco RPMI Medium 1640 GlutaMAX. Both RPMI and DMEM media were supplemented with 10% (v/v) fetal bovine serum (PAA) and penicillin/streptomycin solution (1% v/v, Sigma). The medium for U2OS cells stably expressing shRNF168 was supplemented with 2.5 µg/ml of puromycin. The medium for cells stably expressing mutant variants of RNF168 was supplemented with 600 µg/ml of G418/geneticin. Cells were incubated in a humidified atmosphere at 5 % CO_2 and 37 °C.

4.2.3 Generation of double-strand breaks by irradiation

DNA double-strand breaks were induced using an X-ray source (Xstrahl RS Research Cabinet). X-ray irradiation was done at the following settings: 200 kV, 15 mA, 2.3 Gy/min. Different irradiation dosages were used (4.5 Gy, 5 Gy and 10 Gy). Irradiated cells were incubated for 1hour in standard cultivation conditions prior to further processing.

4.2.4 Polymerase chain reaction

Reaction was carried out in a total volume of 50 µl. The reaction mixture contained the components listed in Table 3. Reaction conditions can be found in Table 4.

Table 3. Reaction mixture components

Components	Final concentration
5x Phusion GC buffer	1x
100% DMSO	10% (v/v)
12.5 mM dNTPs	0.2 mM
10 µM F primer	500 nM
10 µM R primer	500 nM
Template DNA	1 ng
Phusion polymerase 2 000 U/ml	1 U/50 µl PCR
ddH_2O	to 50 µl

Table 4. The PCR program used for inverse PCR

Step		# of cycles	Time	Temperature (°C)
1.	Denaturation	1	30 s	98
2.	Denaturation	30	15 s	98
3.	Annealing		15 s	54,5
4.	Polymerization		2:30 min	72
5.	Final extension	1	5:00 min	72

4.2.5 Bacterial transformation

Transformation was done according to the High efficiency transformation protocol for Stable Competent E. coli (New England Biolabs). In brief, 1 - 100 ng of plasmid DNA was added to 25 µl of competent E. coli suspension, mixture was mixed and placed on ice for 30 minutes. Then the mixture was exposed to heat

shock at 42 °C for 30 s, placed on ice for 5 minutes and subsequently incubated with 900 µl of LB medium in 37 °C under constant agitation for 1 hour. 50 - 100 µl of bacterial suspension were spread onto a pre-warmed selection plates and incubated for 16 - 24 hours in 37 °C.

4.2.6 Plasmid DNA isolation from E. coli

Two different kits were used for plasmid DNA isolations. NucleoBond Xtra Midi/Maxi (Macherey-Nagel) kit was used for larger scale isolations, while some of the isolations were carried out using NucleoSpin Plasmid miniprep kit (Macherey-Nagel). In both cases, isolations were done according to manufacturer's instructions.

4.2.7 DNA extraction from agarose gel

Extraction of DNA from agarose gel was done using Gel Extraction Kit (QIAquick) according to manufacturer's instructions.

4.2.8 Plasmid DNA digestion by restriction endonuclease

Plasmid DNA digestion was usually carried out in a total volume of 20 µl. 1 - 2 µg of pDNA was used per reaction. The mixture was supplemented with reaction buffer to a final 1x concentration. 1 - 10 units of restriction endonuclease were added per µg of DNA. The reaction mixture was then vortexed briefly, centrifuged and incubated for 2 - 4 hours at 37 °C.

4.2.9 Denaturing polyacrylamide gel electrophoresis (SDS-PAGE)

Standard SDS-PAGE was done as described by Laemmli (1970). Samples were lysed in Laemmli sample buffer (LSB) and subsequently incubated at 95 °C with shaking at 1500 rpm for 5 minutes. Proteins were then resolved using 8 % polyacrylamide gels at a constant current of 30 mA in 1x SDS-PAGE running buffer using a PowerPac HC apparatus (Bio-Rad). Gels for subsequent MS analysis were stained in colloid Coomassie Brilliant Blue solution (Bio-Rad).

4.2.10 Immunoblotting

Proteins were first resolved on an SDS-PAGE gel. Then, stack of three 1x transfer buffer pre-wetted gel sized filter papers with nitrocellulose filter on the top was placed on the anode of Bio-Rad TransBlot SD electroblotting apparatus. The gel was then placed onto a membrane and covered by another stack of three 1x transfer buffer pre-wetted filter papers of the same size. The blotting apparatus was then assembled by putting the catode in place and proteins were transferred to the membrane for 1 hour at a voltage of 15 V. After the transfer the membrane was incubated in blocking buffer for 1 hour under agitation at room temperature. Then the blocking buffer was decanted and fresh blocking buffer supplemented with primary antibody was added followed by 1 hour incubation at room temperature. Following primary antibody dilutions were used: RNF168 1:4000, FLAG 1:200, Phospho-T: 1:200, Phospho-S: 1:200, SUMO: 1:400, total ubiquitin: 1:400, α-tubulin 1:300, GAPDH 1:300. After the incubation with primary antibody, the membrane was washed 3 x 5 minutes in 1x TBST buffer. Subsequently, alkaline phosphatase conjugated secondary antibodies in a 1:1000 dilution were added. After 45 minutes of incubation at room temperature the membrane was washed 6 x 5 min with 1x TBST. Signal was developed using WesternBright Sirius HRP substrate (Advansta) detection reagent and detected using the Bio-RadChemiDoc MP system.

4.2.11 Generation of RNF168 mutants

Reference sequence for RNF168 cDNA (NM_152617.3) available from NCBI was used for generation of RNF168 mutants carrying phosphomimetic and phospho-inhibiting mutations. Both predicted (S70, S197, T362) and confirmed (S411, S414, S415) phosphorylation sites were mutated either to glutamic acid (phosphomimetic mutation) or alanine (phosphoinhibiting mutation). Fragments of RNF168 carrying mutations (phosphomimetic C-term., phosphoinhibiting C-term., phosphomimetic N-term., phosphoinhibiting N-term.) were synthesized by Life Technologies (Carlsbad, California, USA). Synthesized fragments were subcloned into pAcGFP1-N1-RNF168 vector using the NEBuilder HiFi DNA Assembly Cloning kit according to manufacturer's instructions. Products of the cloning reactions were transformed into Stable Competent E. coli cells (see "Bacterial transformation" section above).

Five clones of each of the mutant were randomly chosen for confirmation of successful cloning step and two of these clones for each of the mutant were then chosen for sequencing analysis.

4.2.12 Cell transfections

Viromer RED, Lipofectamine 2000 and Lipofectamine RNAiMAX reagents were used. Transfections were done according to manufacturer's instructions.

4.2.13 Immunofluorescence

U2OS cells seeded on coverslips in 6-well plates were irradiated with 4.5 Gy of X-rays and then incubated in standard cultivation conditions for 1 hour. Cells were then washed 2 times in ice-cold PBS and fixed in 4% formaldehyde for 15 minutes at room temperature. Permeabilization was done using 0.2% Triton X-100 in PBS for 5 minutes at room temperature. Samples were washed 3 times in PBS and then incubated for 1 hour with primary antibodies. After 3 washes in PBS, samples were incubated for 30 minutes with secondary antibodies. Following antibody dilutions were used: 53BP1 1:500, γH2AX 1:1000, secondary antibodies 1:1000. In subsequent washing steps, nucleic DNA was stained with Hoechst 33342 for 5 minutes. Final wash with PBS and ddH_2O was performed and coverslips were mounted in VECTASHIELD medium. All images were obtained with the Axio Observer.Z1/Cell Observer Spinning Disc microscopic system (Yokogawa, Tokyo, Japan and Zeiss, Oberkochen, Germany) equipped with an Evolve 512 (Photometrix, Tucson, AZ, USA) EMCCD camera. Zeiss Plan Apochromat 63x and 100x/1.40 NA objectives were used. Signal intensities and foci numbers were quantified using ScanR acquisition and ScanR analysis software (Olympus, Japan) and processed using STATISTICA software (StatSoft, Czech Republic)

4.2.14 Chromatin fraction enrichment

6-well cultivation plates with cells were placed on ice, medium was removed and the cells were covered with 1 ml of Buffer A. After 5 minutes of incubation on ice,

Buffer A was removed and the chromatin enriched fraction was lysed to 400 µl of 1xLSB as described above.

4.2.15 Immunoprecipitation

20 µl of ANTI-FLAG M2 Affinity Gel beads slurry were washed three times in RIPA buffer. RIPA lysed sample was then added to the slurry and the mixture was incubated for 4 hours in 4 °C under constant agitation. After several washing steps with RIPA buffer, immunoprecipitated RNF168 was eluted by addition of LSB followed by incubation at 95 °C for 10 minutes.

4.2.16 Calf intestinal phosphatase dephosphorylation assay

"Protein dephosphorylation protocol" from Abcam (http://www.abcam.com/) was used as a guideline. Components of the reaction mixture can be found in Table 5. 1 U of CIP/µg of total protein in RIPA lysate was added to the mixture and subsequently, the mixture was incubated in 37 °C for 1 hour. In order to maximize the protein concentration, TCA protein precipitation was carried out. Modified "TCA protein precipitation protocol" (http://www.its.caltech.edu/~bjorker/-TCA_ppt_protocol.pdf, originally from Luis Sanchez) was used. In brief, 1 volume of 100% TCA was added to 4 volumes of protein sample and sample was then incubated for 10 minutes at 4 °C. Then centrifugation step at 14 000 rpm for 5 minutes was carried out and supernatant was removed. Pellet was washed with cold acetone 3 times. Subsequently, pellet was dried by placing the tube in 95 °C for 10 minutes. Samples for SDS-PAGE were prepared as described above.

Table 5. Components of the reaction mixture.

Components	Volume
RIPA cell lysate	50 µl (1/8 of the total lysate volume)
CIP buffer (CutSmart)	50 µl
CIP	10 µl (100 U)
ddH_2O	390 µl (total volume 500 µl)

4.2.17 Clonogenic assay

300 cells/well were seeded into 6-well cultivation plates. The next day, cells were irradiated with 2 Gy of X-rays and subsequently let to form colonies for 1 week in standard conditions. Then, cells were fixed in 70% ethanol for 30 minutes and colonies were stained with crystal violet for 1 hour. Total area of colonies and their numbers were quantified using algorithm for colony detection and quantification created by Tomáš Furst (Faculty of Science, Palacky University, Olomouc, 2013).

4.2.18 Mass spectrometric analysis

The mass spectrometric (MS) analysis was performed as described in Shevchenko et al. (2007). In brief, immunoprecipitated FLAG-RNF168 was separated using SDS-PAGE and the gel was then stained with Coomassie brilliant blue solution. Lane in the gel containing separated proteins was then cut into 12 fragments and destaining (Coomassie destain solution), reduction (DTT) and alkylation (iodoacetamide) steps were carried out. Next, in-gel tryptic digestion was performed, peptides were extracted and then purified on C18 reverse phase. Eventually, peptide sequencing (PS) analysis strategy using LC-MS/MS (nanoEASY coupled to an UHR-Q-TOF maXis instrument equipped with online nanoESI source, Bruker Daltonics) was done.

5. RESULTS

5.1 Is RNF168 modified in response to DNA damage?

We have repeatedly observed occurrence of a band with higher molecular weight than the major form when probing RNF168 in several cell lines of different origin (Figure 6A). siRNA knockdown of RNF168 in the indicated cell lines confirmed that the band does not represent a cross-reaction of the antibody used (data not shown). The fact that the shifted band could also be observed upon ectopic expression of RNF168 from plasmids suggests that it most likely represents a post-translationally modified form of RNF168 and not different isoforms of the protein. Our previous observations suggested that the detected modification of RNF168 might be associated with modulation of activity of the protein (Chroma, unpublished). Hence, we set out to investigate the nature of the modification(s) in more detail.

Since RNF168 facilitates the key ubiquitination step in repair of DSBs, we were curious whether these modifications are associated with induction of DNA damage. In order to investigate this, LNCaP cells were irradiated with two different dosages of X-rays and after 1 hour incubation period lysates enriched for chromatin fraction were prepared and the levels of modified forms of RNF168 were investigated using SDS-PAGE and subsequent Immunoblotting analysis (Figure 6B).

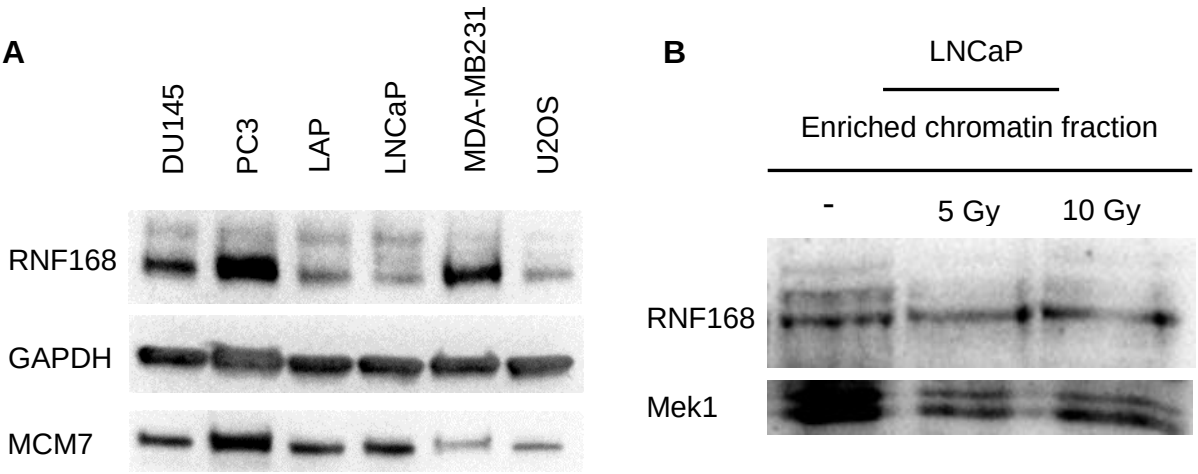


Figure 6. A) Presence of the modified forms (upper bands) of RNF168 has been observed in several cancer cell lines (Chroma, unpublished). **B)** Cells were irradiated by two different dosages of X-rays and lysates enriched for chromatin fraction were prepared and used for immunoblotting analysis.

Despite the higher amount of loaded untreated sample (Figure 6B) we could conclude that modifications of RNF168 are not introduced in response to DNA damage induction, since no significant increase in the levels of modified forms with slower migration were observed.

5.2 RNF168 undergoes various post-translational modifications

We showed that post-translational modifications of RNF168 responsible for the occurrence of the band with shifted mobility are probably not associated with the response to DSBs. However, the exact nature of the modifications responsible for the shift remained unclear. Since little is known about RNF168 post-translational modifications and no commercial antibodies recognizing the modifications are available, immunoprecipation was a necessary step before detecting the modifications. Immunoprecipitated RNF168 was probed for the most common modifications known to be crucial for DSB repair such as ubiquitination, SUMOylation and phosphorylation on two most common residues (Figure 7).

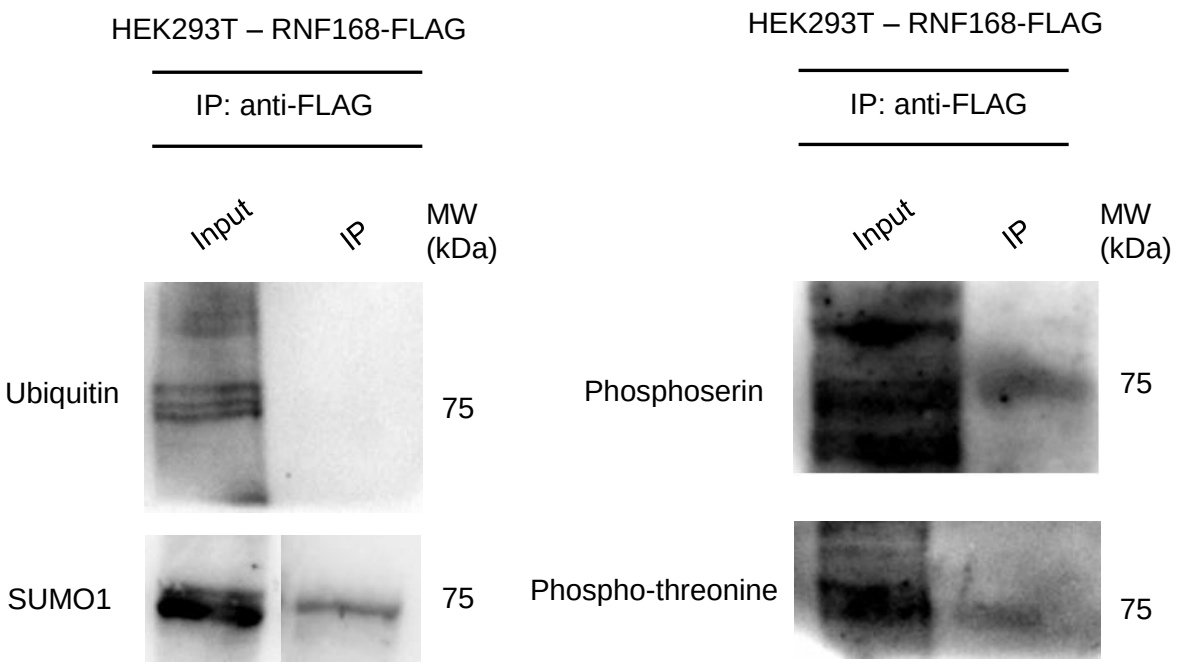


Figure 7. Identification of RNF168 post-translational modifications. HEK293T cells were transfected with a plasmid for ectopic expression of FLAG-tagged RNF168, which was subsequently immunoprecipitated. 1/10 of the whole lysate volume was used as the “input” sample and 1/6 of the total volume eluted after immunoprecipitation was loaded as the “IP” sample. Proteins were separated using SDS-PAGE and transferred on a membrane by western blotting. RNF168 was probed with antibodies against various PTMs. SUMOylation and phosphorylations of serines and threonines were detected. Ubiquitination was not detected.

As shown in Figure 7, SUMOylation and phosphorylations of RNF168 were detected. These findings agree with already published data. In 2012 RNF168 was identified as a target for PIAS4-dependent SUMOylation in response to DNA damage (Danielsen et al., 2012). However, the exact biological role of this modification has not been completely understood yet. In case of phosphorylations, several phosphorylation sites were identified by mass spectrometry analysis, while some were predicted based on homology with mouse and rat RNF168 (Huttlin et al., 2010; Lundby et al., 2012). Nevertheless, the function of these phosphorylations has also been unknown. In contrast, ubiquitination of RNF168 was not detected.

5.3 Phosphorylation might modulate RNF168 stability

Since the putative biological role of RNF168 SUMOylation was already indicated (Danielsen et al., 2012) and further investigation would require complex MS analyses, we decided to point our focus towards biological functions of RNF168 phosphorylations, which have not been investigated in detail, yet.

Our previous experiment (Figure 7) suggested that RNF168 is indeed modified by phosphorylations on both serine(s) and threonine(s). To confirm this observation we performed an *in vitro* dephosphorylation assay. To see whether the bands with slower migration possibly representing the phosphorylated forms disappear after dephosphorylation, whole cell lysates were treated with calf intestinal phosphatase (CIP). RNF168 was then probed by immunoblotting (Figure 8).

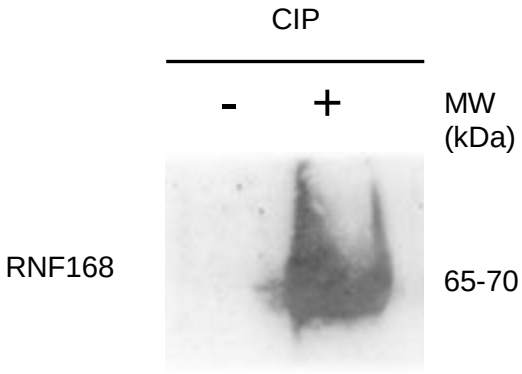


Figure 8. Dephosphorylation assay in whole cell lysates. 10 U of CIP/µg of protein was used for dephosphorylation of lysate in solution. Lysates were prepared in partially denaturing RIPA buffer without addition of protease or phosphatase inhibitors.

Surprisingly, RNF168 was completely degraded in untreated lysates (-), while in lysates incubated with phosphatase (+) RNF168 seemed to be extremely stabilized. Intriguingly, this result suggests that phosphorylations might be involved in destabilization of RNF168 ligase.

5.4 Post-translational modifications do not drive RNF168 proteasome-dependent degradation

To better understand whether the post-translational modifications of RNF168 drive its UPS-mediated degradation, we treated cells with proteasome inhibitor MG132 and then looked at the levels of RNF168 in untreated control and treated samples using immunoblotting (Figure 9).

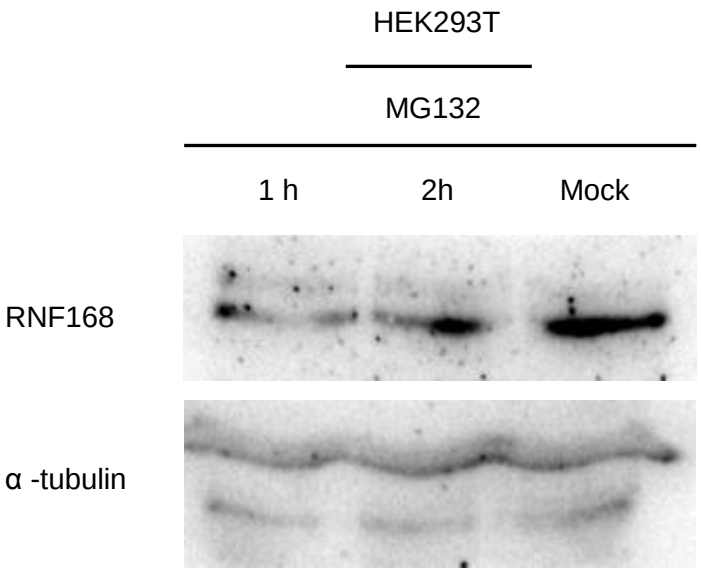


Figure 9. The effect of proteasome inhibition on the stability of modified RNF168. Cells were either mock- or MG132 (5 μM, 1 or 2 hours) treated. Cells were then lysed and probed for the levels of both RNF168 forms.

Experimental data shown in Figure 9 indicate that the putative phosphorylation-mediated destabilization and degradation of RNF168 observed in previous experiment (Figure 8) is not UPS-dependent, since no accumulation of native or modified form of RNF168 was observed after treatment. Probably other mechanism is involved in regulation of RNF168 levels through its degradation, e.g. specific protease degradation seems to be the candidate.

5.5 Generation of RNF168 phosphosites mutants

The above mentioned approach(es) could not be used to selectively map the function of phosphosites within RNF168 (Figure 10A). Hence, to test the intriguing possibility that phosphorylation affects RNF168 stability we generated four different mutants carrying either phosphomimetic or phosphoinhibiting mutations of the predicted and MS identified phosphosites (Figure 10A-E)

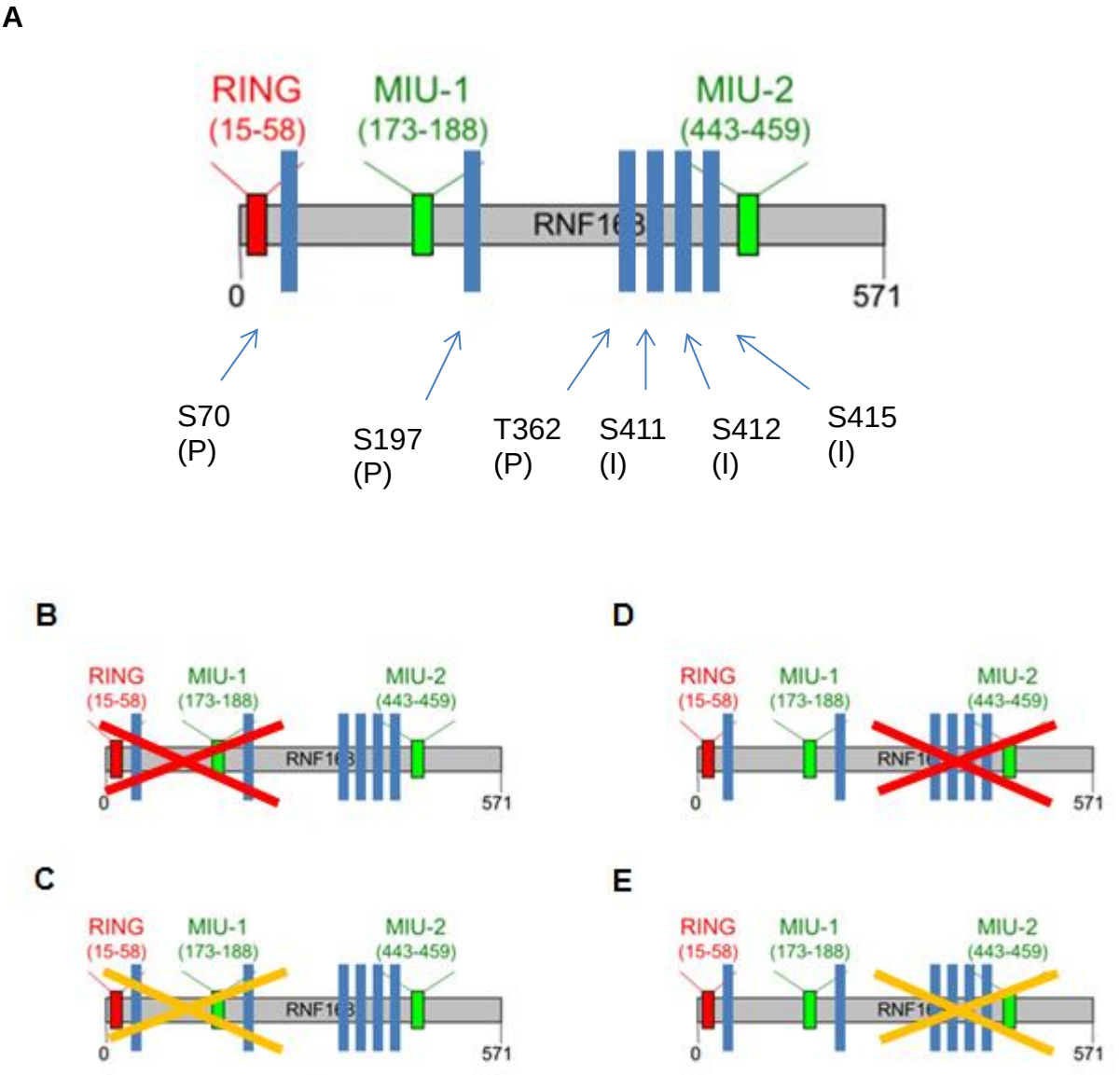


Figure 10. Schematic structure of RNF168 with approximate locations of phosphorylation sites. A) Locations of predicted (P) and identified (I) phosphorylation sites are indicated by blue rectangles. Positions of the catalytic RING domain and the two ubiquitin-interacting motifs are indicated in red and green. **B** – **E**) Schematic representations of the four different phosphomutants. (**B**) shows mutant with phosphoinhibiting mutations (S → A) at the N-terminal half of the protein, picture (**C**) shows mutant with phosphomimetic mutations (S → E) also located at the N-terminal part. Similarly, pictures (**D**) and (**E**) display phosphoinhibiting (**D**) and phosphomimetic (**E**) mutations of the C-terminus proximal sites. Modified from Doil et al., (2009).

Short DNA fragments carrying the indicated mutations were synthesized and subcloned into pAcGFP1-N1-RNF168 vector prepared by inverse PCR (Figures 11, 12A) Efficiency of the insertion of the synthesized fragments was confirmed by restriction analysis using *Bam*HI endonuclease (Figure 12B).

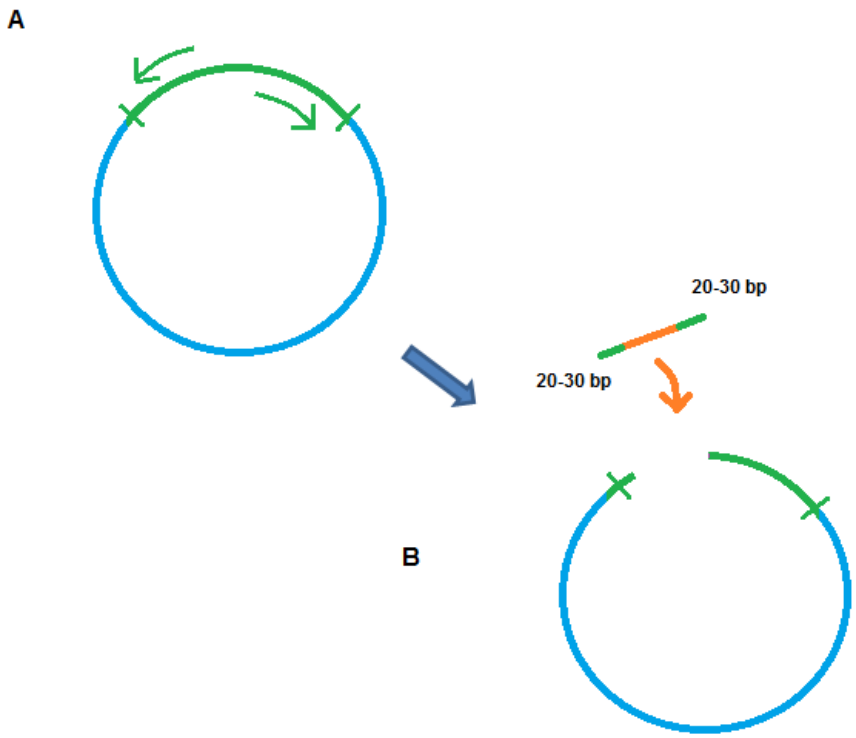


Figure 11. Schematic illustration of the homology-based cloning strategy. A) Inverse PCR with amplification from the regions surrounding the N- or C-terminal phosphosites (not shown) was used to prepare the vector. RNF168 gene and primers are indicated by green color. **B**) Synthesized fragments of RNF168 gene carrying the introduced mutations (NA, NE, CA, CE) were subcloned based on homology of the ends of the vectors (N- or C-terminal amplification) with 20 - 30 bp long homologous regions in the synthesized fragments.

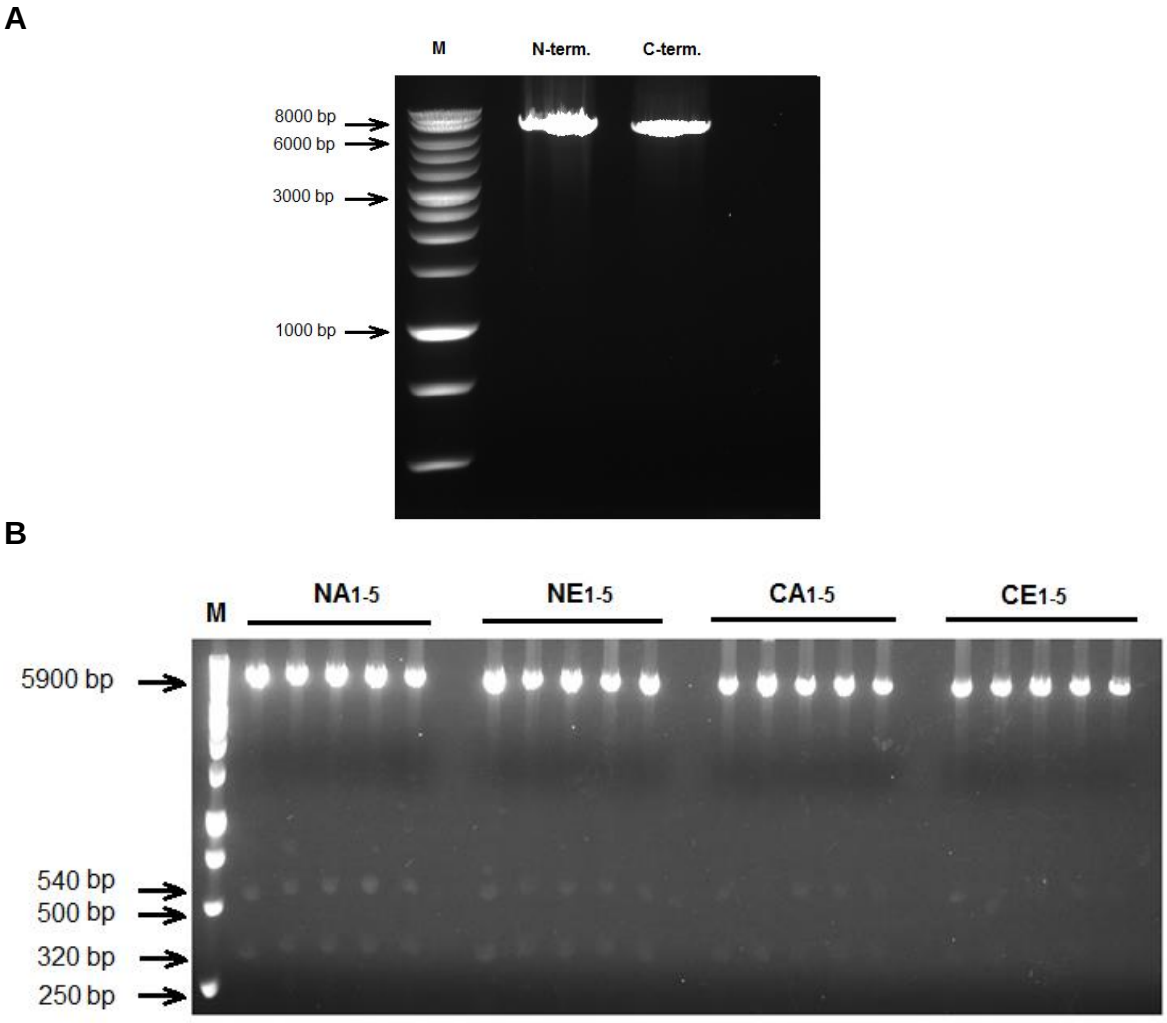


Figure 12. Products of the inverse PCR and restriction analysis of recombinant mutated phosphosites carrying plasmids. A) Two inverse PCR reactions were carried out to prepare the vector (6.5 kb) for subcloning the RNF168 fragments with the N-terminal and C-terminal mutations. M - Molecular weight marker. **B**) Endonuclease digestion of the vectors with the subcloned mutated RNF168 fragments. Five bacterial clones carrying each of the plasmid (NA - N-term. alanine, NE - N-term. glutamic acid, CA - C-term. alanine, CE - C-term. glutamic acid) were randomly selected and the plasmid DNA was isolated and analysed by restriction digest using *Bam*HI.

Subsequent sequencing analysis confirmed that all the substitutions at the targeted phosphorylation sites were introduced successfully and no unwanted mutations were generated (Figure 13).

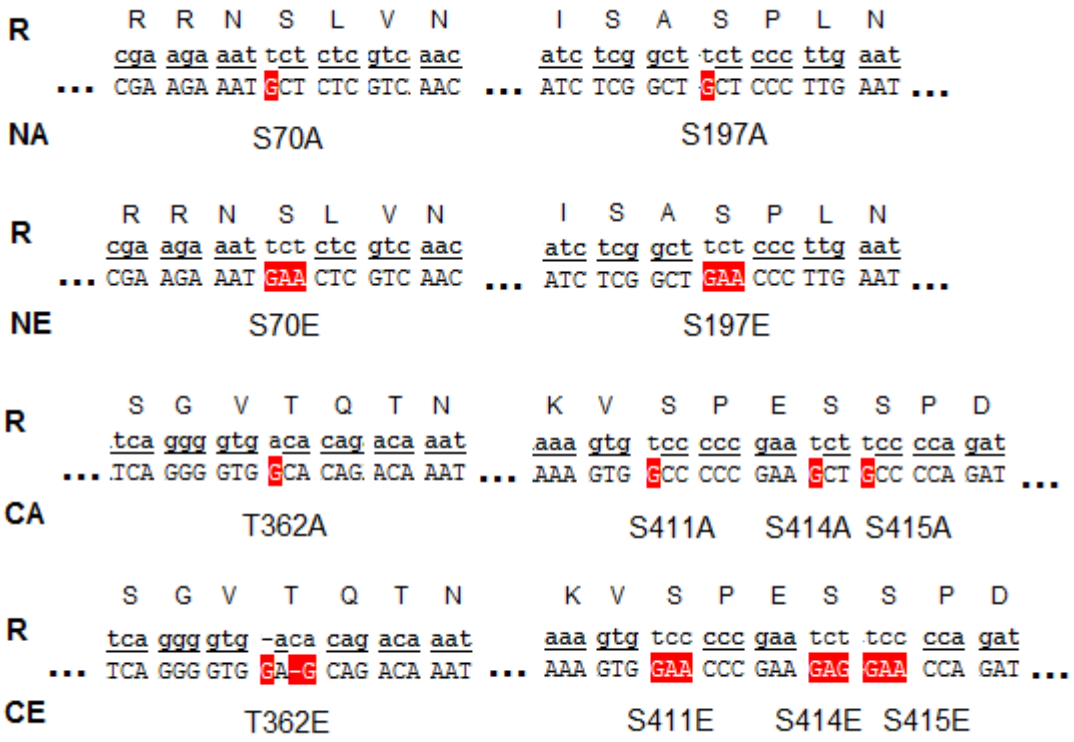


Figure 13. Mutagenesis of the RNF168 phosphorylation sites. Parts of the sequences displaying introduced substitutions with positions of the mutated codons. R - reference cDNA sequence of RNF168; NA, NE, CA, CE - generated mutants

5.6 Manipulation of the predicted and putative phosphosites in RNF168 leads to pronounced changes in the stability of RNF168 *in vivo*

The treatment with calf intestinal phosphatase indicated a potential role of phosphorylations in stability modulation of RNF168. We tried to recapitulate these results *in vivo* utilizing the RNF168 phosphomutants (Figure 14). Apart from confirming the *in vitro* data, this approach might help to identify the exact phosphosites involved in the regulation of RNF168 turnover.

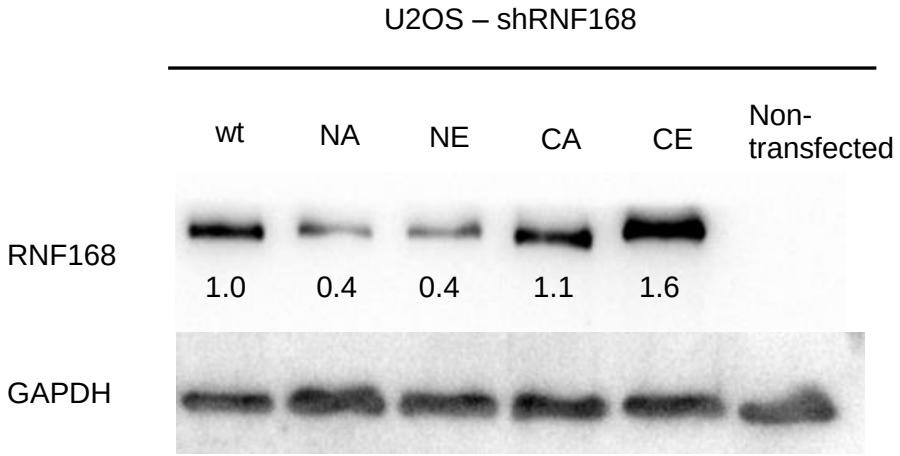


Figure 14. Levels of the wt- and mutRNF168. Significant differences in levels of the protein for each subset of the mutants are displayed on this immunoblot. Constitutive RNF168 knockdown in U2OS cell line was reconstituted with shRNA resistant wtRNF168 or with each of the mutant (NA, NE, CA, CE). ImageJ (https://imagej.nih.gov/ij/) software was used for level quantifications of mutated RNF168 relative to GAPDH and wilt-type RNF168 levels.

While the subset of mutations in the N-terminal half of the protein resulted in a significant decrease in levels of RNF168 (to approximately 40 % of wild-type level), mutations located at the C-terminus showed none or slight (in the case of CA) or substantial increase (in the case of CE) in the RNF168 protein level. Interestingly, we did not observe any differences in the levels of the phosphomimetic and the phosphoinhibiting mutations in the N-terminal half of RNF168. It seems that both N- and C terminal subsets of phosphosites might be important in modulation of stability of RNF168 and that any mutagenesis at these positions (mainly in case of the N-terminal part located sites) deregulates this mechanism.

5.7 Known and predicted phosphorylation sites of RNF168 are not involved in the recruitment of downstream repair factor 53BP1

Mutating the N- and C-terminal subsets of phosphorylation sites allowed us to test whether modifications at these positions possess a role in the response

to DNA damage. Since RNF168 is directly responsible for the recruitment of 53BP1 to IRIFs, monitoring of the recruitment of this protein to sites of damage represents an ideal readout for assessing the function of RNF168 phoshorylation in the DDR. We investigated the recruitment of 53BP1 and both the wild type and phosphomutant RNF168-GFP fusions to IRIFs by immunofluorescence (Figure 15).

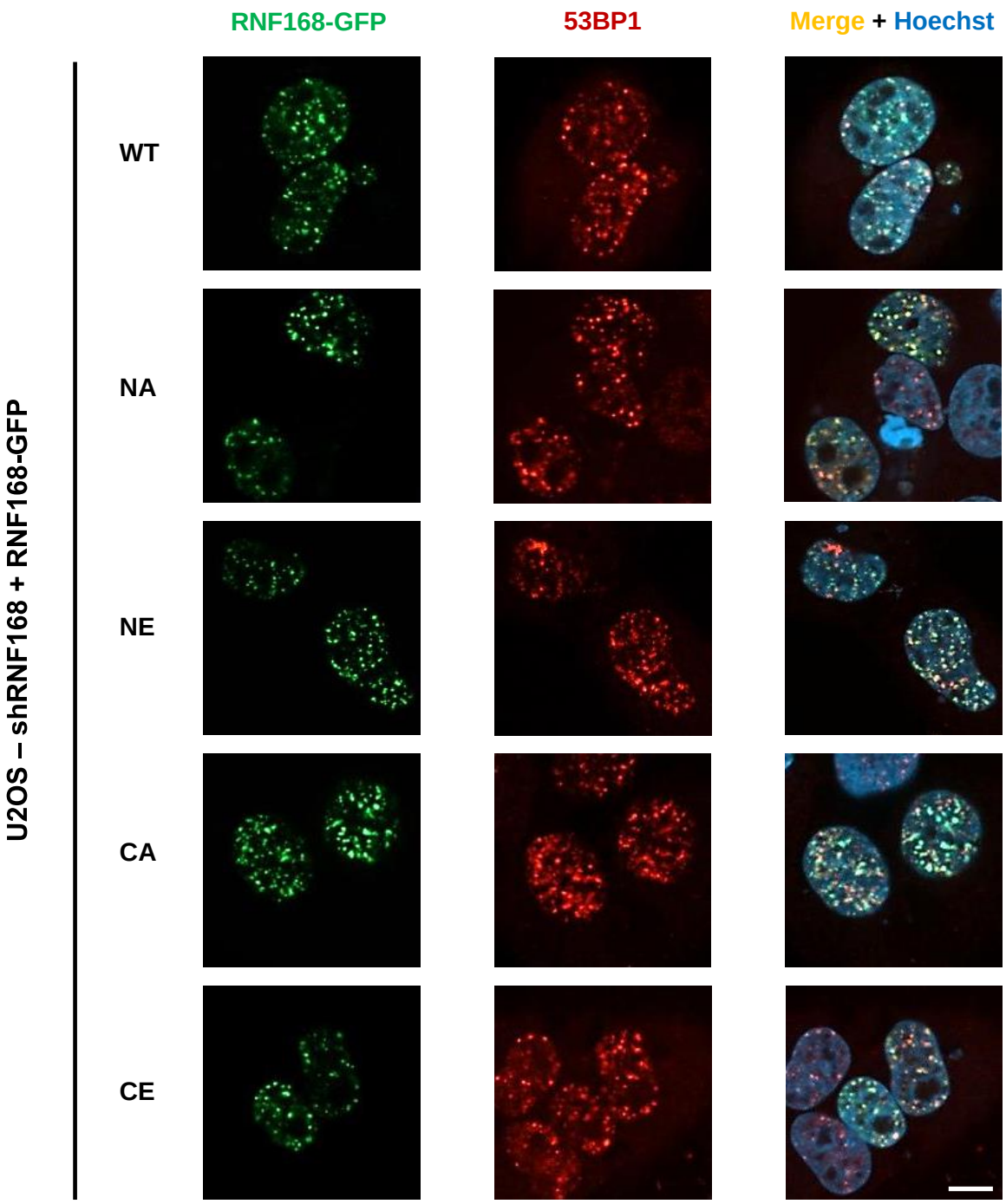
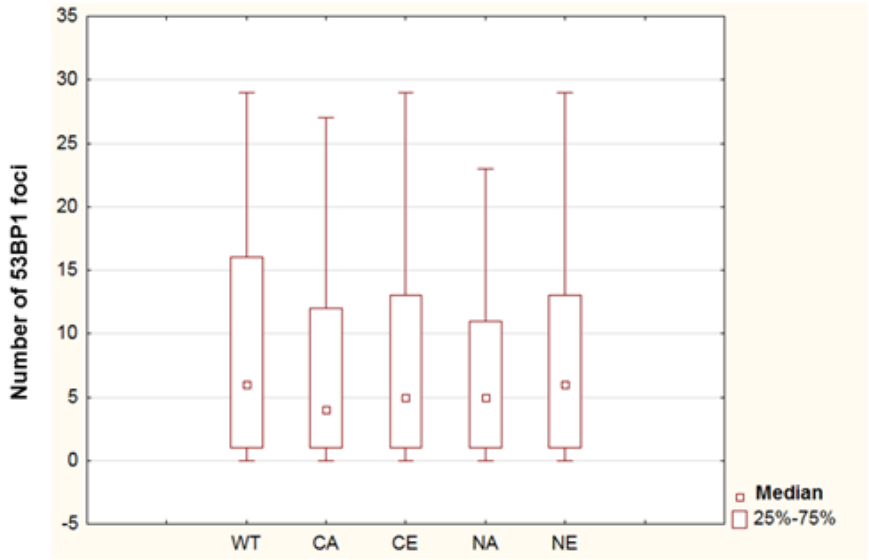


Figure 15. The phenotypes of RNF168 phosphosites mutants. U2OS cells harboring a constitutive RNF168 knockdown were transfected with shRNA resistant wild type and phosphomutant GFP tagged versions of RNF168. The cells were then irradiated and probed for the ability to form RNF168 and 53BP1 IRIF at the damaged chromatin (scale bar 2 μm).

It seems that the six phosphorylation sites are not directly involved in DSB signalling and that mutagenesis at these sites does not have any effect on recruitment of the downstream factor 53BP1. We also did not observe any defects in colocalization of RNF168 and 53BP1 at the sites of irradiation-induced damage (Figure 15). The notion that mutating the phosphosites in RNF168 does not influence the 53BP1 foci numbers and structure was confirmed by quantification of IRIFs and their intensity in irradiated cells transiently expressing the mutant versions and a wild type control (Figures 16A, B).

A



B

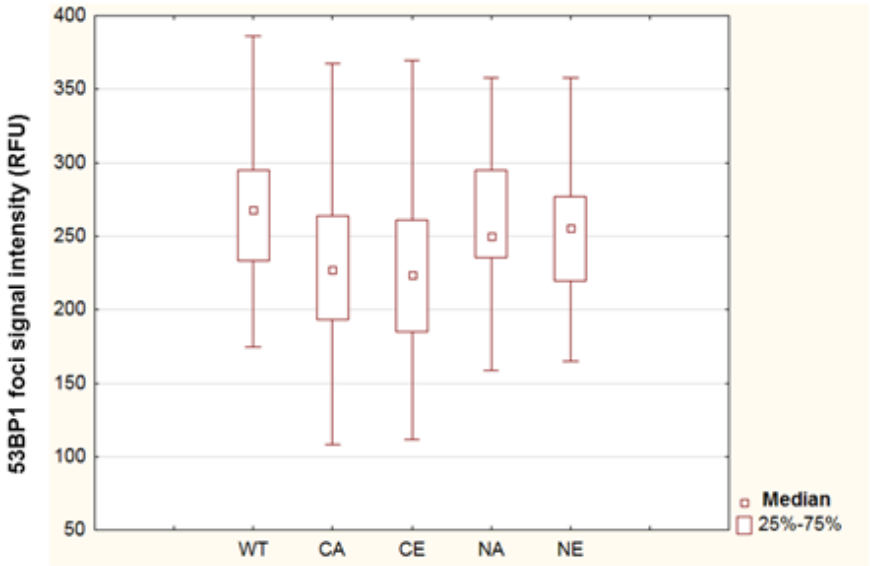


Figure 16. Number and intensity of 53BP1 foci in cells with either mutRNF168 or wtRNF168. Nuclei displaying the high intensity of mutRNF1688-GFP or wtRNF168-GFP IRIFs signal were chosen for the analysis. Number (**A**) and intensity (**B**) were then measured in the gated nuclei using the ScanR system. Results obtained by ScanR where further processed using ScanR acquisition and analysis software (Olympus) and STATISTICA software (StatSoft).

Signal intensity quantification showed some differences between RNF168 forms, especially in the case of C-terminal mutants, however, more replicates would be required to confirm the results. Taken together, quantifications of 53BP1 foci numbers and 53BP1 signal intensities supported our previous observations that phosphorylations at these sites of RNF168 do not regulate the activity of the protein, since no substantial fluctuations in foci numbers or their intensities were observed.

5.8 Mass spectrometric identification of potential RNF168 interactors

To get a clear picture of the PTMs (including the SUMOylation) associated with RNF168, MS analysis was performed as described in Shevchenko et al. (2007). As a by-product we identified several novel candidate RNF168 interactors. The list of the most interesting identified proteins is shown in the Table 6.

Table 6. List of the most interesting identified putative interactors of RNF168. BRCA2 - Breast cancer type 2 susceptibility protein; STK38L - Serine/threonine kinase 38-like; TRIM21 - Tripartite motif containing 21; XRCC5 - X-ray repair cross complementing protein 5.

Identified protein	MASCOT score	Sequence coverage (%)
ATM	87.75	1.87
BRCA2	134.24	1.73
p36	100.72	3.4
p38	576.13	27.53
STK38L	1128.13	50.43
TRIM21	481.97	26.32
XRCC5	136.17	4.23

Although we were not able to detect any PTMs of RNF168 due to insufficient amounts of the immunoprecipitated protein, several promising candidates for potential novel interactors showed up including DDR and stress related kinases and E3 ubiquitin ligases. Since the kinase or kinases responsible for RNF168

phosphorylations still remain(s) elusive, ATM, p36 and p38 MAP kinases and STK38L kinase caught our attention as the potential novel interactors and modifiers of RNF168. Also the two E3 ubiquitin ligases BRCA2 and TRIM21, and part of the regulatory subunit of DNA-PK - XRCC5, have been identified. Interestingly, their direct interactions with RNF168 have not been described yet. Degradation fragments of RNF168 were also detected by the MS analysis, which corresponds to our previous observations and again points to the possibility that RNF168 turnover is regulated by a specific protease. Nevertheless, the MS data are only preliminary and. further effort is required to confirm these findings.

5.9 Ectopic expression of the phosphomimetic RNF168 mutants does not alter colony formation potential of cells

MS analysis revealed p38 MAP kinase as a potential RNF168 interactor. Since this protein is known to be (together with PLK1) responsible for driving the exclusion of 53BP1 from damage chromatin in mitosis, it might be also involved in exclusion of RNF168, which has been previously observed (Lee et al., 2014). Therefore, clonogenic assay was performed to test the colony formation potential of the cells expressing the RNF168 phosphosite mutants (Figure 17).

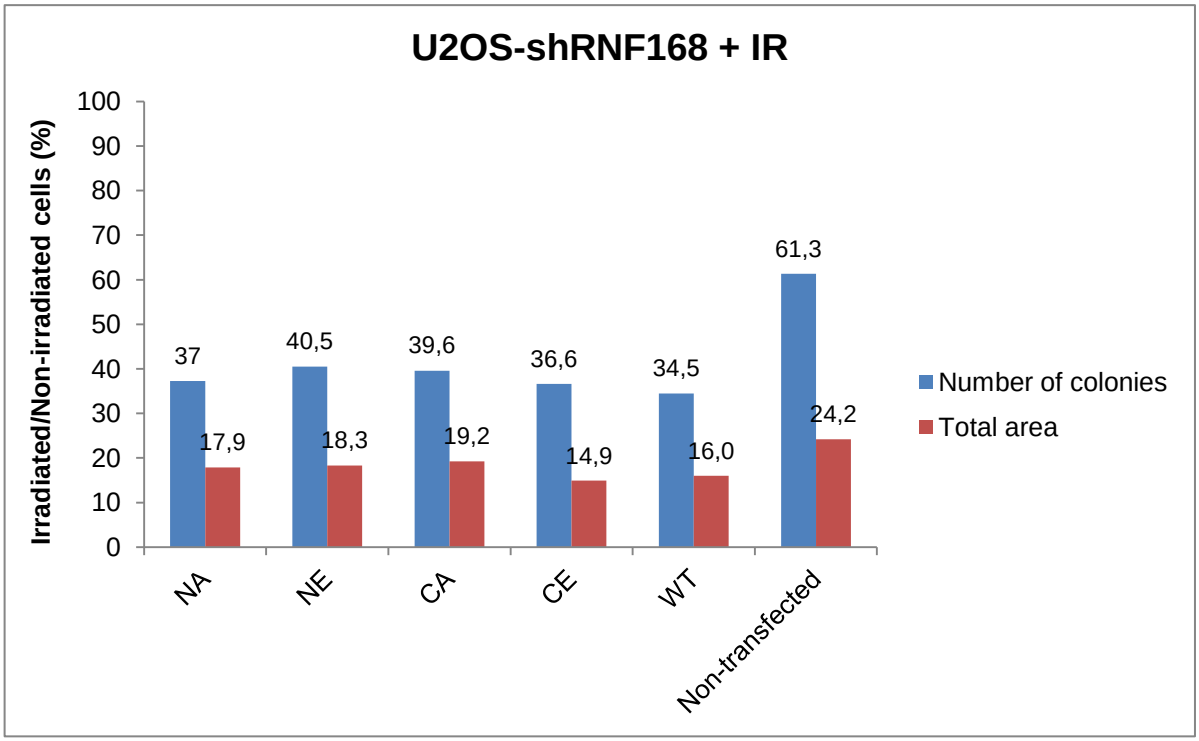


Figure 17. Relative colony formation potential of irradiated cells. Cells were irradiated with 2 Gy of Xray and grown for 1 week. Colonies of irradiated cells were compared to non-irradiated cells. Two parameters of colonies were quantified: number of colonies and total area of colonies.

Quantitative analysis of colonies did not reveal any major differences in cells expressing each of the RNF168 mutant form. However, this might be caused by loss of expression of the mutated proteins in these stable cell lines, since we also microscopically observed intense decrease of the GFP signal. Interestingly, it also seems that cells completely lacking the RNF168 display higher colony formation properties than cells transfected RNF168 mutant variants.

6. DISCUSSION

An impressively complex network of repair factors and their interactions evolved in cells in order to maintain the genome stability after DNA damage occurs. Complexity of the whole system is further increased by plethora of various post-translation modifications and their combinations, which also provides the repair and repair-associated signalization system with the extreme flexibility (Smeenk and van Attikum, 2013; van Attikum and Gasser, 2009). This work is mainly focused on E3 ubiquitin ligase RNF168 and its role in DSB repair, and brings the novel aspects of the mechanisms of its regulation mediated by post-translation modifications.

Since the DNA damage response is essentially dependent on ubiquitin-mediated signalization in cells, proteasome inhibition inducing the proteotoxic stress via the depletion in free ubiquitin levels should normally abolish the DDR, due to accumulation of ubiquitin in conjugated form in cytoplasm. However, we have recently observed that endogenously elevated levels of RNF168 in some cancer cell lines promote level of resistance to the proteasome inhibitor induced proteotoxic stress and that DDR signalization is sustained in these cells, despite the depleted free ubiquitin level. Interestingly, in some lines displaying the sustained DDR phenotype, the level of RNF168 was not elevated significantly, however, the putative modified form of the RNF168 was more prominent Therefore, we hypothesized that activity-increasing post-translational modifications of RNF168 might play a significant role in this phenotype and might be also involved in other, yet non-uncovered RNF168-mediated functions and DDR regulations.

Therefore, the experimental part of this work dealt with RNF168 PTMs and their role in modulation of activity and stability of RNF168. First, we wanted to investigate, whether modifications introduced to RNF168 are DNA damage-associated and surprisingly, our experiments did not confirm this hypothesis (Figure 1B). Hence, it seems that the putative post-translational modifications do not have any modulating functions directly connected to the ubiquitin-based signalization upon DNA damage. In contrast to our findings, Danielsen and colleagues showed the essential role of SUMOylation of DDR associated factors in response to damage induction. Evidence they gathered suggest that PIAS4-mediated SUMOylation of HERC2, RNF8 and RNF168 is increased upon irradiation of cells and function

as a “molecular glue” enhancing the HERC2-RNF8-UBC13 complex formation and also stimulates the accumulation of RNF168 at the damage site (Danielsen et al., 2012). They quantified levels of RNF168 in FLAG-SUMO1 immunoprecipitates prior and after irradiation and interestingly, they observed intensive increase in RNF168 levels, while electrophoretic mobility stayed unchanged. This suggests that the increase in level of RNF168 is probably caused by elevation in SUMOylated protein fraction and not by hyperSUMOylation of RNF168. Surprisingly, despite we detected SUMOylation of RNF168 in our samples, we did not observe any substantial accumulation of any form of RNF168 protein. Hence, detailed MS analysis of each of the RNF168 forms would be required in order to better understand their PTM profile.

Next, we also focused on other PTMs that are most commonly involved in functional regulations in the mammalian cells. In addition to SUMOylation, using the immunoprecipitation approach we also confirmed the presence of phosphorylations on RNF168. Phosphorylation of RNF168 was reported before, but not all phosphorylated residues have been mapped and the function of this modification of RNF168 remains unknown (Dephoure et al., 2008). On the other hand, we found out that RNF168 does not undergo regulating (auto)ubiquitination, which can be commonly observed in case of some other E3 ubiquitin ligases e.g. BRCA1 (Chen et al., 2002) or MDM2 (Ranaweera and Yang, 2013) .

Since the biological role of RNF168 phosphorylations is still unclear and it represented the interesting direction for further investigation, we focused on their detailed characterization and their biological function in RNF168 activity and stability modulation. Several phosphorylation sites within the RNF168 amino acid sequence have been previously described, three of them have been identified in MS-based large-scale proteomic study (Dephoure et al., 2008), while another three were predicted based on homology with mouse and rat RNF168 (Huttlin et al., 2010; Lundby et al., 2012).

To selectively map the biological function of the specific phosphorylation sites *in vivo*, we generated four phosphomimetic and phosphoinhibiting mutants and screened them using immunofluorescence experiments and immunoblot analysis. Surprisingly, no defects in formation of RNF168 IRIFs were observed in these mutants and also recruitment of downstream repair factor 53BP1 did not display any significant changes compared to wtRNF168. Nevertheless,

our immunoblot analysis indicated that phosphorylations at these specific sites might play an important role in regulation of RNF168 at the level of protein stability modulation (Figure 14). Several examples of phosphorylation-dependent protein stability regulations have been already described and well characterized in cells, e.g. in the DNA repair, or the cell proliferation regulations. As an example of phosphorylation-dependent degradation may serve e.g. the HIPK2 (homeodomain-interacting protein kinase 2)-phosphorylation-dependent destabilization and degradation of WIP1 (wild-type p53-induced phosphatase 1), the important ATM homeostatic regulator (Choi et al., 2013). Cyclin E-CDK2-mediated degradation of CDK inhibitor p27 (also known as Kip1) can be used as another example of phosphorylation-driven destabilization. In this case, T187 phosphorylation destabilizes p27 and drives it proteasome degradation (Sheaff et al., 1997; Vlach et al., 1997). These examples demonstrate the importance of phosphorylation-mediated protein destabilization in cells. In case of RNF168, this regulatory mechanism might be utilized in cell cycle phase-dependent activity regulation, as it was already shown for some DDR factors (Lee et al., 2014; Orthwein et al., 2014).

We also carried out the MS analysis of a FLAG-RNF168 immunoprecipitate separated by SDS-PAGE and identified few promising novel RNF168 interactors. Couple of kinases potentially involved in RNF168 phosphorylations have been detected such as p36 and p38 MAP kinases, ATM and STK38L. While the key roles of ATM in DDR have been the subject of intensive research for many years now (Ali et al., 2004; Bakkenist and Kastan, 2003), the direct association of other mentioned kinases with DDR have not been extensively studied so far. Interestingly, proline-directed kinase p38, together with PLK1, has been shown to be crucial for phosphorylation-dependent exclusion of 53BP1 from the chromatin during mitosis, which prevents cells from mitotic defects and genomic instability. It is also known, that recruitment of RNF8 and RNF168 to the damage site is during mitosis also abrogated and that their recruitment is reconstituted in the late mitosis during the anaphase/telophase transition (Lee et al., 2014; Orthwein et al., 2014). Intriguingly, S411 (one of the identified C-terminal phosphosites) of RNF168 is located in the same amino acid context (V-[T/S]-P) as the known p38-phosphorylated T1609 residue of 53BP1 (one of two residues essential for mitotic exclusion of 53BP1) and S415 residue of RNF168 is also followed by a proline

(53BP1 RefSeq. NM_001141979, RNF168 RefSeq. NM_152617.3). Therefore, some of the RNF168 C-terminal phospho-sites might also be the p38 targets, and their phosphorylation might drive the mitosis-dependent exclusion of RNF168 from the chromatin, same as in case of 53BP1. Interestingly, Giunta et al. showed that RNF8, RNF168, 53BP1 and BRCA1 (all excluded from chromatin in mitosis) protein levels do not substantially fluctuate throughout the cell cycle (Giunta et al., 2010). Therefore, together with our experimental data, it seems that stability modulation of RNF168 observed in our phosphosite mutants is uncoupled from the mitotic-associated DDR regulations and might operate in different, mitotic-independent mode. Therefore, further extensive research would be required to fully understand the nature of RNF168 post-translational modifications and their biological functions.

7. CONCLUSION

The goal of the theoretical part of this thesis was to summarize the latest knowledge about the double-strand break repair, indicate its overwhelming complexity and to briefly describe its role in carcinogenesis and also opportunities for targeting the DNA repair machinery in cancer therapy. The second half of the theoretical part focuses in more detail on the key factors involved in DNA double-strand break signalization cascade with strong emphasis on an essential role of the RNF168 E3 ubiquitin ligase in DNA damage repair and the maintenance of genome integrity.

The experimental part of this thesis dealt with identification of post-translational modifications of RNF168 and their role in activity and stability modulation of this protein, with emphasis on phosphorylations. We showed that both identified and predicted phosphorylations of RNF168 are probably not directly involved in modulation of the activity of the protein in interphase cells, however, our results pointed to an intriguing possibility that phosphorylations regulate RNF168 protein stability and activity during mitosis.

8. LITERATURE

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9. ABBREVIATIONS

53BP1	–	Tumour suppressor p53-binding protein
ATM	–	Ataxia telangiectasia mutated
ATR	–	Ataxia telangiectasia mutated and Rad3 related kinase
BER	–	Base excision repair
BLM	–	Bloom syndrome protein
BRCA1	–	Breast cancer type 1 susceptibility protein
BRCA2	–	Breast cancer type 2 susceptibility protein
BRCC36	–	BRCA1/BRCA2-containing complex subunit 36
BRCC45	–	BRCA1/BRCA2-containing complex subunit 45
BRCT	–	BRCA1 C-terminal
BRE	–	Brain and reproductive organ-expressed protein
BSA	–	Bovine serum albumin
CCDC98	–	Coiled-coil domain-containing protein 98
CDK1	–	Cyclin-dependent kinase 1
CHD4	–	Chromodomain-helicase-DNA-binding protein 4
CHIP	–	Carboxy terminus of Hsp70-interacting protein
CHK1	–	Checkpoint kinase 1
CIP	–	Calf intestinal phosphatase
CtIP	–	CtBP-interacting protein
DDR	–	DNA damage response
DMSO	–	Dimethylsulfoxide
DNA	–	Deoxyribonucleic acid
DNA-PK	–	DNA-dependent protein kinase
DNA-PKcs	–	DNA-dependent protein kinase catalytic subunit
dNTPs	–	Deoxyribonucleotide triphosphates
DSB	–	Double-strand break
DTT	–	Dithiothreitol
EDTA	–	Ethylenediaminetetraacetic acid
EXO1	–	Exonuclease 1
FHA	–	Forkhead associated
FRAP	–	Fluorescence recovery after photobleaching

GFP	–	Green fluorescent protein
HELB	–	DNA helicase B
HERC2	–	HECT domain and RCC1-like domain-containing protein 2
HIPK2	–	Homeodomain-interacting protein kinase 2
HR	–	Homologous recombination
IP	–	Immunoprecipitation
IR	–	Ionizing radiation
ROS	–	Reactive oxygen species
IRIF	–	Ionizing-radiation-induced foci
LRM	–	Leucine-rich motif
LSB	–	Laemmli sample buffer
MAPK	–	Mitogen-activated protein kinase
MDC1	–	Mediator of DNA damage checkpoint 1
MERIT40	–	Mediator of RAP80 interactions and targeting subunit of 40 kDa
MIU	–	Motif interacting with ubiquitin
MMEJ	–	Microhomology-mediated end joinig
MMR	–	Mismatch repair
MRE11	–	Meiotic recombination 11 homolog 1
MRN	–	MRE11-RAD50-NBS1
MS	–	Mass spectrometry
MUM1	–	Mutated melanoma-associated antigen 1
MW	–	Molecular weight
NBA1	–	NAP1 and bud neck-associated protein 1
NBS1	–	Nijmegen breakage syndrome 1
NER	–	Nucleotide excision repair
NHEJ	–	Non-homologous end-joining
OTUB1	–	OUT domain ubiquitin aldehyde –binding 1
P	–	Phosphate
PARP	–	Poly-(ADP-ribose)-polymerase
PARPi	–	Poly-(ADP-ribose)-polymerase inhibitor
PIAS1	–	Protein inhibitor of activated STAT protein 1
PIAS4	–	Protein inhibitor of activated STAT protein 4
PIKK	–	Phosphatidylinositol-3 kinase-like kinase
PLK1	–	Polo-like kinase 1

PS	–	Peptide sequencing
PTIP	–	PAX transactivation activation domain-interacting protein
PTM	–	Post-translational modification
RAD6	–	Radiation sensitivity protein 6
RAD6A	–	Radiatin sensitivity protein 6 homolog A
RAD6B	–	Radiation sensitivity protein 6 homolog A
RAP80	–	Receptor-associated protein 80
REV7	–	Revertibility protein 7
RIDDLE	–	Radiosensitivity, immunodeficiency, dysmorphic features and learning difficulties
RIF1	–	RAP1-interacting factor 1
RING	–	Really interesting new gene
RIPA	–	Radioimmunoprecipitation assay
RNF168	–	RING finger protein 168
RNF8	–	RING finger protein 8
RNS	–	Reactive nitrogen species
RPA	–	Replication protein A
SCID	–	Severe combined immunodeficiencies
s-CIN	–	Structural chromosomal instability
SDS	–	Sodium dodecyl sulphate
SDS-PAGE	–	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
shRNA	–	Short hairpin ribonucleic acid
SMC1	–	Structural maintenance of chromosomes protein 1A
SSB	–	Single-strand break
STK38L	–	Serine/threonine kinase 38-like
SUMO	–	Small ubiquitin-like modifier
Tip60	–	60 kDa Tat-interactive protein
TRAF6	–	TNF receptor-associated factor 6
TRIM21	–	Tripartite motif containing 21
TRIP12	–	Thyroid receptor-interacting protein 12
Ub	–	Ubiquitin
UBC13	–	Ubiquitin-conjugating enzyme E2 N
UbcH7	–	Ubiquitin conjugating enzyme H7
UBR5	–	Ubiquitin protein ligase E3 component n-recognin 5

UDM1	–	Protein-interacting motif 1
UDR	–	Ubiquitination-dependent recruitment
UIM	–	Ubiquitin-interacting motif
UMI	–	UIM- and MIU-related ubiquitin binding domain
UPS	–	Ubiquitin proteasome system
USP16	–	Ubiquitin-specific protease 16
USP3	–	Ubiquitin-specific protease 3
UV	–	Ultraviolet light
w-CIN	–	Whole chromosomal instability
WEE1	–	Wee1-like protein kinase
WIP1	–	Wild-type p53-induced phosphatase 1
X4-L4	–	X-ray repair cross-complementing protein 4 – DNA Ligase IV
XLF	–	X-ray repair cross-complementing protein 4-like factor
XRCC4	–	X-ray repair cross-complementing protein 4
XRCC5	–	X-ray repair cross complementing protein