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**Regulation of the DNA damage response by R2TP mediated MRN complex assembly  
and control of 53BP1 localisation.**

PhD thesis

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I declare that I wrote this work independently and that I did my best to acknowledge all people and literature. I did not use this work or a substantial part of it to obtain another academic degree or equivalent.

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## List of abbreviations

53BP1	tumor suppressor p53 binding protein 1
ATLD	ataxia-telangiectasia-like disorder
ATM	ataxia telangiectasia mutated kinase
ATR	ataxia telangiectasia and Rad3 related kinase
BRCA1	breast cancer type 1 susceptibility protein
CDK	cyclin dependent kinase
Chk1	checkpoint kinase 1
Chk2	checkpoint kinase 2
CtIP	CtBP-interacting protein
CK2	casein kinase 2
Co-IP	CO-Immunoprecipitation
DSB	double strand break
DDR	DNA damage response
DNA-PK	DNA-dependent protein kinase
G1	Gap phase 1 of the cell cycle after mitosis
G2	Gap phase 2 of the cell cycle after DNA duplication
GFP	green fluorescent protein
HR	homologous recombination
M phase	Mitotic phase, actual cell division
MRN	Protein complex consisting out of MRE11, RAD50 and NBS1
NHEJ	non-homologous end joining
NBS1	nijmegen breakage syndrome protein 1
NLS	nuclear localisation signal
p21	CDK inhibitor
p53	“guardian of the genome” which function is affected in many tumors
PIKKs	phosphatidylinositol 3-kinase related kinases
PLK1	polo like kinase 1
PTIP	PAX transactivation activation domain-interacting protein
RB	retinoblastoma protein
RIF1	RAP1-interacting factor 1 homolog
RING	really interesting new gene, zing finger type of protein domain
RNF8	RING finger protein 8

RNF168

RING finger protein 168

RPA

replication protein A

S phase

Synthesis phase, in which the DNA is duplicated

Abbreviations of amino acids:

Full name	3 letter code	1 letter code	Full name	3 letter code	1 letter code
Alanine	Ala	A	Isoleucine	Ile	I
Arginine	Arg	R	Leucine	Leu	L
Asparagine	Asn	N	Lysine	Lys	K
Aspartate	Asp	D	Methionine	Met	M
Aspartate or Asparagine	Asx	B	Phenylalanine	Phe	F
Cysteine	Cys	C	Proline	Pro	P
Glutamate	Glu	E	Serine	Ser	S
Glutamine	Gln	Q	Threonine	Thr	T
Glutamate or Glutamine	Glx	Z	Tryptophan	Trp	W
Glycine	Gly	G	Tyrosine	Tyr	Y
Histidine	His	H	Valine	Val	V

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## **Abstract**

DNA double strand breaks are the most dangerous type of DNA damage. The MRN complex and 53BP1 have essential functions in the repair of DNA double strand breaks and are therefore important for maintaining genomic stability and preventing cancer. DNA double strand breaks are repaired by two main mechanisms - homologous recombination and non-homologous end joining. The MRN complex senses DNA double strand breaks and activates a cascade of posttranslational modifications that activates and recruits other effector proteins. In addition MRN mediated resection is important for removing adducts in non-homologous end joining and creating single stranded DNA required for homologous recombination. 53BP1 is recruited to DNA double strand breaks by site specific ubiquitinations and inhibits DNA resection, thereby promoting non-homologous end joining at the expense of homologous recombination. In this thesis we show that MRE11 binds to the R2TP chaperone complex through a CK2 mediated phosphorylation. Knockdown of R2TP or mutating the MRE11 binding site leads to decreased MRE11 levels and impaired DNA repair. Similar phenotype has been observed in cells from patients with ataxia-telangiectasia-like disorder (ATLD), containing MRE11 deletion mutation which is missing the R2TP complex binding site. Based on R2TP complex function as a molecular chaperone, we conclude, that R2TP complex is important for MRN complex assembly/quality control. Moreover, we explored the processes important for regulating localisation of DNA repair protein 53BP1 to the nucleus and DNA damage site. We investigated PLK1 and CDK1 mediated phosphorylations in 53BP1 ubiquitin binding domain and discovered that they inhibit 53BP1 recruitment to the DNA damage site in mitosis. Finally we discovered the sequence of 53BP1 localisation signal and explored regulatory mechanisms of 53BP1 nuclear localisation by post translational modifications. In conclusion, we deepened our understanding of MRN complex assembly and pathophysiology of a specific MRE11 mutation leading to ATLD. In addition we found new ways of regulation of 53BP1 localization and function.

## **Abstract (Czech)**

Dvouvláknové zlomy jsou nejnebezpečnějším typem poškození DNA. Proteinový komplex MRN a protein 53BP1 hrají důležitou roli při opravě dvouvláknových zlomů DNA a jsou proto nezbytné pro udržení stability DNA a prevenci rakoviny. Dvouvláknové zlomy mohou být opraveny dvěma hlavními způsoby - homologní rekombinací a nehomologním spojením konců DNA. MRN detekuje dvouvláknové zlomy DNA a aktivuje signální kaskády vedoucí k opravě DNA nebo buněčné apoptoze. Zároveň komplex MRN pomáhá během nehomologního spojení konců DNA začistit konce DNA a vytvořit jednořetězcové úseky DNA potřebné pro homologní rekombinaci. 53BP1 inhibuje resekci DNA, čímž podporuje nehomologní spojení konců na úkor homologní rekombinace. V této práci ukazujeme, že MRE11 se váže na šaperonový komplex R2TP prostřednictvím místa fosforylovaného CK2. Snížení hladiny R2TP komplexu nebo zmutování vazebného místa pro MRE11 vede k destabilizaci MRE11 a k narušení opravy DNA. Podobný fenotyp byl pozorován v buňkách pacientů s dědičným syndromem ataxie-telangiectasie (ATLD), které obsahují zkrácený protein MRE11, v němž chybí vazebné místo pro komplex R2TP. Vzhledem k funkci R2TP jako molekulárního šaperonu předpokládáme, že R2TP je důležitý pro sestavování nebo kontrolu kvality komplexu MRN. Dále jsme prozkoumali procesy důležité pro regulaci jaderné lokalizace 53BP1 a k jeho vazbě na poškozenou DNA. Ukázali jsme, že fosforylace 53BP1 zprostředkované kinázami PLK1 a CDK1 zabraňují vazbě 53BP1 k poškozené DNA během mitózy. V poslední části této práce jsme identifikovali umístění jaderného lokalizačního signálu v 53BP1 a vysvětlili jak je jaderná lokalizace 53BP1 regulovaná posttranslačními modifikacemi. Tato práce vedla k prohloubení našich vědomostí o sestavování komplexu MRN a patofyziologii specifické mutace MRE11 vedoucí k ATLD syndromu. Zároveň jsme objevili nové způsoby regulace jaderné lokalizace a funkce 53BP1.



# **1. Introduction**

## **The cell cycle**

Our DNA contains the hereditary information with which we are born. During development and normal body homeostasis, successive rounds of DNA replication and subsequent cell divisions ensure that all cells contain a copy of the genomic DNA present in the fertilized oocyte. The cell cycle harbours all the events that are needed for one round of cell duplication and is divided in four phases. The first phase is a gap phase (G1) in which cells reside and grow until they have received sufficient external or internal signals to initiate the second phase, the synthesis phase (S phase), during which the genetic material is duplicated creating two sister chromatids. The third phase is again a gap phase (G2) that allows the cells to prepare for the nuclear division, mitosis (M phase), which is the last phase of the cell cycle (1). The cells must be able to detect errors (such as damaged DNA) occurring during the cell cycle progression and correct them. They have therefore evolved surveillance mechanisms called cell cycle checkpoints, which are integrated with the core cell cycle machinery and continuously monitor and safeguard successful completion of cell cycle events. (2).

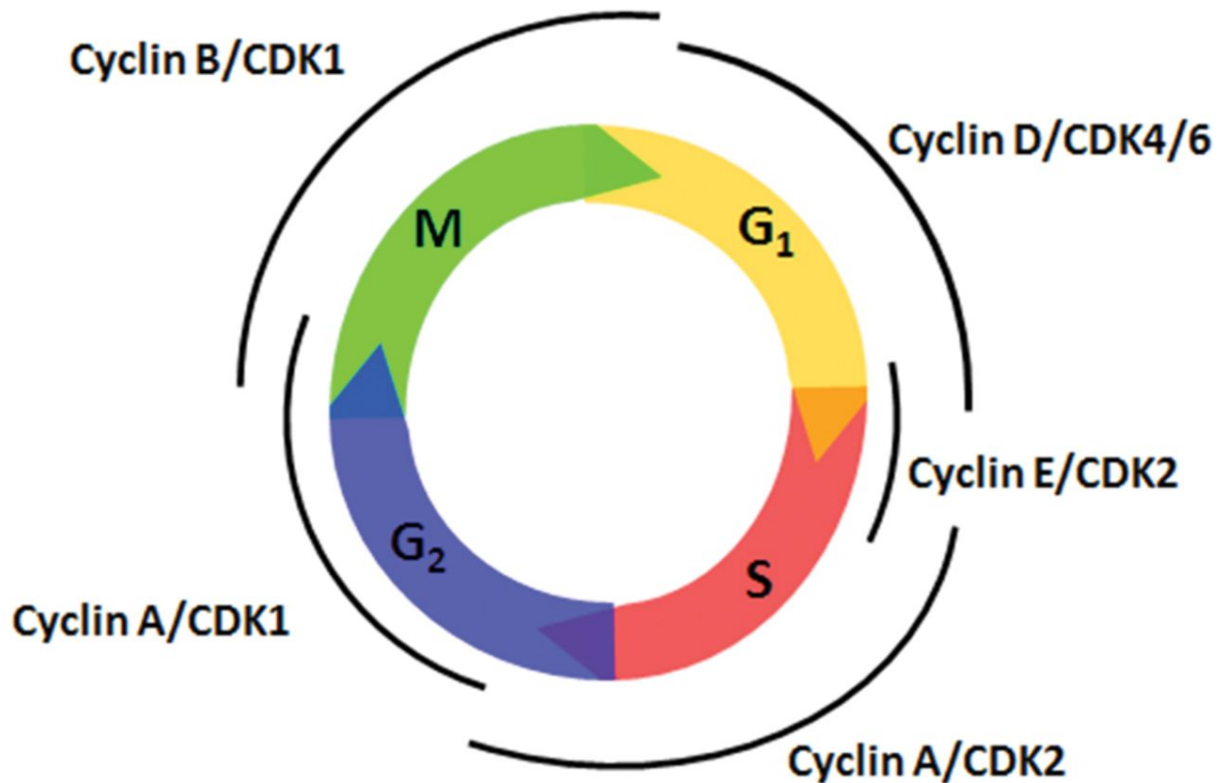
## **Molecular control of the cell cycle by protein phosphorylation**

Phosphorylation is a well-studied post translational modification with important roles for cell cycle regulation and repair of damaged DNA. Phosphorylation can quickly change the (catalytic) activity of proteins and modulate protein-protein interactions.

The cell cycle is for a large part regulated by cyclin dependent kinases (CDKs) that act in complex with cyclins (see figure 1). CDKs phosphorylate and regulate the activity of many proteins involved in DNA replication and mitosis while cyclins are important for the timing and specificity of CDKs activity (3). Cyclins are degraded upon moving to the next cell cycle phase to restrain CDKs activity and assure the directionality of the cell cycle (4, 5). In early

G1 cyclin D is expressed in response to growth factors and activates CDK4 and CDK6. These CDKs stimulate cell cycle progression by phosphorylating the retinoblastoma protein (RB) (6, 7). RB hyperphosphorylation removes this protein from its targets, which include the transcription factor E2F (inhibited by RB and activated by Myc) and the histone deacetylase protein 1 (recruited to the DNA by RB) (8-10). CDK2 promotes DNA synthesis and plays a role later in G1 and during the S phase. First, at the G1/S transition, CDK2 is activated by cyclin E while later, during the S phase, CDK2 forms a complex with cyclin A (11). CDK1 coordinates the M phase by phosphorylating many substrates involved in mitosis, including proteins involved in spindle assembly (12). CDK1 is activated by cyclin A in G2 phase and by cyclin B during M phase (13). CDKs are controlled by phosphorylations, for example CDK1 is inhibited through phosphorylation by WEE1 which is counter balanced by CDC25 dephosphorylation (14).

Other kinases with important roles in the cell cycle include the Polo like kinases. Polo-like kinase 1 (PLK1) is one of the five Polo like kinases and is particularly important for mitosis (15). PLK1 has an N-terminal serine/threonine kinase domain and a C-terminal polo box domain enabling the binding to its substrates (16). PLK1 polo box domain binding is controlled by priming phosphorylations by either itself or other kinases, such as CDK1 (17, 18). Auora-A and Bora activate PLK1 by phosphorylating threonine 210 within the T-loop located in its kinase domain (19, 20). PLK1 activity builds up during G2 and peaks during mitosis. Cells depleted of PLK1 have faulty centrosome maturation and are therefore unable to form a proper bipolar spindle, leading to mitotic failure (21, 22). Additional functions of PLK1 include: inhibiting WEE1 and activating Cdc25 to promote mitotic entry through CDK1 activation and stabilizing 53BP1, reported to be important for the centrosomes (19, 23-25).

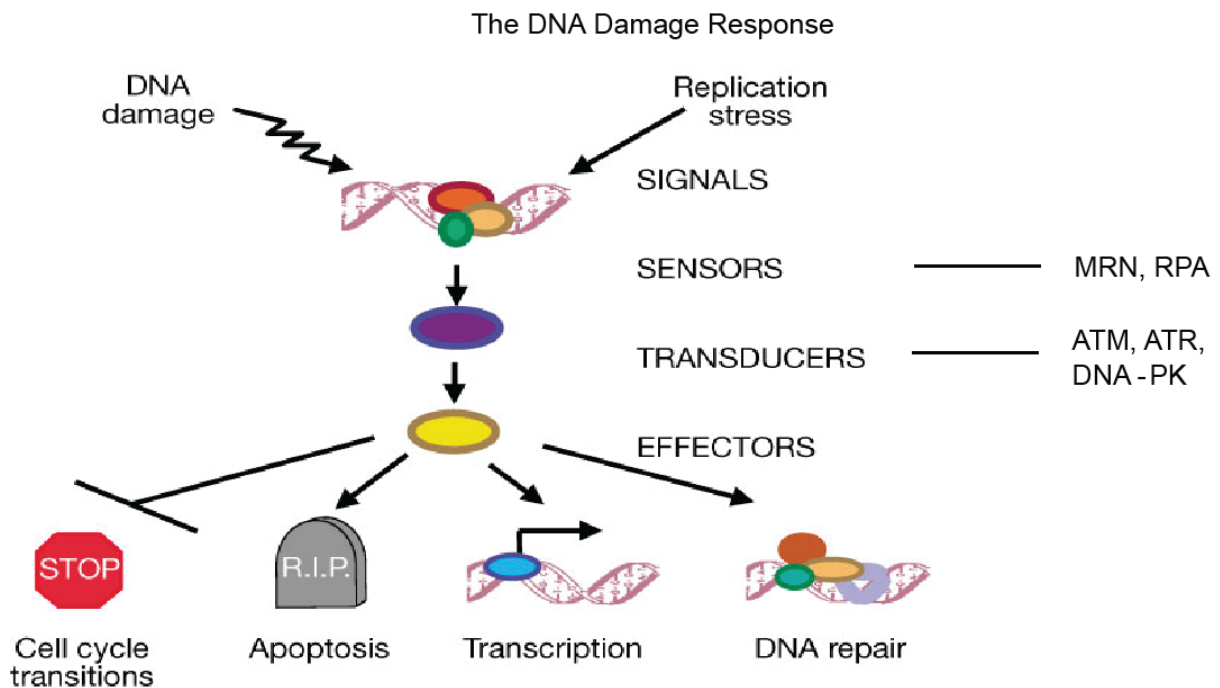


**Figure 1. CDKs and cyclins control the cell cycle.** Cyclin-CDK complexes regulate the cell cycle and their activity is restricted to distinct cell cycle phases. In the figure is depicted for each of the cell cycle phases which cyclins activate which kinases.

### The DNA damage response

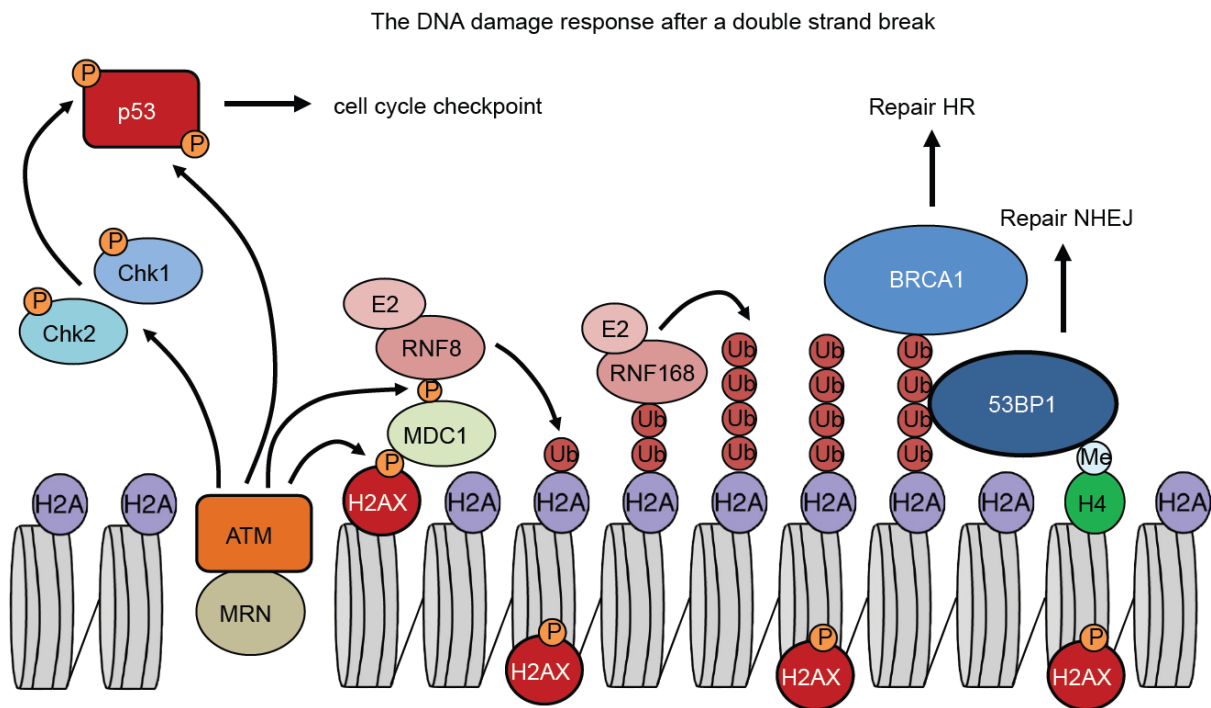
DNA is a fragile molecule that is constantly damaged by both external (UV, IR, chemicals) and internal (replication, cellular metabolism) factors. Damaged DNA is problematic for cellular processes such as transcription and replication, and can result in mutations. These mutations, when present in the gametes, can affect the fitness of the offspring (genetic disease, evolution) or lead to genomic instability in somatic cells, which may cause diseases in the affected individual, such as cancer. The DNA damage response is a set of pathways that become activated after the occurrence of DNA damage and the main function of these pathways is to detect the DNA damage, induce DNA damage cell cycle checkpoint and repair the damage, thereby protecting genomic stability and preventing development of cancer (26).

The response to DNA damage is in broad lines similar for different types of DNA damage (see figure 2). First the damage is recognized by DNA damage sensors, subsequently this signal is transduced to effector proteins that stop the cell cycle (checkpoint activation), repair the damaged DNA or induce programmed cell death (apoptosis) (27). Here I will focus on the recognition and repair of DNA double strand breaks. When left unrepaired, a single DNA double strand break can lead to cell death or the loss of many genes simultaneously while imperfect repair also leads to the loss of genetic information or chromosome fusions (28). Double strand breaks are recognized by the MRN complex, KU (recognizing DNA ends) and RPA (recognizing single stranded DNA) which respectively activate kinases from the phosphatidylinositol 3-kinase related kinase family (PIKKs) ATM, DNA-PK and ATR (29-31). PIKKs transduce the signal by phosphorylating hundreds of substrates, thereby orchestrating the recruitment of repair proteins and activation of the cell cycle checkpoint (32).



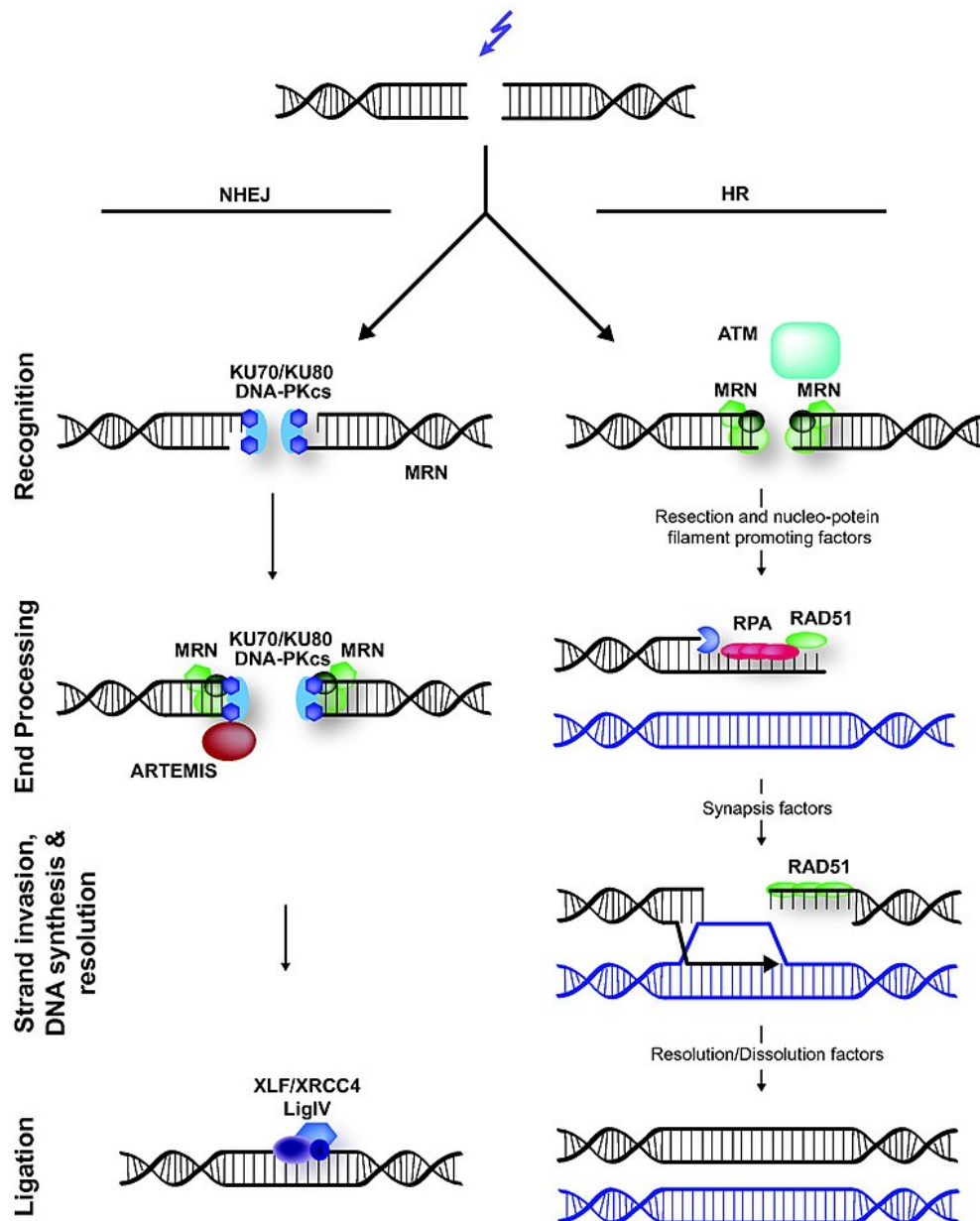
**Figure 2. The general mechanisms of the DNA damage response.** After the formation of DNA damage the damage is recognized by sensors (MRN complex for DNA ends and RPA for single stranded DNA), subsequently the signal is transduced by PIKKs (ATM, ATR and DNA-PK) to effector proteins that stop the cell cycle, initiate apoptosis, alter transcription and execute DNA repair. (Adapted with modifications from (27))

In response to DNA damage a cascade of posttranslational modifications starting with the PIKKs ensures that all important effectors come into play (see figure 3). After a double strand break KU and MRN complex bind and activate DNAP-PK and ATM, respectively. During S and G2 phase single stranded DNA is created by MRN mediated resection of the two ends of the DNA double strand break (29, 33). The single stranded DNA gets quickly coated with RPA, which leads to ATR activation (34, 35). While DNA-PK phosphorylates mainly substrates directly involved in repair of DNA double strand breaks (36), ATR and ATM activate kinases CHK1 and CHK2, which stop the cell cycle progression. For example, CHK2 phosphorylates and activates CDC25 (involved in CDK2 activation) and targets it for degradation (37). Moreover, phosphorylation of MDC1 by ATM enables MDC1 binding to ubiquitin ligases RNF8 and subsequent recruitment of ubiquitin ligase RNF168 to the site of DNA damage. RNF8 and RNF168 ubiquitinate histone H2A and facilitate recruitment of important DNA repair proteins such as 53BP1 and BRCA1 (38-40). Also, as a deadly backup in case DNA repair fails, phosphorylation of the transcription factor p53 on serine 15 leads to protein stabilization and activation of its target genes, including the ones that induce apoptosis (41, 42).



**Figure 3. DNA damage signalling after DNA double strand break formation.** MRN complex binds at the site of a double strand break and stimulates ATM activation. ATM phosphorylates a multitude of proteins involved in the DDR. These include CHK2 and p53, important for the induction of a cell cycle checkpoint and promoting programmed cell death. H2AX and MDC1, phosphorylated by ATM, enable the recruitment of the E3 ligase RNF8 that mono ubiquitinates H2A. E3 ligase RNF168 subsequently poly ubiquitinates H2A enabling the recruitment of BRCA1 and 53BP1 which are involved in choosing between repair by NHEJ and homologous recombination. (Adapted with modifications from Jan Benada)

The repair of DNA double strand breaks occurs mainly through one of two distinct mechanisms; non homologous end joining (NHEJ) or homologous recombination (see figure 4). The need for a template restricts homologous recombination to the cell cycle phases where the sister chromatid is present (S and G2 phase). NHEJ is the dominant way of repairing DNA double strand breaks; the repair is initiated by recognition of the break by KU70 and KU80 and, when needed, minor processing of the break after which the ends are ligated together by a complex of XRCC4 and DNA ligase 4. NHEJ usually leads to small deletions at the site of the break (43). The use of a template enables error free repair of DNA double strand breaks by homologous recombination. After recognition of the break by the



**Figure 4. Repair of double strand breaks by NHEJ or Homologous recombination.**

Repair of double strand breaks occurs mainly through two distinct pathways. NHEJ is the major repair mechanism during all phases of the cell cycle, while homologous recombination is exclusively used during S and G2 phase when a sister chromatid is present. NHEJ minimally processes the break by removing adducts and trimming of the overhangs upon which the two ends are ligated together. Homologous recombination requires long range resection followed by invasion of the sister chromatid and template directed repair. Finally the intermediate complex has to be resolved which can occur either without any crossover or with a crossover. (Adapted from (44))

MRN complex resection is initiated creating stretches of single stranded DNA. These short stretches of single stranded DNA are extended by long range resection mediated by nucleases EXO1 and DNA2 (45, 46). The single stranded DNA is initially coated with RPA which is later replaced by the recombinase RAD51 (29, 33). RAD51 catalyses strand exchange between the newly created single stranded DNA and the sister chromatid allowing template directed DNA synthesis (33). After DNA synthesis by DNA polymerase delta the newly formed intermediate can be resolved either by synthesis-dependent strand annealing or through a double holiday junction intermediate; the latter can in some cases result in a crossover where one side of the double strand break is exchanged with the sister chromatid (47, 48).

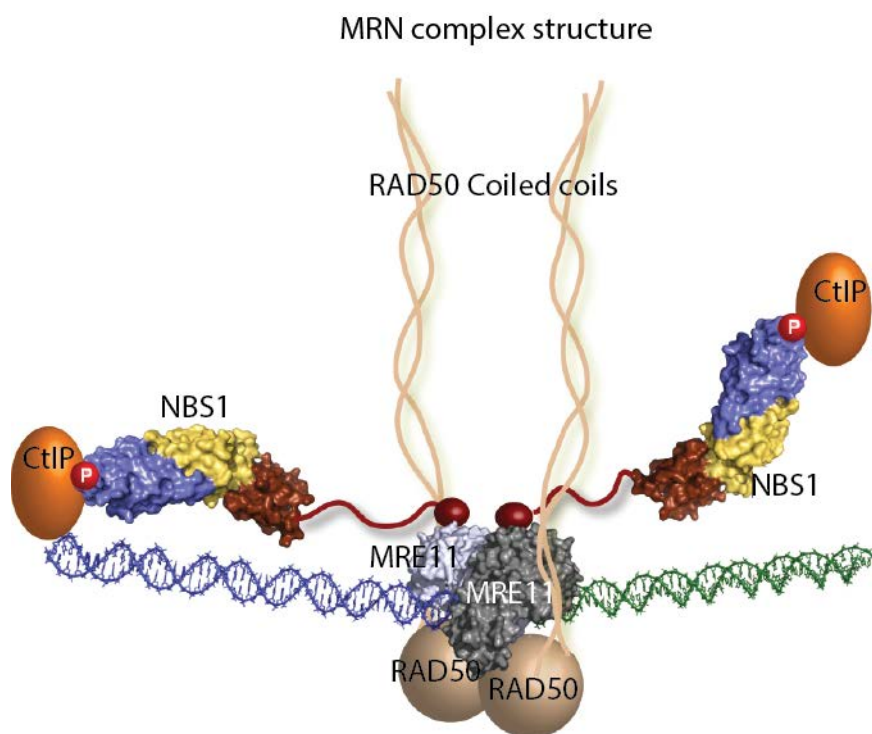
The decision between NHEJ and homologous recombination is governed by several checks and balances to ensure that NHEJ is used in the absence of a sister chromatid. Controlling resection at the site of the double strand break is key to pathway choice, with resection stimulating homologous recombination while at the same time inhibiting NHEJ. KU binds quickly to the DNA ends and protects them against resection. Phosphorylation of multiple substrates by CDK1, including MRN complex, stimulates resection in the S and G2 phase of the cell cycle and leads to nucleolytic removal of KU (49-51). An additional important layer of control is the balance between 53BP1 and BRCA1: 53BP1, together with its binding partners RIF1 and PTIP, inhibits resection while BRCA1 competes with 53BP1 and promotes resection (52-54).

### **MRE11 and the MRN complex**

The MRN complex is important for the repair of DNA double strand breaks both through NHEJ and homologous recombination. The complex consists out of MRE11, RAD50 and NBS1 and is conserved from yeast to mammals (see figure 5). MRN complex recognizes DNA double strand breaks and promotes activation of ATM and ATR (55). MRE11 binds



DNA ends at the site of DSBs and DNA branches and possesses 3' to 5' endonuclease activity (51, 55, 56). Dimers of MRE11 can keep the two ends of the double strand break together (55). RAD50 is an ATPase with DNA binding activity and its two coiled coil regions can form zinc hooks with their coiled coils connecting to other RAD50 molecules. These zinc hooks have roles in keeping the two sides of the double strand break together and enabling interaction with the sister chromatid (57). At the same time the ATPase activity of RAD50 can unwind the DNA and thereby stimulates the nuclease activity of MRE11 (58). NBS1 is needed for the nuclear localisation of MRE11 and its FHA and BRCT phospho-protein binding domains are important for recruiting other DNA damage response proteins to the site of the DNA damage including CtIP and MDC1 (59-61).



**Figure 5. The structure of the MRN complex.** The MRN complex forms dimers and binds at DNA ends, keeping the two sides of a broken DNA molecule together. MRE11 and RAD50 poses DNA binding activity and the coiled coils of RAD50 enable interaction with the sister chromatid. NBS1 is connected with a flexible linker to the MRE11 and RAD50 core complex and provides interaction with other repair proteins including CtIP. (Adapted with modifications from (61))

The MRN complex nuclease activity is important for homologous recombination where it, together with CtIP, initiates short range resection of the two ends of the broken DNA, resulting in the removal of KU from the DNA ends and the creation of a 3' single stranded DNA overhang. After the initial short range resection MRN complex further stimulates long range resection driven by EXO1 and DNA2, needed for the sister chromatid exchange (51, 55, 56). During NHEJ, MRN complex nuclease activity is required to remove DNA bound adducts, such as topoisomerase II adducts, that are preventing the repair of double strand breaks by the NHEJ machinery (62-64). Therefore the MRN complex plays pivotal role in both NHEJ and homologous recombination and also in the choice between these two pathways.

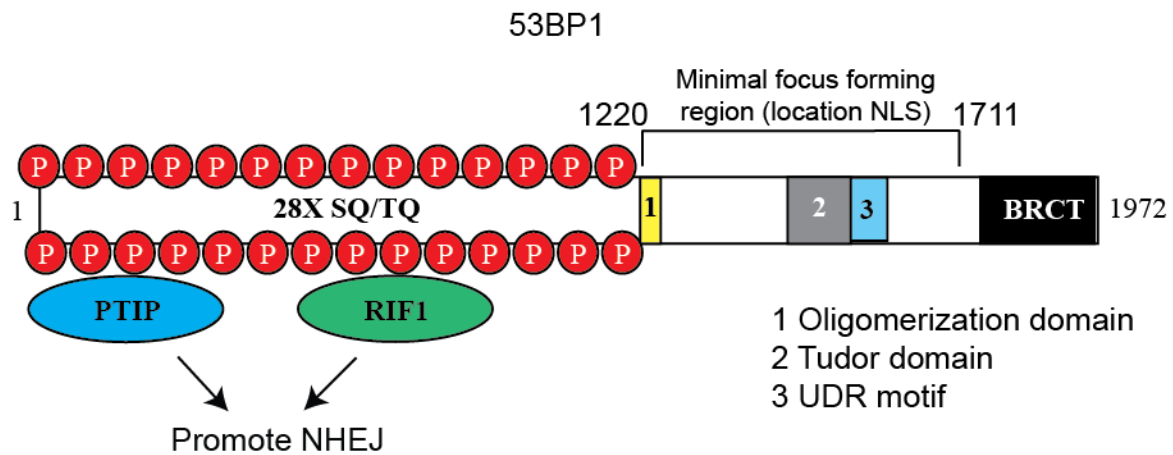
Besides a role in the repair of DNA double strand breaks the MRN complex is important for telomere maintenance and at stalled replication forks. The MRN complex is recruited to dysfunctional telomeres and promotes telomere maintenance (65-67). The role of MRN at stalled replication forks is two sided. MRE11 mediated resection is important for fork restart after release from replication blocks, but at the same time this activity leads to fork instability and is responsible for much of the toxicity observed after replication fork stalling (68, 69).

The N-terminus of MRE11 comprises its core domain which contains the nuclease domain and a first DNA binding domain. The core domain is sufficient for MRE11 dimerization (70). The MRE11 C-terminus contains a Glycine Arginine rich motif (GAR domain) and a second DNA binding domain (see figure 9). The GAR domain is important for the recruitment of MRE11 to the site of DNA damage and regulation of MRE11 nuclease activity (71). The very C-terminus of MRE11 interacts with CDK2 and is important for the cell cycle dependent activation of MRE11 nuclease activity (72). The interaction site with NBS1 is located within the first DNA binding domain and the interaction with RAD50 is mediated by MRE11 C-terminus (70).

Mutations in MRE11, NBS1 and RAD50 lead to severe hereditary cancer prone disorders, namely ataxia-telangiectasia-like disorder (ATLD) for MRE11, Nijmegen breakage syndrome for NBS1 and a Nijmegen breakage syndrome-like disorder for RAD50 (73-75). In accordance with the role of MRN in the DNA damage response, these patients suffer from neurodegeneration and cellular sensitivity to irradiation. In addition, patients with Nijmegen Breakage syndrome have 50 times increase in cancer incidence and decreased immunity. ATLD patients suffer from cerebellar ataxia, chromosomal instability and DNA-damage dependent checkpoint defects (76, 77). ATLD patients have low levels of the MRN complex components. In the main part of this thesis we investigate the molecular mechanism leading to the low levels of MRN complex proteins.

### **53BP1**

53BP1 is a large protein (nearly 2000 amino acids) that acts as a molecular platform for the recruitment of other DNA damage response proteins to the sites of DNA damage. 53BP1, together with its binding partners RIF1 and PTIP, inhibits both the initiation and the extent of resection (see figure 6) and is therefore important for the choice between NHEJ and homologous recombination and preventing over resection during homologous recombination (78-80). The C-terminal part of 53BP1 enables its recruitment to sites of DNA damage while the N-terminal part of 53BP1 is important for promoting NHEJ (53, 81). The N-terminus contains 28 S/TQ sites that can be phosphorylated by ATM and/or ATR after the occurrence of DNA damage. These phosphorylations enable 53BP1 interactions with RIF1 and PTIP and are essential for 53BP1 function in promoting NHEJ, including the inhibition of resection and stimulating the mobility of the DNA ends (53, 54, 82).



**Figure 6. Domain organization of 53BP1.** 53BP1 has 1972 amino acids. The N-terminus of 53BP1 is heavily phosphorylated in the DDR by ATM and ATR. These phosphorylations enable the interaction with two of its effectors PTIP and RIF1 that stimulate NHEJ. The minimal focus forming region contains an oligomerization domain, a Tudor domain (binding methylated H4K20) and a UDR motif (binding ubiquitinated H2A(X)K13/K15). The C-terminus of 53BP1 contains a BRCT domain important for interaction and stimulation of p53.

The central and C-terminal parts of 53BP1 are important for its cellular localisation; they contain a putative nuclear localisation signal and two binding domains: the Tudor domain and an UDR motif. The Tudor domain binds methylated histone H4 at lysine 20, while the UDR motif binds ubiquitinated histone H2A(X) at lysine 13 and 15 (81, 83). These two binding domains together regulate the recruitment of 53BP1 to the DNA damage site. The C-terminal BRCT domain mediates interaction with p53 and stimulates p53 mediated transcription activation (84).

53BP1 blocks resection, but during the S and G2 phases of the cell cycle resection needs to be allowed to enable homologous recombination. BRCA1 and CtIP stimulate resection and homologous recombination in these cell cycle phases by competing with 53BP1 functions (85) and this competition is regulated by cell cycle-dependent manner. For example, phosphorylation of CtIP on T847 by CDK1/2 suppresses 53BP1 mediated RIF1 recruitment (86). BRCA1 competes with 53BP1 localisation and activity and is suppressed by a cell cycle specific ubiquitination of its effector PALB2, which inhibits BRCA1 and PALB2 interaction

(87, 88). Consequently, in the presence of 53BP1 and the absence or mutation of BRCA1, resection is also blocked in S and G2 cells which leads to increased repair by NHEJ and more genetic instability (88).

The initial recruitment of 53BP1 to the sites of DNA damage depends on ubiquitination of H2A by RNF168, which is restricted to the site of the DNA damage. Methylated histone H4 is present throughout the genome and is important for the retention of 53BP1 at the site of DNA damage (54). Interestingly, 53BP1 foci formation is inhibited during mitosis while there is normal recruitment of more upstream DNA repair proteins such as MDC1. This inhibition is mediated by CDK1 phosphorylation of MDC1, which prevents the recruitment of RNF8 and therefore downstream interactors such as 53BP1 are also not recruited and activated. In addition, during mitosis 53BP1 localizes to the kinetochores and is hyperphosphorylated probably by mitotic kinases PLK1 and cyclinB-CDK1 (89). This raises an important question, addressed in this thesis, whether phosphorylation of 53BP1 by both PLK1 and CDK1 could influence 53BP1 recruitment to sites of DNA damage and what is the rationale for preventing 53BP1 recruitment during mitosis.

A pre requirement for 53BP1 recruitment to sites of DNA damage is its localisation in the nucleus. Nuclear import of 53BP1 depends on the nuclear transporter importin  $\beta$  and nucleoporin NUP153 (90, 91). Importin  $\beta$  often recognizes its substrates through adaptor protein importin  $\alpha$ , which binds to nuclear localisation signals present in its target proteins (92-94). The nuclear localization of 53BP1 is dependent on its C-terminal part, but the exact nuclear localization signal within 53BP1 is not known. At the same time, it is unknown how the localization of 53BP1 is regulated. These open questions are addressed in this thesis.

### **DNA damage induced cell cycle checkpoints**

Cell cycle checkpoints ensure that only when certain requirements are met the cell progresses

further in the cell cycle. The DNA damage response pathways induce a cell cycle arrest to ensure time for repair of the damaged DNA. In the case of extensive DNA damage, a more permanent cell cycle arrest, cell senescence or apoptosis is induced. To arrest the cell cycle the CDKs need to be inactivated (2). This occurs in a fast phase solely dependent on post-translational modifications and a slower phase in which transcription is required. After a double strand break is formed, CHK2 and CHK1 are activated by ATM and ATR. ATM is mainly involved in the activation of CHK2 while ATR activates CHK1, however crosstalk between the two pathways exist (35). CHK1 is important for the fast phase of the cell cycle arrest and inhibits protein phosphatase CDC25 and activates protein kinase WEE1, thereby increasing the phosphorylation of CDKs and repressing their activity (95-97). CHK2 (but also CHK1) is important for the slower phases of the cell cycle arrest and together with ATM stabilizes p53 by post translational modifications (98, 99). P53 acts as a transcription factor and upregulates, among others, the expression of p21 which acts as a more stable repressor of CDK activity (100, 101).

After DNA is damaged, PLK1 is rapidly inactivated in an ATM dependent manner and subsequently degraded by APC/C/Cdh1 signalling pathway, which becomes activated in G2 by DNA damage. In turn ATR activation leads to degradation of Bora, which is needed for PLK1 activation (102-105). PLK1 inactivation ensures a time frame in which cells can repair their damaged DNA and PLK1 reactivation is required for recovery from a DNA damage induced G2 checkpoint (19, 106). The timing of cell cycle restart depends on the balance between the DNA damage signalling and PLK1 stimulation of the cell cycle. Rapid reversal of ATM phosphorylations by the phosphatase WIP1 allows reactivation of PLK1 and subsequent re-entry into the cell cycle (106). In the case of extensive damage the expression of p53 and its transcriptional target p21 can lead to a permanent cell cycle arrest or apoptosis (107, 108).

## **The DNA damage response and cancer**

Defects in the DNA damage response or high DNA damage load can lead to genomic instability. Genomic instability is important for tumour development and is considered one of the “enabling characteristics” that leads to cancer (109). According to the model of multistep tumorigenesis, cells have to acquire several genetic changes that would turn a normal cell into a cancer cell (110, 111). In addition to genetic alterations, non-mutational changes such as changes in expression, can also be one of the steps towards cancer (112). After every subsequent alteration clonal expansion of the cell will lead to a population of cells in which the next step could occur.

Normally the rate of spontaneous genetic alterations is very low, leading to a long lag time until a given individual develops cancer. The rate in which one acquires mutations can be increased by elevated DNA damage load or by altered repair capacity (113). DNA damage load can be increased by external sources, such as smoking, or internal sources, including oncogene induced replication stress and improper repair of eroded telomeres (114, 115). The rate of DNA repair can be influenced by inherited genetic variation in DDR genes leading to hereditary cancer syndromes or by mutations occurring early on in these genes (116).

Evidence from hereditary cancers indeed suggests a strong link between mutations in DDR genes and cancer development. For example, BRCA1 mutations, leading to defects in homologous recombination, are strongly associated with increased breast cancer risk, while mutations in mismatch repair are associated with early onset of intestinal cancer (117-119). Interestingly loss of 53BP1 can partly rescue the repair defects seen in BRCA1 deficient cells (120).

MRN complex has known roles in cancer and cancer development. Carriers of the allele for Nijmegen breakage syndrome have increased risk of developing cancer, while for ATLD

patients this has not yet established (121). Mice models recapitulating the human syndromes do not directly show increased tumour incidence, but Nijmegen breakage syndrome mutations in combination with p53 mutations do (122, 123). In addition MRN is important for telomere maintenance in tumour cells by stimulating telomerase activity and enabling alternative lengthening of the telomeres (67, 124). Therefore inhibition of MRE11 can lead to a crisis by shortening the telomeres.

DNA damage and the DDR are common targets of cancer treatment. Radiotherapy and various types of the conventional chemotherapeutics induce DNA damage, thereby targeting highly proliferating and DNA repair-deficient cells (125). More recent therapies exploit the fact that many cancers have defects in certain DNA repair pathways which are often compensated by other repair pathways. Inhibiting these other repair pathways can therefore more specifically target the cancer cells (126). The first of such targeted therapy are PARP inhibitors, which disrupt base excision repair and are approved for treatment of breast cancers deficient in DNA double strand break repair due to BRCA1 and 2 mutations. Inhibitors for other repair factors such as MRE11 are currently in development (125).

PARP inhibitors trap PARP onto the DNA and thereby inhibit single strand break repair and increase replication stress and fork stalling (127-131). The toxicity from stalled replication forks comes largely from MRE11 mediated degradation of these stalled forks. Patients with BRCA1 and BRCA2 mutations have defective homologous recombination and increased MRE11 dependent degradation of stalled replication forks (69, 127, 132, 133). Resistance to PARP inhibitors can occur when BRCA deficient cells reduce fork degradation and or restore their ability to perform homologous recombination. Loss of PTIP reduces the recruitment of MRE11 to stalled replication forks and is one mechanism in which fork degradation can be reduced (134). The balance between BRCA1 and 53BP1 regulates the choice between



homologous recombination and NHEJ after a DNA double strand break (52). 53BP1 deficiency can restore homologous recombination in BRCA1 negative cells and these tumour cells also reduce their sensitivity to parp inhibition (52, 120, 135, 136).

### **The R2TP complex**

For proper assembly and function, repair complexes often require interaction with chaperones and co-chaperones. In the absence of these chaperones incorrect assembly of protein complexes is associated with their instability. The R2TP complex is a HSP90 co-chaperone involved in the DNA damage response. Established targets of the R2TP complex include: RNA polymerase II, the snoRNPs, snRNPs, ECD, and, important for DNA damage signalling, the phosphatidylinositol 3-kinase related kinase family (PIKKs) including ATM, ATR and DNA-PK (137-141). The complex consists of four main subunits; PIH1D1 and RPAP3 that are specific for the complex and RUVBL1 and RUVBL2, which can be found in many cellular protein complexes (142). The R2TP complex interacts with the prefoldin-like complex, which consists of the prefoldin and prefoldin-like proteins PFDN2, PFDN6, UXT, WDR92, URI and PDRG1 (143).

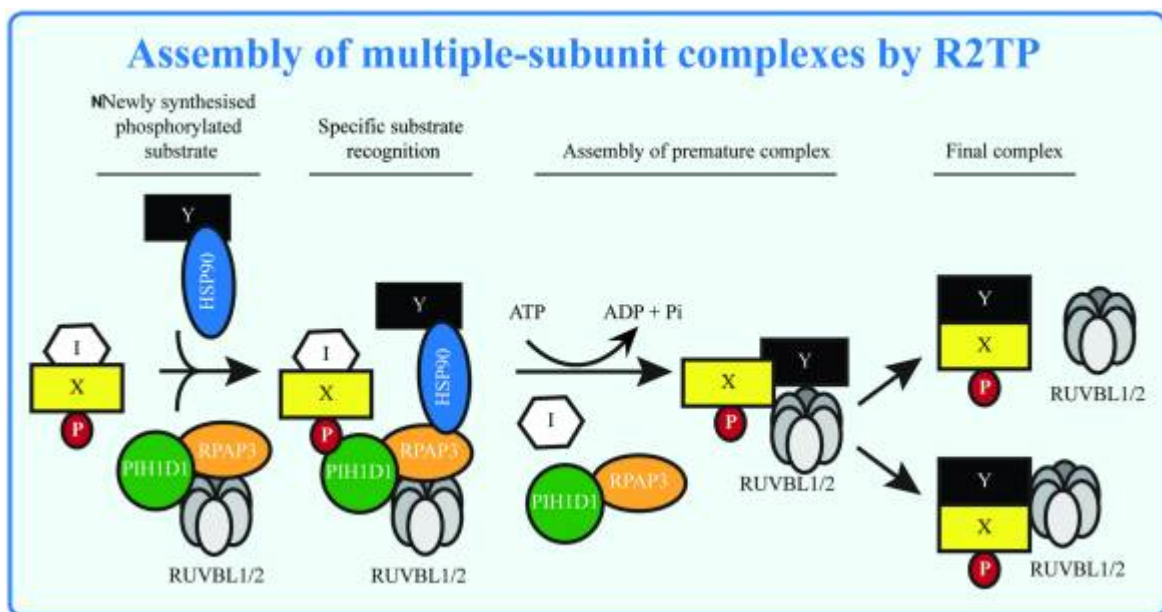
RUVBL1 and RUVBL2 belong to the AAA+ ATPases family and possess both ATPase and helicase activity (144). RUVBLs have been reported to form both hexamers and dodecamers and changing between these different conformations could provide them with chaperone like activity (145). RPAP3 has two tetratricopeptide domains that are important for binding HSP90 and it has been suggested that this interaction is needed to keep PIH1D1 stable (142, 146). PIH1D1 contains a basic N terminal phosphor peptide binding domain (PIH-N domain) that can bind to TEL2 and potentially other substrates (147).

## **R2TP complex working mechanism**

Currently there is a limited understanding of how the R2TP complex is aiding in complex assembly (see figure 7). In order to carry out its function the R2TP complex has to recognize its substrates. PIH1D1 is the most likely R2TP complex subunit which recruits the R2TP complex to its substrates via the PIH-N phosphor-binding domain. The consensus binding sequence of PIH-N domain is phosphorylated acidic motif DpSDD (147). Binding of the PIH-N domain to TEL2 recruits the R2TP complex to the PIKKs and is needed for the stability of SMG1, mTOR and other PIKKs. Other substrates interacting with the PIH-N domain are ECD, the U5 snRNP subunit SNRP116 and RNA polymerase II subunit RPB1. However, PIH1D1 was not essential for the interaction between SNRP116 or ECD and the other R2TP complex subunits (140, 141, 147). Therefore R2TP complex binding with its substrates is likely to be dependent on combined binding of the PIH-N domain and other interaction surfaces provided either by PIH1D1 or the other R2TP complex components.

Casein kinase 2 (CK2) phosphorylates serines and threonines in acidic motifs and it is likely that CK2 phosphorylates many of the PIH1D1 binding sites. Indeed, the TEL2 site responsible for PIH1D1 binding is phosphorylated by CK2 (137). CK2 is considered a promiscuous kinase that is constitutively active and phosphorylates hundreds of substrates. Therefore CK2 is important for numerous cellular processes including the cell cycle and the DNA damage response. Sites phosphorylated by CK2 often form protein–protein interaction motifs that are, among others, critical for regulation of DNA damage response pathways (137, 147-151). For example CK2 mediated phosphorylation of NBS1, following a priming phosphorylation by PLK1, enables interaction between NBS1 and RAD51 and thereby stimulates homologous recombination (150).

Once binding to its substrates, the R2TP complex may serve to recruit HSP90 to them. Nevertheless, it becomes clear that the R2TP complex often functions independently of HSP90. Studies on snoRNP assembly suggest that the R2TP complex could be bringing and/or loading the RUVBLs onto its target complexes (152). This interesting hypothesis is supported by the fact that the RUVBLs within one of the potential target complexes (INO80) have been shown to poses chaperone like activity (145). Additionally, the RUVBLs, together with the other R2TP complex components, are able to remove inhibitory proteins from intermediate complexes, thereby allowing the assembly of the substrate complexes (153).



**Figure 7. R2TP complex functions.** The R2TP complex recognizes its substrates through the PIH-N phosphor binding domain in PIH1D1. The R2TP complex brings proteins to its substrates, removes inhibitory factors from pre-complexes and can load the RUVBLs on its targets. (From (154))

Assembly of the R2TP complex itself is regulated as well. Nutrient starvation leads to relocation and disassembly of the R2TP complex and this directly affects snoRNP and mTORC1 assembly and activity (155, 156). Since snoRNPs are important for ribosome

biogenesis, and mTORC1 is a central player in regulating cellular metabolism (156-158), the R2TP complex plays a role in the cellular stress response upon starvation. If the R2TP complex can be regulated by other means, such as DNA damage, has yet to be determined.

### **The R2TP complex and the DNA damage response**

The R2TP complex stabilizes the phosphatidylinositol 3-kinase related kinase family members and is therefore important for DNA damage signalling. Interestingly, replacing endogenous PIH1D1 with a PIH1D1 binding mutant unable to bind phosphorylated substrates decreases overall levels and phosphorylation of p53, a central protein in the DNA damage response, at time points when levels of ATM and DNA-PK are still unaffected (137). This suggests that the R2TP complex is able to regulate the activity of DNA-PK and ATM or that it could be important for other parts of the DNA damage response.

The chromatin remodelling complexes TIP60, INO80 and SWI/SCRAP all contain RUVBL proteins and are associated with the DNA damage response (159, 160). The role of the RUVBLs could lie within the assembly of these complexes, but whether PIH1D1 and the R2TP complex are involved in the loading of the RUVBLs to these complexes, is currently unknown (145, 154). The same applies to the DNA inter-strand cross link repair Fanconi Anaemia Core complex, which also interacts with the RUVBL proteins (161).

Identification of the consensus binding sequence of PIH1D1 allows prediction of other substrates of the R2TP complex within the DNA damage response. Possible other targets that could influence p53 phosphorylation includes ECD and the E3 ubiquitin ligase UBR5. Both contain PIH1D1 binding sites, ECD stabilizes p53 by preventing it from binding to its negative regulator MDM2 and UBR5 regulates signal transduction in the DNA damage response (162, 163). ECD interacts with the R2TP complex but this interaction is not strictly dependent on PIH1D1 binding and its function might not be affected by mutating the PIH1D1

binding site (141). Last but not least the MRN complex component MRE11 contains at least three potential PIH1D1 binding sites and loss of the C terminal part, which contains one of them, results in MRN complex stability. PIH1D1 could therefore affect DNA damage signalling by regulation of MRN complex assembly.

## 2. Aims

The DNA damage response protects genomic stability and prevents development of diseases such as cancer. 53BP1 and the MRN complex are important for the repair of DNA double strand breaks. Quality- and assembly-control of protein complexes and protein localisation are pivotal for the function of the proteins involved in DNA damage response. The aim of this thesis was to increase our understating of the DNA damage response by investigating the suggested role of the R2TP complex in MRN complex assembly and function. In addition we explored the processes that regulate localisation of DNA repair protein 53BP1 to the nucleus and to the sites of DNA damage.

The particular aims of this thesis are the following;

1. To determine the role of the R2TP complex in MRN complex assembly (main objective):
  - 1.1 Confirm interaction between the R2TP complex and MRN complex
  - 1.2 Examine the importance of the R2TP complex for MRN complex stability
  - 1.3 Investigate the role of the R2TP complex in MRN mediated repair
2. To investigate regulation of the cellular localisation of 53BP1 and its recruitment to sites of DNA damage:
  - 2.1 Identify the nuclear localisation signal of 53BP1
  - 2.2 Examine ways in which post translational modifications can affect 53BP1 localisation
  - 2.3 Investigate if the nuclear import and recruitment of 53BP1 to sites of DNA damage is regulated during the cell cycle

### 3. Research papers and manuscripts

MRE11 stability is regulated by CK2-dependent interaction with R2TP complex; **von Morgen P**, Burdová K, Flower TG, O'Reilly NJ, Boulton SJ, Smerdon SJ, Macurek L, Hořejší Z; **Oncogene** 2017 Aug 24;36(34):4943-4950

Substrate recognition and function of the R2TP complex in response to cellular stress; **von Morgen P**, Hořejší Z, Macurek L; **Frontiers in Genetics** 2015 Feb; 6:69

Nuclear import of 53BP1, discovery of 53BP1s NLS and regulation of nuclear import **von Morgen P**, Lidak T, Hořejší Z, Macurek L; **Submitted manuscript**

Polo-like kinase 1 inhibits DNA damage response during mitosis. Benada J, Burdová K, Lidak T, **von Morgen P**, Macurek L. **Cell Cycle** 2015 Jan; 14(2):219-231

## **MRE11 stability is regulated by CK2-dependent interaction with R2TP complex**

**von Morgen P**, Burdová K, Flower TG, O'Reilly NJ, Boulton SJ, Smerdon SJ, Macurek L, Hořejší Z; **Oncogene** 2017 Aug 24;36(34):4943-4950

In this publication we addressed the main aim of this thesis. We showed that the R2TP complex is important for the MRN complex stability and function. We uncovered that the R2TP complex binds via PIH-N domain to two phosphorylated acidic motifs within the C-terminus of MRE11. The acidic site within MRE11 containing phosphorylated serine 688 and 689 was found to be most important for the observed interaction with PIH1D1 with an additional smaller contribution of the acidic site containing phosphorylated serine 558 and S561. We identified CK2 as the target kinase phosphorylating serine 688 and 689. We found that long-term depletion of PIH1D1 decreases stability of the MRN complex components. ATLD patients with a C-terminal truncating mutation in MRE11 (ATLD1) miss one of these PIH1D1 binding sites, making the MRN complex unstable. Our findings offer for the first time a rationale for the MRN instability observed in these patients.

In accordance with the lower levels of the MRN complex after PIH1D1 depletion, we observed defects in repair of DNA double strand breaks and removal of TOPOII adducts after etoposide poisoning, two processes in which the MRN complex plays an important role.

We conclude that the R2TP complex is important for the MRN complex stability and function and that the MRN complex instability in ATLD1 patients can be explained by reduced interaction with the R2TP complex

P. von Morgen designed and performed most of the experiments, created the figures and was actively involved in writing the manuscript.



## **Substrate recognition and function of the R2TP complex in response to cellular stress**

von Morgen P, Hořejší Z, Macurek L; **Frontiers in Genetics** 2015 Feb; 6:69

In this review we describe the current knowledge of the function of the R2TP complex and its four main components PIH1D1, RPAP3, RUVBL1 and RUBL2. We describe the structure of the yeast and the human R2TP complex. We discuss its role in the assembly of snoRNPs, RNA polymerase II and PIKKs, which are known substrates of the R2TP complex. We describe the PIH-N phospho-peptide binding domain and the identified consensus binding sequence. Based on the consensus PIH-N binding motif and on the presence of RUVBLs in many protein complexes we continue to speculate on other potential substrates of the R2TP complex that remain to be confirmed. Both with the known substrates and potential substrates we focus on protein complexes that play a role in the DNA damage response and cancer. We conclude with a list of questions on R2TP complex function and working mechanism that still needs to be answered.

P von Morgen wrote the manuscript and created most of the figures.

## **Nuclear import of 53BP1, discovery of 53BP1s NLS and regulation of nuclear import**

**von Morgen P**, Lidak T, Hořejší Z, Macurek L; submitted manuscript

In this manuscript we discovered that 53BP1 binds to importin  $\alpha$  and has a classical nuclear localisation signal. We identified the nuclear localisation signal as a bipartite NLS 1666-**GKRKLITSEEERSPAKRGRKS**-1686 with both basic stretches (in bold) important for nuclear import of 53BP1. The bipartite NLS is solely sufficient for efficient nuclear import since a GFP construct containing 1658-RASMGVLSG**KRKLITSEEERSPAKRGRKSA**-168 localizes exclusively to the nucleus. Since several sites within the NLS have been reported to be phosphorylated in mass spectrometry screens, we explored potential regulatory mechanisms of the nuclear import of 53BP1. An especially interesting posttranslational modification within 53BP1 NLS is S1678 phosphorylation, which we found to be phosphorylated by cyclinB-CDK1 *in vitro* and which is reported to be phosphorylated in mitosis by mass spectrometry screens. We found that phospho-mimicking mutation of this site strongly decreases the interaction with the importins and leads to decreased nuclear localisation of 53BP1. In addition, we tested the effect of acetylation of K1667 by mutating this site and we found that even very mild alterations of this site severely decreases the nuclear localisation of 53BP1.

P von Morgen performed the majority of the experiments and wrote the manuscript.

## **Polo-like kinase 1 inhibits DNA damage response during mitosis.**

Benada J, Burdová K, Lidak T, **von Morgen P**, Macurek L. **Cell Cycle** 2015 Jan; 14(2):219-231

In this article we addressed the question why the recruitment of 53BP1 is repressed during mitosis. We found that phosphorylation of 53BP1 is increased in mitosis and the phosphorylation depends on PLK1 and CDK1 activity. We showed that PLK1 phosphorylates 53BP1 *in vivo* on S1618 and that CDK1 can phosphorylate T1609 and S1678. S1618 and T1609 are located within the UDR domain of 53BP1, which is important for binding of 53BP1 to ubiquitinated histones. 53BP1 with T1609 and S1678 substituted by the phospho-mimicking Aspartic acid leads to decreased binding to ubiquitin and reduced 53BP1 recruitment to the sites of DNA damage in G1 cells. Similarly, inhibition of PLK1 and therefore reduction of S1618 phosphorylation in mitosis, increases the repair capability of mitotic cells although not completely.

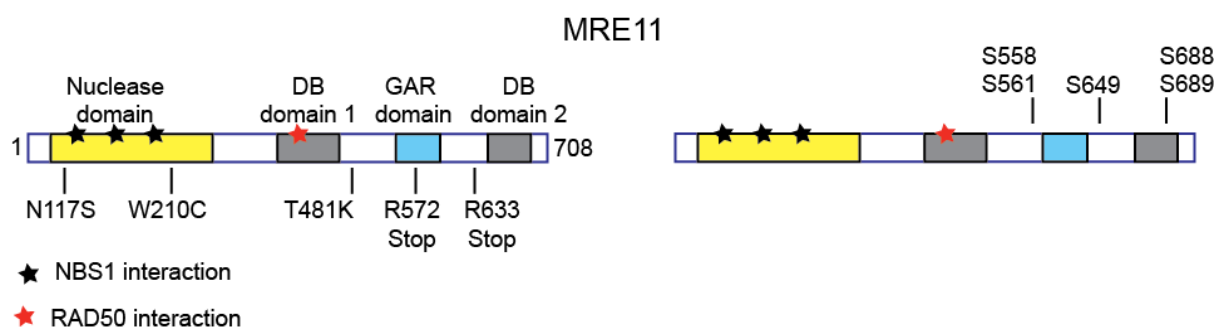
We conclude that 53BP1 recruitment and function is suppressed during mitosis by PLK1 and CDK1 phosphorylation in 53BP1 UDR domain.

P von Morgen purified proteins for the *in vitro* experiments.

## 4. Discussion

### ATLD mutations lead to MRN complex instability

The importance of the MRN complex for the repair of DNA double strand breaks has been clearly established and mutations in MRN complex components lead to cancer-prone syndromes associated with DNA repair defects. Mutations in *MRE11* are the genetic cause of ATLD, a rare autosomal recessive disorder associated with cerebellar degeneration, genome instability and radiosensitive cells (75, 77). Several distinct mutations in *MRE11* that affect patient health have been previously described (see figure 8). For some of these mutations the functional effect is known: truncating mutation of *MRE11* at R572 leads to nonsense mediated decay of the transcript and N117S and W210C mutations that are located in the *MRE11* nuclease domain affect *MRE11* interaction with NBS1 (75, 164-166). Truncation of *MRE11* from R633 (known as ATLD1) leads to MRN complex instability in both human patients and a mouse model, but *MRE11* binding to RAD50 and NBS1 is unaffected by this mutation (75, 123). In our study we provide for the first time a rationale why ATLD1 truncating mutation leads to MRN complex instability through the loss of the MRN complex interaction with the R2TP chaperone complex.



**Figure 8. ATLD mutations in *MRE11*.** The domain organization of *MRE11* is depicted. The N-terminus contains the Nuclease Domain and is important for the interaction with NBS1. The middle and C-terminus contain two distinct DNA binding domains and a Glycine Arginine rich motif (GAR domain). ATLD mutations are depicted on the left and acidic regions that could function as PIH1D1 binding sites on the right. Notice that two of these binding sites are missing in the R633Stop mutation (ATLD1)

### **The stability of the MRN complex depends on a C-terminal PIH1D1 binding site**

The C-terminus of MRE11 from residues 633 to 708 contains two important features: the second DNA binding domain and a CDK2 binding site (72). The CDK2 binding site regulates MRE11 nuclease activity in a cell cycle dependent manner. In addition, Serine 676 and Serine 678 that are located within this region can be phosphorylated by ATM and ribosomal S6 kinase and the phosphorylation affects the processing of the DNA double strand breaks by the MRN complex (167). Importantly, within the C-terminus of MRE11 are also localized two acidic motifs that resemble PIH1D1/R2TP complex binding sites.

Our initial data using isothermal titration calorimetric analysis revealed that binding between PIH-N domain and phosphorylated S558/561 was stronger than interaction between PIH-N domain and phosphorylated S688/689. Similarly, the binding of PIH1D1 to pS558/561 appeared to be stronger than the binding to pS688/689 in a truncated variant of MRE11 consisting out of the C-terminal part of MRE11 containing the three potential R2TP complex binding sites (MRE11 537-708). However, the interaction between full length MRE11 and PIH1D1 was mostly dependent on phosphorylated S688/689, suggesting that in the context of the full protein, S688/689 is either more phosphorylated or better accessible for PIH1D1 binding. While S688/689 is missing, S558/561 is present in the ATLD1 cells. The additional binding of PIH1D1 to S558/561 may be important in maintaining the low levels of MRN complex observed in ATLD1 patients and preventing complete degradation of the MRN complex.

The R2TP complex is important for protein complex assembly and incorrect assembly of protein complexes is often associated with their instability. To test the importance of the R2TP complex for MRN complex stability we depleted PIH1D1 using siRNA. From previous studies it has become clear that PIH1D1 has to be depleted for several days to very low levels

in order to observe an effect on the stability of its substrates (137). We have observed a major decrease of MRE11 levels and a slight decrease of NBS1 and RAD50 levels after prolonged (7-10 days) treatment with PIH1D1. The PIH1D1 siRNA effects can be rescued by expressing WT PIH1D1 but not by expressing a mutation of PIH1D1 that is unable to bind the phosphorylated substrates. To be able to see better effect on stability of all MRN complex subunits, we would have to treat the cells with PIH1D1 siRNA for longer time, however the 10 days treatment is the maximum time the cells can survive with PIH1D1 downregulation. Alternatively a CRISPR-mediated PIH1D1 knock-out could reveal a stronger effect on stability of MRN complex subunits, however PIH1D1 is an essential gene and therefore the knock-out cells are not viable (168).

The R2TP complex has been shown to be important for both the assembly and stability of many different complexes including RNA polymerase II and the snoRNPs which are important for ribosomes (154). It is therefore possible that the effect we see on MRN complex stability is an indirect consequence of an incorrect assembly of other R2TP complex substrates. However, the levels of other proteins used in our experiments as loading controls stay constant after the prolonged PIH1D1 knock down and therefore we think that the reduced levels of the MRN complex components are not a consequence of reduced protein synthesis. However, to exclude these indirect effects we decided to study the importance of this interaction by replacing endogenous MRE11 with GFP tagged wild type MRE11 protein or GFP tagged MRE11 mutated at the two R2TP interaction sites. After we depleted the endogenous MRE11 by siRNA from cells stably transfected with GFP MRE11 constructs, we saw reduced stability of GFP MRE11 with S688/689 or MRE11 with S558/561 and S688/689 mutated to alanine but not in cells expressing WT GFP MRE11 or MRE11 with only S558/561 mutated to alanine. GFP MRE11 could be stabilized by dimerizing with the

endogenous MRE11, allowing interaction with R2TP, in cells that are not treated with siMRE11.

Interestingly transfection with control siRNA also leads to decreased stability of GFP MRE11 S688/689A, leading us to speculate that the MRN complex instability could be a combined consequence of incorrect assembly and cellular stress. Indeed we observed more  $\gamma$ H2AX foci in cells treated with the control siRNA suggesting that the RNAi mediated transfection of siRNA could cause DNA damage and cellular stress.

The R2TP complex is important for the assembly of the PIKKs and thus for DNA repair and DNA damage signalling, To test if the R2TP complex is also important for DNA repair via regulation of assembly of the MRN complex, we assessed two MRN complex-regulated repair pathways in the presence and absence of PIH1D1. The first one, TOP2-adducts removal from DNA, may be considered a direct function of the MRN complex that mainly occurs upstream of PIKK activation. The second one, homologous recombination, is also directly regulated by ATM and ATR. As can be expected from the decreased stability of the MRN complex after PIH1D1 depletion, we find that both repair pathways are affected, however the effect on homologous recombination can be seen already before the levels of the MRN complex are significantly decreased. These results indicate that PIH1D1 could have other roles in regulation of homologous recombination or it is already important for MRN complex or ATM/ATR function before their instability can be observed.

There are some indications that MRN complex function could be affected by PIH1D1 depletion before the stability of the MRN components are affected. R2TP is a HSP90 co-chaperone and a recent paper describes an increase in interaction between the MRN complex and HSP90 shortly after irradiation, suggesting that HSP90 and maybe also the R2TP complex could be important for the acute DNA damage response (169). Therefore the quality

of the assembly of the MRN complex could already be affected before the complex becomes unstable.

Our work revealed that the phosphorylation of S688/689 is dependent on CK2. Others have reported that S688 phosphorylation depends on combined action of CK2 and PLK1, with PLK1 phosphorylating MRE11 at S649 and priming it for CK2 phosphorylation at S688 (170). However, with our antibody that recognizes the doubly phosphorylated S688/689 we were unable to reproduce these results (unpublished results). It could therefore be that the double phosphorylation on S688/689, which we believe is required for PIH1D1 binding, is regulated differently from phosphorylation of the single S688 site.

Taking everything together, we believe that CK2 mediated phosphorylation of S688/689 and subsequent binding of the R2TP complex is important for the stability of the MRN complex, possibly due to R2TP complex involvement in MRN complex assembly. These findings are in agreement with the fact that a truncating mutation of MRE11, which leads to loss of S688/689, causes MRN complex instability in ATLD1 patients. The presence of an additional R2TP complex binding site preserved in ATLD1 patients could provide weak interaction with PIH1D1 and help to maintain low levels of the MRN complex, required for patient survival. The role of the R2TP complex lies in the assembly and/or quality control of protein complexes, but further studies would be needed to determine how the R2TP complex acts on the MRN complex.

### **Novel R2TP complex substrates**

We identified the MRN complex as a novel substrate of the R2TP complex. In recent years it is becoming clear that the R2TP complex is involved in the assembly/quality control of many protein complexes. Loading the RUVBLs during snoRNP assembly suggests that the R2TP complex could function to load the RUVBLs onto other protein complexes (152). Work on



the INO80 complex shows that the RUVBLs possess chaperone like activity which corresponding to R2TP complex function (145). INO80 and other RUVBL-containing complexes have so far not been identified as R2TP complex substrates and it can therefore be expected that in the coming years even more R2TP complex substrates will be identified.

### **Regulation of nuclear import of 53BP1**

Protein complex assembly and nuclear transport are essential for proper function of the DNA damage response. These processes can often be regulated depending on the cells need, making them attractive study objects. In this thesis we identify the nuclear localisation signal of 53BP1 and explore potential regulatory mechanisms of 53BP1 nuclear import. It was already known that the nuclear import of 53BP1 depends on the nuclear transporter importin  $\beta$  and nucleoporin 153 kDa (NUP153) (90, 91). Nucleoporin NUP153 forms part of the nuclear basket of the nuclear core complex (171). Nuclear pore complexes enable the transport of proteins into the nucleus; they consist out of nucleoporins that together form a ring perforating the nuclear membrane. Small molecules (below 40kDa) are able to diffuse through the nucleoporins, while bigger molecules, such as 53BP1, require active transport by nuclear transporters which use a RANGTP gradient to provide the energy for directed transport (172).

We have discovered that 53BP1 has a classical bipartite nuclear localisation signal, 1666-**GKRK**LITSEEERS**PAKRGRKS**-1686, which enables the binding of importin  $\alpha$  binding. Importin  $\alpha$  functions as an adaptor protein that recognizes the cargo of importin  $\beta$  (92-94). We identified that acetylation and phosphorylation within the nuclear localization signal region can regulate 53BP1 nuclear import. Lysine 1667 is located within the nuclear localization signal and it has been found to be acetylated in mass spectrometry screens and its mutation significantly reduces nuclear localisation of 53BP. Similarly, phospho-mimicking

mutation of 53BP1 Serine 1678, a site reported to be phosphorylated by CDK1, resulted in reduced nuclear import of 53BP1.

Since the nuclear envelope is not present in mitosis, CDK1-mediated phosphorylation of 53BP1 likely does not affect function of 53BP1 in mitosis. However, early in the subsequent G1, 53BP1 is needed to localize to the sites of endogenous DNA damage that occur during unperturbed mitosis (OPT bodies) (173). Therefore, we propose that S1678 needs to be rapidly dephosphorylated at mitotic exit to allow efficient transport of 53BP1 to the nucleus. At present we can only speculate which phosphatases might be involved in this process. In general, PP2A protein phosphatase activity is very high during mitotic exit and is able to efficiently remove the majority of phosphorylations executed by CDK1 (174, 175). Another candidate is PP4, which has been shown to associate with 53BP1 and remove S1618 during mitotic exit (176). More work will be needed to validate which phosphatase is responsible for dephosphorylation of 53BP1 to allow its efficient nuclear transport.

### **Polo-like kinase 1 inhibits 53BP1 recruitment to DNA double strand breaks in mitosis**

During mitosis DNA repair is suppressed (177). The molecular mechanisms behind these observations are only recently uncovered and it appears that sites of DNA damage are still marked by the DNA damage response during mitosis but only repaired in the subsequent interphase (178, 179). The repair of double strand breaks by NHEJ is inhibited by various mechanisms. For example the function of XRCC4, important for the final ligation step in NHEJ, is inhibited by CDK1 and PLK1 phosphorylations (180). In addition the recruitment of 53BP1 to the sites of double strand breaks is impaired during mitosis.

We and others have identified phosphorylation of 53BP1 UDR domain on S1609 and S1618 by PLK1 and CDK1 as one of the mechanisms responsible for the inhibition of 53BP1 recruitment in mitosis (176, 181, 182). These phosphorylations are subsequently removed by

PP4C/R3 $\beta$  phosphatase during interphase to allow 53BP1 recruitment to the (already marked) sites of double strand breaks (176). However, even though the 53BP1 S1609A and S1618A mutant is able to localize to the site of double strand breaks in interphase cells it still does not localize to DNA damage foci in mitosis (unpublished results and (181)). Therefore there must be an alternative mechanisms for inhibiting 53BP1 recruitment in mitosis.

These alternative mechanisms could be affecting the levels of ubiquitination at the site of DNA double strand breaks during mitosis. When RNF8 and RNF168 recruitment to sites of DNA damage is inhibited by PLK1 and CDK1 activity and at the same time deubiquitinating enzymes are active (183-186). Indeed, when both RNF8-T198A and 53BP1-T1609A-S1618A are introduced into cells, the mutated 53BP1 can localize to the sites of DNA damage (181).

Restoring of 53BP1 localization to the sites of DNA damage in mitosis leads to hypersensitivity to irradiation in mitosis and genomic instability (176, 181). In addition these cells have increased rates of sister telomere fusion (181). Telomeres, the ends of the chromosomes, resemble DNA double strand breaks and the shelterin complex normally protects them against activation of the DDR (187). Remarkably, during prolonged mitosis the shelterin complex dissociates from the telomeres and the DDR gets activated (188).

To summarize, there are multiple ways to suppress NHEJ and the formation of 53BP1 foci in mitosis and the suppression of NHEJ in mitosis is important to protect genomic stability and prevent telomere fusion. Cells achieve this by a multitude of mechanisms with major roles for CDK1 and PLK1: these kinases phosphorylate and inhibit 53BP1, XRCC4 and RNF8. Major questions that remain to be answered include why and how mitotic telomeres get de-protected and if additional mechanisms exist to suppress the activation of the DDR in mitosis.

## **5. Conclusions**

In this thesis we determined a CK2 mediated interaction between the R2TP and MRN complex and we showed that the R2TP complex is important for MRN complex stability and MRN mediated repair. We determined the nuclear localization signal of 53BP1 and we found that posttranslational modifications can regulate 53BP1 localisation. Furthermore, we found that CDK1 and PLK1 mediated phosphorylations of 53BP1 UDR domain inhibit the recruitment of 53BP1 to sites of DNA damage. This work therefore brought new insights into the function and regulation of the highly important DNA damage repair factors MRN complex and 53BP1.



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## **7. Supplement**

**R2TP complex is important for MRN complex stability**

**Review-substrate recognition and function of the R2TP complex**

**Nuclear import of 53BP1, discovery of 53BP1s NLS and regulation of nuclear import**

**Polo-like kinase 1 inhibits DNA damage response during mitosis**