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Natálie Palková

Role of the MRN complex in the nucleolar DNA damage response

Úloha MRN komplexu v odpovědi na poškození jadérkové DNA

Bachelor thesis

Supervisor:

MUDr. Libor Macůrek, PhD.

Prague, 2021

Declaration of authorship

I hereby declare that I am the sole author of this bachelor thesis „Role of the MRN complex in the nucleolar DNA damage response“. I have mentioned all the sources used and that I have cited them correctly according to established academic citation rules. I further declare that I have not submitted this thesis neither its substantial part at any other institution in order to obtain a degree.

Prague, 4.5. 2021 Natálie Palková

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Abstrakt

Zachování integrity genomu je zásadní pro správné fungování a přežití všech organismů, a to zejména, je-li buňka konstantně vystavena nejrůznějším genotoxickým vlivům. Z tohoto důvodu existují mechanismy, které detekují poškození DNA, zajišťují signalizaci a podporují opravu postiženého místa. Tyto mechanismy se souhrnně označují jako odpověď na poškození DNA (DDR). Nezbytnou součástí těchto procesů je také MRE11-RAD50-NBS1 komplex (MRN) složený z proteinů, jako je nukleáza MRE11, ATPáza RAD50 a regulační adaptérový protein NBS1. MRN komplex má nezastupitelnou roli v detekci a časné resekci dvouřetězcových zlomů (DSBs), přenosu signálu, aktivaci ataxia telangiectasia mutated (ATM) kinázy a následných efektorů potřebných pro DDR. Složky MRN komplexu jsou zapojeny v procesech stěžejních pro přesnou opravu poškození DNA, přežití buňky a zachování stability genomu.

Hlavním cílem této práce je popsat méně známou úlohu MRN komplexu v jadérku, jaderné organele bez membránového obalu, která se zakládá kolem kopií genů kódujících rRNA. V této práci je rozebráno, jakým způsobem je MRN komplex transportován do jadérka, jak figuruje v opravě dvouřetězcových zlomů rDNA, dočasném zastavení transkripce rRNA nebo v segregaci jadérka. Práce dává do souvislostí nejnovější poznatky v oblasti jadérkové odpovědi na poškození DNA (n-DDR) a zdůrazňuje důležitost celého MRN komplexu v odpovědi na poškození jadérkové DNA. Tato pozorování jsou v kontrastu s původní představou, že následkem poškození rDNA se jaderný MRN komplex rozpadá a v jadérku působí pouze NBS1, zatímco MRE11 a RAD50 zůstávají v nukleoplasmě.

Na základě nejnovějšího poznání se MRN komplex zásadním způsobem uplatňuje v odpovědi na poškození jadérkové DNA a pomáhá zachovávat stabilitu genetické informace buňky. Současné znalosti, uspořádané v této práci, přinášejí další otázky týkající se transportu DDR faktorů do jadérka, modifikací jadérkového chromatinu nebo případné role fosfatáz v n-DDR a otevírají nová zákoutí výzkumu DDR i jadérka obecně.

Klíčová slova: n-DDR, MRN komplex, poškození rDNA, TCOF1, segregace jadérka

Abstract

Genome integrity maintenance is crucial for proper functioning and survival of all organisms, especially if the cell is constantly exposed to various genotoxic agents. For that reason, there are specific mechanisms that detect DNA damage, facilitate signalling and promote repair of the damaged region. These processes are referred to as DNA damage response (DDR). Necessary part of the DDR is also the MRE11-RAD50-NBS1 complex (MRN), comprised of the nuclease MRE11, ATPase RAD50 and regulatory docking protein NBS1. The MRN complex has an indispensable role in the detection and immediate resection of double-strand breaks (DSBs), signal transduction and activation of ataxia telangiectasia mutated (ATM) kinase with its downstream effectors necessary for the DDR. The compounds of the MRN complex are involved in processes crucial for efficient DNA repair, cell survival and maintenance of genomic stability.

The main aim of this work is to elucidate less known functions of the MRN complex in the nucleoli, nuclear membrane-less organelles formed around the copies of genes coding rRNA. This work discusses how the MRN complex is involved in the repair of rDNA double-strand breaks, transient inhibition of rRNA transcription or nucleolar segregation. Thereafter, this work puts into context the latest knowledge in the field of the nucleolar DNA damage response (n-DDR) and emphasises the importance of the entire MRN complex in response to nucleolar DNA damage. These findings are in contrast with the previous opinion that upon rDNA damage, the nuclear MRN complex falls apart and only NBS1 operates in the nucleolus while other components, MRE11 and RAD50, remain in the nucleoplasm.

Based on the recent research, the MRN complex makes a significant contribution in the nucleolar DNA damage response and maintains the stability of the genetic information of the cell. Current knowledge, reviewed in this work, raises new questions about transport of DDR factors into the nucleolus, modifications of nucleolar chromatin or possible role of phosphatases in the n-DDR, and opens new possibilities for the research of the n-DDR and also nucleolus in general.

Keywords: n-DDR, MRN complex, rDNA damage, TCOF1, nucleolar segregation

Abbreviations

4-OHT – 4-hydroxytamoxifen
53BP1 – p53 binding protein 1
5-EU – 5-ethynyluridine
a-EJ – alternative end joining
ABC – ATP binding cassette
ActD – actinomycin D
ADAR2 – adenosine deaminases that act on RNA
ADP – adenosine diphosphate
APE1 – apurinic/apyrimidinic (AP) endonuclease
ARF – ADP-ribosylation factor
ATLD – ataxia telangiectasia like disorder
ATM – ataxia telangiectasia mutated
ATP – adenosine triphosphate
ATR – ataxia telangiectasia and Rad3-related protein
ATRIP – ATR interacting protein
BER – base excision repair
BIR – break-induced replication
BLM – Bloom syndrome helicase
BRCA1 – breast cancer associated 1
BRCA2 – breast cancer associated 2
BRCT – BRCA1 C-terminal
CARM1 – coactivator-associated arginine methyltransferase 1
Cas9 - CRISPR associated protein 9
cDNA – coding DNA
ChIP – chromatin immunoprecipitation
CHK1 – checkpoint kinase 1
CHK2 – checkpoint kinase 2
CK2 – casein kinase 2
CPDs – cyclobutane pyrimidine dimers
CtIP – CtBP interacting protein
CtBP – C-terminal binding protein
DDR – DNA damage response
DFC – dense fibrillar component
DJ – distal junction
DNA – deoxyribonucleic acid
DNA-PK – DNA-dependent protein kinase
DNA-PKcs – DNA-dependent protein kinase, catalytic subunit
DSB – double-strand break
dsDNA – double-stranded DNA
ER – estradiol receptor
EXO1 – exonuclease 1
FC – fibrillar centre
FHA – fork-head associated
Fig. – Figure
FISH – fluorescent *in situ* hybridization
GC – granular component
GFP – green fluorescent protein
GST – glutathione S-transferase
Gy – Gray
HA – hemagglutinin
HDAC1 – histone deacetylase 1
HP1 – heterochromatin protein 1
HR – homologous recombination
hTERT – telomerase reverse transcriptase
HUSH – human silencing hub
INB – intranucleolar bodies
I-PpoI – inducible endonuclease Physarium polycephalum
IR – ionizing radiation
KAP1 – KRAB-associated protein-1
kb – kilobase
kDa – kilodalton
LIG4 – DNA ligase 4
LINC – linker of nucleoskeleton and cytoskeleton
LMB – leptomycin B
LTGC – long-tract gene conversion
Mb – megabase
MDC1 – mediator of DNA damage checkpoint protein 1
MDM2 – mouse double minute 2 homolog
MRE11 – meiotic recombination 11 homolog 1
MRN – MRE11-RAD50-NBS1
mRNA – messenger RNA
MS – mass spectrometry
NBS – Nijmegen breakage syndrome
NBS1 – Nijmegen breakage syndrome 1
NCL - nucleolin
NCs – nucleolar caps
n-DDR – nucleolar DNA damage response
NHEJ – non-homologous end joining
NLS – nuclear localization signal
NoLS – nucleolar localization signal
NOP58 – nucleolar protein 58
NOR – nucleolar organizing region
NPM1 – nucleophosmin 1
PARP – poly (ADP-ribose) polymerase
PAXX – paralogue of XRCC4 and XLF
p53 – tumour protein 53
PJ – proximal junction
PLA – proximity ligation assay
rDNA – DNA kódující rRNA
RING – really interesting new gene
RNA – ribonucleic acid
RNA Pol I – RNA Polymerase I
RNA Pol II – RNA polymerase II
RNF20 – ring finger protein 20
RNF8 – ring finger protein 8
RPA – replication protein A
RPA34 – DNA-directed RNA polymerase I subunit
rRNA – ribosomal RNA
RT-PCR – reverse transcription polymerase chain reaction
S – svedberg
siRNA – small interfering RNA
SDSA – synthesis-dependent strand annealing
SMC – structural maintenance of chromosomes
snoRNP – small nucleolar ribonucleoprotein
SSA – single-strand annealing
ssDNA – single stranded DNA
SV40 – simian virus 40
T – transcription terminator
TAF1C – TATA box-binding protein-associated factor RNA polymerase I subunit C
TCOF1 – treacle ribosome biogenesis factor 1
TIP60 – Tat-interactive protein, 60 kD
TOPBP1 – DNA topoisomerase II binding protein 1
TRE – tetracyclin responsive element
TTF1 – transcription termination factor 1
UBF – upstream binding factor
UV – ultraviolet
WIP1 – wt-p53-induced phosphatase
WRN – Werner syndrome ATP-dependent helicase
XLF – XRCC4-like factor
XRCC1 – X-ray repair cross-complementing protein 1
XRCC4 – X-ray repair cross-complementing protein 4
XRCC5 – X-ray repair cross-complementing pro

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1. Introduction

The cell is constantly exposed to intrinsic or extrinsic DNA damage agents, and to the danger of genome instability. For that reason, specific mechanisms have been evolved to ensure the maintenance of the genetic information. In addition to DNA repair processes, the damage occurring in the genome induces the DNA damage response (DDR). DDR is a signal transduction pathway which detects DNA damage and replication stress. In response to these stress conditions, the DDR triggers orchestrated processes protecting the cell from the loss of genomic integrity. This signalling activates checkpoint, arrests the cell cycle, promotes efficient DNA repair, inhibits transcription or regulates chromatin remodelling.

The main players in the DDR are proteins of phosphatidylinositol 3- kinase-like protein kinases (PIKKs) family, ataxia telangiectasia mutated (ATM), ataxia telangiectasia and Rad3-related (ATR) protein and DNA-dependent protein kinase (DNA-PK). Both ATM and DNA-PK are activated by double-strand DNA breaks (DSBs) which can be induced by ionizing radiation (IR), such as X-ray, ATR is usually activated by single-strand breaks (SSBs) which can be generated by UV irradiation or at stalled replication forks. While DNA-PK phosphorylates a rather smaller group of targets involved in DSB end joining, ATM regulates hundreds of substrates and orchestrates DDR processes (described in Scully *et al.,* 2019). Additionally, the DDR relies on the MRE11-RAD50-NBS1 (MRN) complex which senses DSBs, facilitates immediate repair and activates ATM and ATR. ATR is recruited with its interacting partner ATRIP to ssDNA regions coated by the replication protein A (RPA) complex (reviewed in Cimprich *et al.,* 2008 and Wold, 1997). These mechanisms have been intensively studied for decades. However, only little is known about specific DDR processes in the individual nuclear compartments. Recently, there were multiple attempts to describe nucleolar DNA damage response (n-DDR) and how it differs from the canonical pathway.

Nucleoli represent another target challenged by the threat of genomic instability. rDNA contains a number of early replicating fragile sites which are unequivocally unstable and prone to inter-chromosomal recombination, accumulation of three-stranded RNA:DNA hybrids (R-loops) or potential loss of protective silent rDNA (Hage *et al.,* 2010). For that reason, nucleolar DNA damage response (n-DDR) represents a safeguard for genomic stability. n-DDR is special in some respects due to specific nucleolar architecture, different histone modifications, liquid droplet behaviour of the nucleolus, necessity of nucleolar localization signal (NoLS) or the nature of rDNA repeats themselves. Moreover, some canonical DDR factors are substituted by nucleolar proteins or gain specific nucleolar functions. Many of the n-DDR processes have not been completely elucidated until now. One example is the nucleolar role of the MRN complex which is meticulously discussed in this work.

2. DNA damage response

2.1. Biochemistry of the MRN complex

In the cell, the sensing of double-strand breaks, immediate steps of repair and signal transduction is facilitated by the MRN complex, an important DDR player comprised of the MRE11 endo/exonuclease dimer, two RAD50 ATP binding cassette proteins, and the NBS1 regulatory docking protein (Käshammer *et al.,* 2019), as is shown in **Fig. 1**. The MRN complex is among the first responders to DNA damage and has a pivotal role in the decision of which repair strategy is chosen (Shibata et al., 2014). The compounds of the MRN complex regulate both signalling and damage response to various types of cellular stress, such as DSBs, stalled replication fork, viral invasion (Stracker *et al.,* 2002) or dysfunctional telomeres (Zhu *et al.,* 2000). The MRN complex cooperates on various repair pathways.

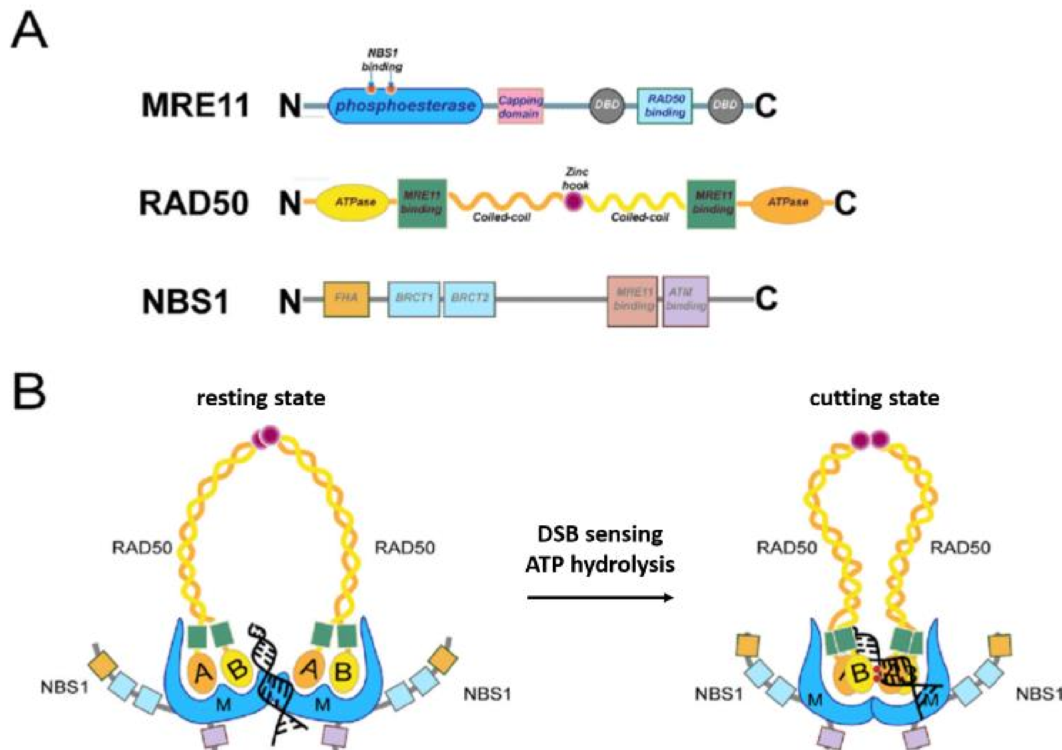

Figure 1*. Structure of the MRN complex. A – essential motifs and domains of the MRN complex subunits. MRE11 has both single strand endonuclease and double strand exonuclease activity. RAD50 contains antiparallel coiled-coil domain and dimerizes through Zinc hook domain. NBS1 possesses phosphobinding motifs and activates ATM/ATR. B – conformation of the entire complex and its structural changes. Upon ATP binding, RAD50 ATPase domains (A/B Walker motifs) form a head-to-tail dimer. This compact, rigid, and closed conformation blocks MRE11 active sites. When DSB is sensed, ATP is hydrolysed, the MRN complex switches to an open form and exposes the active sites of MRE11. Adapted and edited from Bian et al., 2019.*

While the catalytic core of the MRN complex, MRE11 and RAD50, is evolutionarily conserved among all domains of life, NBS1 (also known as Xrs2 in *S. cerevisiae*) is specific only for eukaryotes. The important role of the MRN complex in cellular physiology is underlined by the fact that mutations in the components of the MRN complex are associated with severe disorders. For instance, mutated MRE11 is associated with ataxia telangiectasia like disorder (ATLD) (Stewart *et al.,* 1999), dysfunctional

NBS1 is related to Nijmegen breakage syndrome (NBS) (Varon *et al.,* 1998) and RAD50 deficiency is the cause of the NBS-like disorder (Waltes *et al.,* 2009).

Meiotic recombination 11 homolog 1 (MRE11) operates as a DNA structure-specific endo/exo/hairpin nuclease (Lobachev *et al.,* 2002) and is the structural core of the MRN complex as it binds DNA, RAD50 and NBS1 (Liu *et al.,* 2016, Sung *et al.,* 2014 and Schiller *et al.,* 2012). RAD50 is determinative for promoting facilitated diffusion along the DNA coated with nucleosomes, while MRE11 searches for the DNA ends (Myler *et al.,* 2017). RAD50, a member of the structural maintenance complex SMC protein family, is the largest subunit of the MRN complex (de Jager *et al*., 2004).

RAD50 ATPase domains join together upon binding two Mg^{2+}-ATP molecules in their interface and block the DNA binding cleft of the MRE11 dimer (Käshammer *et al.,* 2019). Thus, ATP promotes stable head complexes with dimeric MRE11 by joining the two RAD50 ATPase domains, so DNA is not cleaved. In the cell, the MRN complex is constantly exposed to ATP but hydrolyses it only with 0.008 ATP/s/active site (Saathoff *et al.,* 2018). For that reason, the predominant conformation of the MRN complex is the ATP-binding autoinhibited resting state because MRE11 nuclease sites are blocked (Käshammer *et al.,* 2019). DNA end sensing induces large conformational changes into the cutting state, ATP is hydrolysed, and ADP bound. The coiled-coil domains of RAD50 zip up into a rod and form tight clamp around dsDNA. The MRE11 dimer moves from the auto-inhibited position, binds the DNA end and together with RAD50 assemble a DNA-cutting channel. The conformational changes of the MRN complex are depicted in **Fig. 1B**

Nijmegen breakage syndrome 1 (NBS1) is a modular protein possessing several distinct domains and interaction sites recognized by various proteins involved in the DDR, chromatin remodelling, checkpoint activation and translesion DNA synthesis pathway. Starting with the N-terminal region, there are situated phosphobinding FHA and BRCT domains (Varon *et al.,* 1998) responsible for the interaction with the mediator protein MDC1 (Chapman *et al.,* 2008), DNA topoisomerase II binding protein 1 (TOPBP1) (Morishima *et al.,* 2007) or the exonuclease CtIP (Chen *et al.,* 2008). At the C-terminus, there are several interaction motifs recognized by RAD18, RNF8, MRE11 and ATM (Lu *et al.,* 2002, Uziel *et al.,* 2003 and Schiller *et al.,* 2012). NBS1 recruits the RING finger E3 ubiquitin ligase RAD18 in response to UV irradiation and possibly regulates Pol η-dependent translesion DNA synthesis (Yanagihara *et al.,* 2011). NBS1 ubiquitination by the ring finger factor RNF8 provides optimal binding to the DSBs. NBS1 binds the entire MRN complex via MRE11-binding domain and it is translocated into the nucleus by importin KPNA2 (Tseng *et al.,* 2005).

The MRN complex is indispensable for ATM/ATR activation ATM (Difilippantonio *et al.,* 2005). Through the interaction between NBS1 and ATM, the MRN complex is in charge of phosphorylation of

approximately >900 sites on >700 proteins (Matsuoka *et al.,* 2017). MRE11 generates ssDNA which is an activation signal for ATR (Cimprich *et al.,* 2008). ATM and ATR activate the downstream effectors, particularly checkpoint kinases CHK1 and CHK2, then CK2 or p38 are phosphorylated as well. One of the most important targets in the DDR signalling cascades is the transcription factor p53, activated by ATM and CHK2 (reviewed in Zou & Elledge, 2003). p53 induces apoptosis, cell cycle arrest, and senescence. The DDR functions as a regulator of physiological processes that involve multiple layers of decisions, such as the determination whether the cell undergoes apoptosis or senescence, enhancing immune surveillance or, after all, the choice of the repair strategy itself. Through ATM and ATR activation, the MRN complex is involved in the orchestration of these fascinating networks.

2.2. Description of two major pathways of double-strand break repair

There have been described numerous types of DNA damage and an elaborate network of repair mechanisms. Because this work is purely focused on the DDR following DSBs which represent the most toxic DNA lesions endangering genomic stability, it is appropriate to characterize the repair pathway and DDR signalling cascade induced by DSBs. At least four distinct strategies of DSBs repair have been identified: homologous recombination (HR), classical non-homologous end joining (c-NHEJ), alternative end joining (aEJ) and single-strand annealing (SSA). The final choice is based on the extent of DNA end processing. For example, c-NHEJ does not demand DNA end resection, a-EJ requires limited resection in the range of 5-25 nucleotides while HR and SSA rely on more extensive resection, but (Hartlerode *et al.,* 2009).

cNHEJ occurs within the cell cycle. The repair is initiated when Ku70/80 heterodimer, which has high affinity for blunt ends or short ssDNA overhangs, binds to DSB ends. Ku70/80 then recruits other repair factors, like the catalytic subunit of DNA-dependent protein kinase (DNA-PKcs), DNA ligase IV (LIG4) and the associated scaffolding factors XRCC4 stabilizing LIG4, XRCC4-like factor (XLF) and paralogue of XRCC4 and XLF (PAXX) (Gottlieb *et al.,* 1993, McElhinny *et al.,* 2000, Ahnesorg *et al.,* 2006 and Ochi *et al.,* 2006). For illustration, see **Fig. 2.** End processing is provided by the nuclease Artemis, specialized DNA polymerases λ and μ and other enzymes ensuring compatibility of the ligated ends or accessory factors supporting cNHEJ, including the MRN complex (Stinson *et al.,* 2019). Finally, the repair is finished with ligation (Mimori *et al.,* 1986).

The second major repair strategy of DSBs is **HR**, a multistep process. HR is restricted to specific phases of the cell cycle, namely S and G2 when DNA is replicated and the copy of damaged DNA region is accessible (Takata *et al.,* 1998). The usually error-free HR pathway uses the sister chromatid as a template. The molecular basis of the template choice is the sequence identity, spatial alignment, and physical cohesion of the two sister chromatids (Kadyk *et al.,* 1992). The crucial step in the repair is

resection facilitated by the MRN complex. For efficient initiation of the resection, CtBP interacting protein (CtIP) is needed (Limbo *et al.,* 2007, Sartori *et al.,* 2007 and Garcia *et al.,* 2011). The next step of resection is mediated by the exonuclease 1 (EXO1), the endonuclease Dna2 and the Bloom syndrome helicase (BLM) (Nimonkar *et al.,* 2011). As a result, the 5' strand is unwinded and digested, so a long 3' ssDNA tail is formed. The emerging strand is coated with the RPA complex comprising RPA1, 2 and 3, to protect the strand from undesirable pairing with other ssDNA and open secondary structures in the strand. RPA is displaced by the breast cancer associated 2 (BRCA2) protein and replaced by the recombinase RAD51 (Yang *et al.,* 2005), see **Fig. 2** for illustration. Breast cancer associated 1 (BRCA1) protein is involved in both these processes (Anderson *et al.,* 1997).

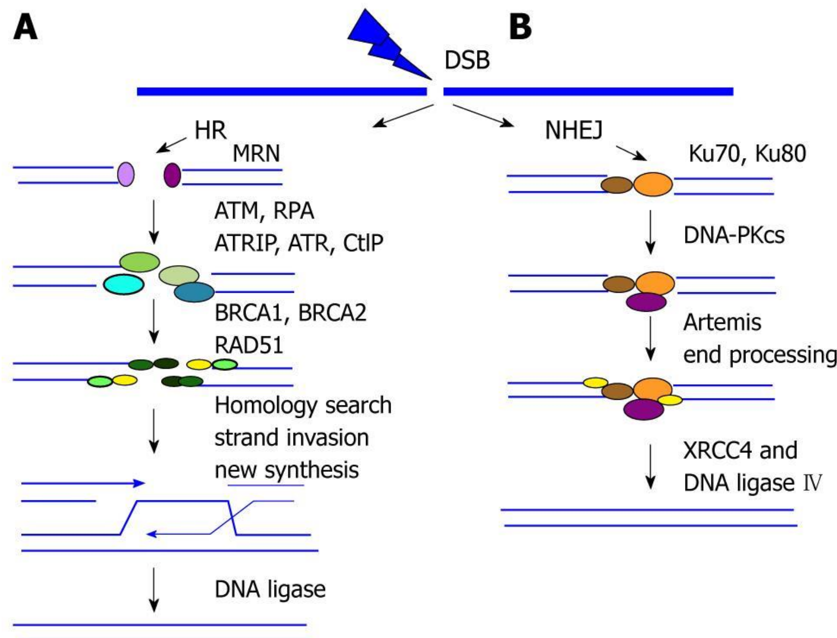

***Figure 2**. Overview of two major DSB repair pathways (adapted from Peng et al., 2011). A – main steps of HR, B – cNHEJ. More details are available in the text.*

The resulting nucleoprotein filament is responsible for the homology search and the strand invasion. Subsequently, the synaptic complexes comprising a three-stranded DNA helix intermediate are formed. If there is a successful base-pairing in the synaptic complex, the synapse is stabilized and the non-base-paired strand of the invaded molecule is displaced to form a displacement loop (D-loop). This process is triggered by RAD51-facilitated ATP hydrolysis and subsequent disassembly of the RAD51 filament (van der Heijden *et al.,* 2008). DNA polymerase δ is recruited to the free 3' end of the invading strand to extend the nascent strand using the invaded donor DNA molecule as a template for gene conversion (Johnson *et al.,* 2015). This process can result in different intermediates, for that reason, several types of HR and their modifications have been described, for instance, double Holliday junction with or without crossover, synthesis-dependent strand annealing (SDSA) conservative and nonconservative, long-tract gene conversion (LTGC) or break-induced replication (BIR). The result is precise DNA repair with low probability of emerging mutations (reviewed in Scully *et al.,* 2019).

3. Nucleolar DNA damage response

3.1. Brief characterization of the nucleolus

Most of the genetic material of the eukaryotic cell is stored in the nucleus. Nucleus has highly specialized and tightly controlled architecture: chromatin, nuclear bodies, and nucleoli. The strict regulation of nuclear organization contributes to precise regulation of genetic expression, DNA repair processes or other genome-related functions.

Nucleoli, firstly observed in 1781 (reviewed in Montgomery, 1898), are nuclear membrane-less self-organising compartments which are set up around nucleolar organizing regions (NORs). The connection between nucleolus and specific chromosomal loci was revealed by Barbara McClintock. These regions are made up of tandemly repeated rDNA clusters (McClintock *et al.,* 1934). While *Drosophila* or *Xenopus* possess a single nucleolar organizer, humans have five NORs on five different chromosomes, namely the shorter arm (p-arm) of acrocentric chromosomes 13, 14, 15, 21, and 22. In the region near the centromere, there was identified a short proximal junction (PJ) sequence. PJ is followed by a long rDNA array encompassing up to 6 Mb of repeated sequence. At the telomeric side, there is a shorter distal junction (DJ) with a cluster of satellite DNA made up of a 48-bp repeat (van Sluis & McStay, 2019). For illustration, see **Fig. 3**. The sequencing results demonstrated that short arms of these five chromosomes were almost identical. The reason could be that umpteen inter-chromosomal exchanges have happened in the past and doubtlessly continue to do so.

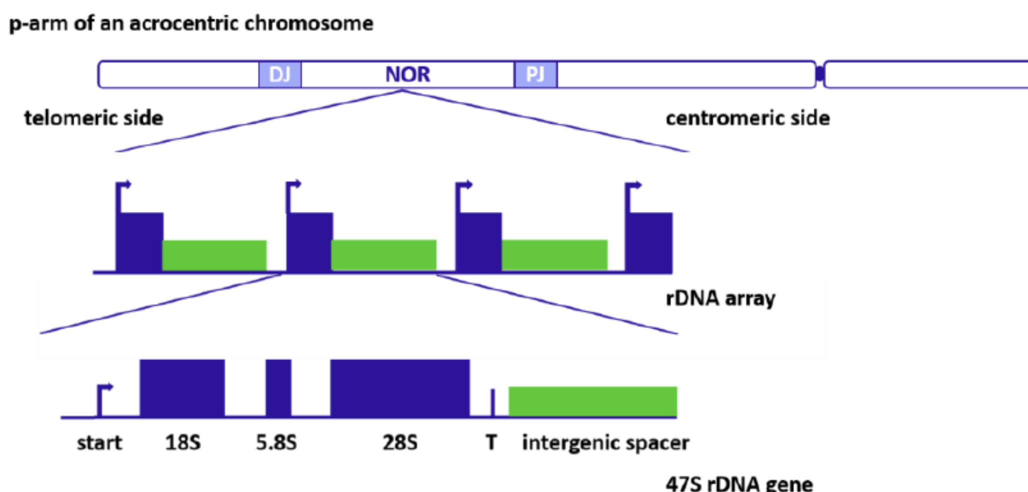

Figure 3. *Mammalian rDNA organization. rDNA units are organized as a tandem of head-to-tail repeats. Each rDNA unit consists of an rRNA coding region (dark violet) and an intergenic spacer (green). The coding region encodes the 18S, 5.8S and 28S rRNAs separated by two internal transcribed spacers (gaps between 18S, 5S and 28S). T is a terminator sequence.*

rDNA, containing 200 to 400 copies of rDNA repeats in the human genome, codes genes for ribosomal RNA. The essential role of the nucleolus is ribosomal biogenesis, including production of rRNAs, small and large ribosomal subunits. These housekeeping processes were estimated to consume up to 80%

of the cellular energy (Schmidt *et al.,* 1999). rRNA transcription starts when nucleoli reassemble around NORs after mitosis and takes place throughout the interphase (Gébrane-Younès *et al.,* 1997 and Muro *et al.,* 2010). rRNA accounts for approximately 80% of the transcription in mammalian cells (Lodish *et al.,* 2000). 47S precursor ribosomal RNA (pre-rRNA) transcript produced by RNA polymerase I (RNA Pol I) is spliced into 28S, 18S, and 5.8S rRNAs, the process is both co- and post-transcriptional and provided by snoRNPs (small nucleolar ribonucleoproteins). Mature rRNA contains snoRNP-mediated modifications, such as 2′-O-methylation and pseudouridine formation (described in Matera *et al.,* 2007 and Aubert *et al.,* 2018). Finally, the 28S, 18S, 5.8S and 5S rRNAs cooperate with ribosomal proteins to form the small and large preribosomal subunits. Each of the subunits is then separately exported to the cytoplasm and undergoes final processing steps to become the mature 40S or 60S ribosomal subunits.

Apart from polycistronic 47S array transcribed by RNA Pol I in the nucleolus, monocistronic 5S rRNA repeat transcribed by RNA Pol III belongs to ribosomal RNAs as well (Sorensen *et al.,* 1991). 5S rRNA is synthetized outside of the nucleolus and the sequence coding 5S rRNA is a part of chromosome 1. Regarding to current knowledge, breaks in 5S result in milder phenotype. It is easier to repair DSBs in 5S, maybe because 5S repeats are located on only one chromosome and are more accessible for repair factors due to their localization (Warmerdam *et al.,* 2016).

The nucleus usually comprises one to several nucleoli, constituting up to 25% of the nuclear volume. Nucleoli are clearly observable in interphase nuclei and filled with transcribed rDNA and assembling ribosomal subunits. The growing knowledge of nuclear architecture and improving technology bring novel models of how nucleoli are constituted. Recent studies indicate that nucleoli are formed through phase separation. Nucleoli exhibit a liquid-droplet behaviour where different compartments of nucleoli represent immiscible liquid phases (Grob *et al.,* 2014). It was suggested that intrinsically disordered proteins or low complexity sequences induced phase transitions underlying nucleolar assembly. The self-organizing structure is driven by rRNA transcription and the liquid-like nature of the nucleolus might promote its function in ribosomal biogenesis (Berry *et al.,* 2015).

Nucleoli are composed of three distinct tripartite subnucleolar compartments: the dense fibrillar component (DFC), the fibrillar centre (FC) and the granular component (GC) (Farley *et al.,* 2015). DFC contains pre-rRNA processing factors, such as the snoRNAs and snoRNP proteins, fibrillarin and NOP58. FC harbours components of the RNA Pol I machinery like UBF transcription factor. Finally, GC surrounds DFC and FC and it is place where preribosomal subunit assembly takes place. pre-rRNA is transcribed from rDNA either in the FC or at the border between the FC and DFC, the modification of pre-rRNA occurs through the DFC and GC region (Koberna *et al.,* 2002 and Cheutin *et al.,* 2002). These

nucleolar structures and their functions are depicted in **Fig. 4**. Only a small part of rDNA genes is localized directly in the nucleolus and actively transcribed, the inactive NORs are situated in the nucleolar periphery and form inactive perinucleolar heterochromatin regions (Pontvianne *et al.,* 2013). rRNA transcription is the target of many signalling pathways associated with growth factors and nutrients availability. There is a correlation between nucleolar size and amount of rRNA transcription or cellular proliferation (Derenzini *et al.,* 2000). Proliferating cells are entitled to intense protein synthesis which is a process dependent on proper assembly of the ribosomes. Number of functional ribosomes is determined by the amount of rRNA which is produced in the nucleoli, downregulated rRNA transcription leads to a decrease in nucleolar size (Hayashi *et al.,* 2014). Typically, dividing cells possess larger nucleoli than non-dividing cells.

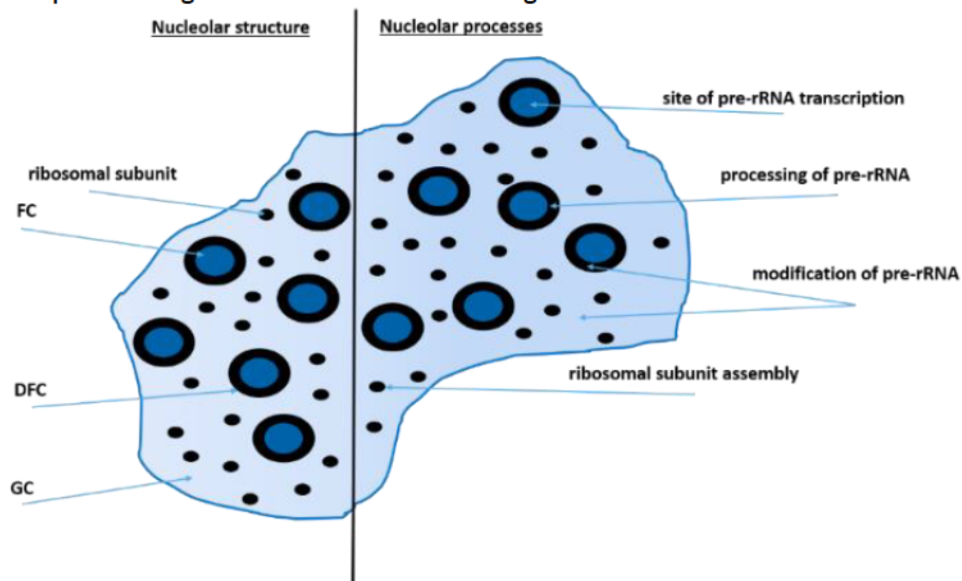

***Figure 4**. Structure of the nucleolus with respect to nucleolar processes. The individual steps of ribosomal biogenesis are associated with specific parts of the nucleolus. pre-rRNA is transcribed in the FC and processed in the DFC. Subsequent modifications occur both in the DFC and GC. Finally, ribosomal subunit assembly takes place in the GC. More details are available in the text.*

Nucleolar abnormalities represent the cause of some diseases, such as Diamond-Blackfan anaemia. This neonatal-pediatric disorder is triggered by mutations in ribosomal proteins and affects bone marrow. The haploinsufficiency for structural proteins of either ribosomal subunit results in abortive ribosomal assembly and induces nucleolar stress. Free ribosomal proteins interact with p53 negative regulator MDM2, so the rate of apoptosis is increased (Zhou *et al.,* 2013). Historically, the number of nucleoli was associated with tumour development (Pianese *et al.,* 1896). Nucleoli found in tumours are enlarged, multiplied and irregularly shaped. These features were considered markers of aggressive malignancies (Busch *et al.,* 1970) and prominent nucleoli relate to poor prognosis in cancer patients (Boulon *et al.,* 2010). Many cancers demonstrate pronounced nucleolar instability and diminished number of rDNA copies in comparison to healthy tissues. However, it is still not possible to clearly state whether the nucleolar DNA damage has an important role in cancer development or is only a side effect of the global genome instability and cellular stress.

3.2. Methods for studying nucleolus and n-DDR

To induce DNA damage specifically in the nucleolus, several methods for specific targeting of rDNA or RNA Pol I have been adopted. There are two types of nucleolar response to DNA damage: *in trans*, induced by DNA damage outside the nucleolus, and *in cis*, triggered by DSBs directly in rDNA (Larsen *et al.,* 2014). It is desirable to distinguish between these two types of response and choose adequate stress agents. In particular, if cells are irradiated by X-ray, which is a commonly used treatment in the DDR research, the damage occurs randomly within the entire nucleus, including both genomic DNA and rDNA. Therefore, treatment inducing DSBs throughout the nucleus can be used, but only for studying nucleolar DNA damage response *in trans*. Under these conditions, it is not possible to apply the impact of rDNA damage simply on the cell physiology because nucleolar damage is only a minority in the nuclear context. For that reason, some treatments commonly used in the DDR research are not suitable for studying the impact of DSBs in rDNA.

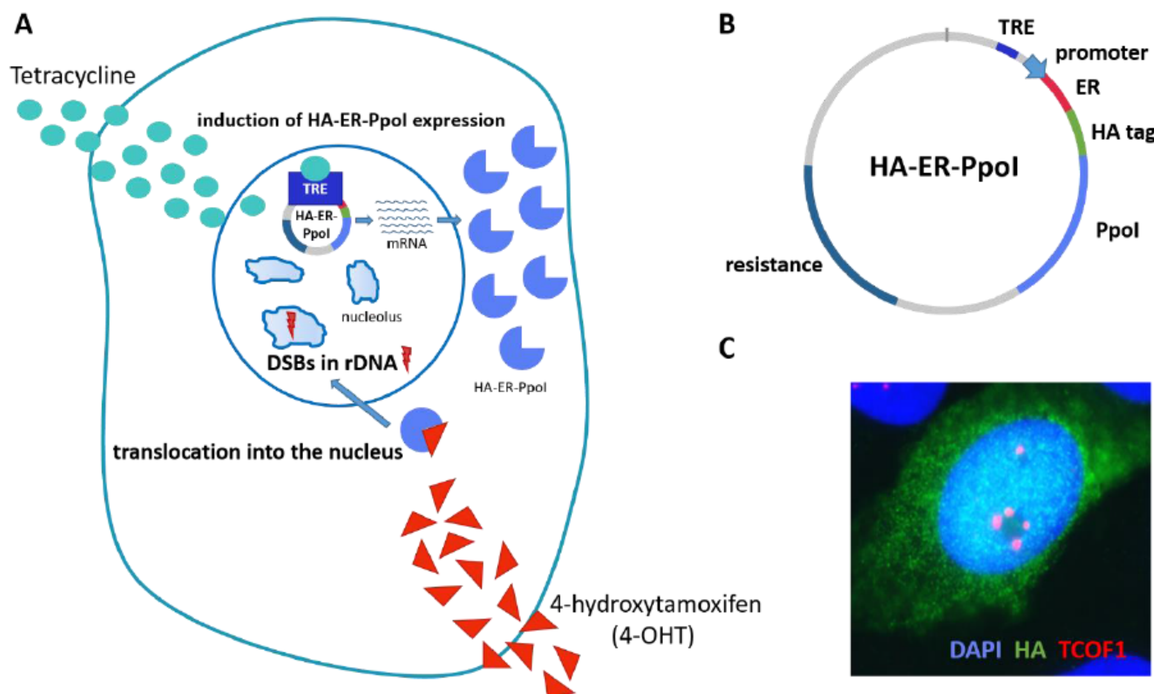

Figure 5. *Example of inducible Ppo-I system (I-PpoI). A – the expression system. Cells stably transfected with HA-ER-PpoI plasmid are treated with tetracycline and 4-hydroxytamoxifen (4-OHT). Tetracycline binds tetracycline responsive element (TRE) in the plasmid sequence and induces the expression of HA-ER-PpoI. This protein cannot enter the nucleus without 4-OHT. In the nucleolus, PpoI recognizes sites in rDNA and generates DSBs. B – plasmid map. The plasmid codes fusion protein comprised of the estradiol receptor (ER) binding 4-OHT, HA tag and endonuclease PpoI. C – I-PpoI in U2OS cells (unpublished data obtained by the author of this thesis). Cells transfected with the described plasmid were fixed after overnight tetracyclin treatment and 3 hours after 4-OHT addition. The green cell is expressing HA-ER-PpoI, and nucleolar protein TCOF1 is forming specific foci in response to DSBs in rDNA.*

One of the best strategies for inducing nucleolar DSBs is cleavage by endonucleases recognizing specific sites in rDNA repeats. Deepening knowledge of protist proteomes has provided the advantage of the homing intron-encoded endonuclease I-PpoI cleaving CTCTCTTAAGGTAGC sequence within the 28S portion of rDNA (Muscarella *et al.,* 1990). Endonuclease I-PpoI was isolated from amoeba *Physarum polycephalum* and where it mediates the mobilization of intron 3 in rDNA (Muscarella *et al.,*

1989). It was estimated that I-PpoI expression in human cells generated about 30 DSBs in rDNA which was equivalent to damage introduced by exposure to approximately 0.8 Gy (Monnat *et al.,* 1999). In addition, a few targets are located elsewhere in the genome as well, so non-nucleolar DNA damage can also be slightly induced (Berkovich *et al.,* 2007). There are several alternatives of this approach: stable cell line inducibly expressing I-PpoI, transient cell transfection with I-PpoI mRNA (Mooser *et al.,* 2020) or plasmid coding I-PpoI sequence (Berkovich *et al.,* 2007). One option of the I-PpoI system is shown in **Fig. 5**.

Another tool used for rDNA breaks induction represents DIvA cell line (**D**SB **I**nducible **v**ia **A**siSI). These cells carry AsiSI restriction enzyme which allows the cutting of DNA at ~150 annotated positions across the human genome, some of them present in rDNA (Iacovoni et al, 2010; Massip et al, 2010, Aymard et al, 2017, Clouaire et al, 2018). The disadvantage is lower number of rDNA DSBs in favour to non-nucleolar DSBs. Apart from that, CRISPR/Cas9 technology can be applied for inducible cleavage of rDNA repeats (van Sluis & McStay, 2015 and Korsholm *et al.,* 2019). This can be achieved by stable cell lines expressing Cas9, the system is regulated by transfection with gRNA targeting rDNA repeats.

Laser micro-irradiation can precisely target individual nucleoli or subnucleolar areas and induce DSBs (Celeste *et al.,* 2003). This technique is essential for studying n-DDR processes *in trans* or *in cis*. On the other hand, laser micro-irradiation has requirements for appropriate technical equipment, live-cell imaging is necessary. Generally, exploring nucleolar architecture, segregation or nucleolar translocation of various factors has increased demands on the microscopy technique. For that reason, a novel method for studying nucleolar processes was developed. 3D immunoFISH, combining immunofluorescence with DNA FISH taking place on 3D-preserved interphase nuclei (Chaumeil *et al.,* 2013). The method facilitates the visualization of nuclear architecture in context with chromosomal sub-regions, especially NORs, and specific nuclear proteins.

Concerning drugs, actinomycin D (ActD) is used in some studies for inducing the nucleolar stress. ActD, the first antibiotics shown to have anti-cancer activity (Gregory *et al.,* 1956), was isolated in 1940 from soil bacteria of the genus *Streptomyces* (Walksman *et al.,* 1940). The mechanism of action had been uncertain until the drug's target, RNA synthesis, was identified (Reich *et al.,* 1961). The key finding was that low concentrations of ActD selectively inhibited RNA Pol I (Roberts *et al.,* 1966) but the standardly used dosage blocked all three RNA polymerases (Iapalucci-Espinoza *et al.,* 1979). ActD triggers nucleolar segregation, but not recruitment of HR repair factors (van Sluis & McStay, 2015). Nucleoli are also sensitive to leptomycin B (LMB), this drug inhibits intranucleolar traffic of nucleophosmin 1 (NPM1), nucleolar protein regulating ribosomal biogenesis (Muro *et al.,* 2012). LPM destabilizes NPM1 protein complexes (Hingorani *et al.,* 2000).

3.3. Nucleolus as a storage depot for DDR proteins

Apart from accumulation of factors essential for rRNA transcription and processing or ribosomal subunit assembly, nucleolus was suggested to function as a storage depot for non-ribosomal proteins, such as factors involved in DNA repair or DDR (Andersen *et al.,* 2005). Fascinatingly, an extensive proteomic study (Ahmad *et al.,* 2009) identified about 166 DNA repair proteins localized in the nucleolus independently on stress and DNA damage (Moore *et al.,* 2011). However, little is still known about whether these proteins operate in the nucleolus or have their specific nucleolar functions. DDR proteins are mostly abundant in the intranucleolar body (INB) (Hutten *et al.,* 2011) and, upon DNA damage, in the structure at the nucleolar periphery referred to as nucleolar caps (NCs) (van Sluis & McStay, 2015 and Harding *et al.,* 2015), which is the concern of this work.

The key requirement for nucleolar recruitment is nucleolar localization signal (NoLS) or interaction with a protein possessing NoLS. The sequence is constituted by several positively charged lysine and arginine residues interacting with negatively charged RNA molecules (Endo *et al.,* 1989). Remarkably, NoLS does not interact with DNA as its negative charge is neutralized by histones, polyamines or bivalent ions. There is no NoLS consensus sequence, but usually, about 50% of amino acids in the NoLS sequence are positively charged arginines and lysines. Possibly, it could be the lower specificity of NoLS what enables protein accumulation in the nucleolus.

Nucleolar localization was confirmed for RecQ Werner syndrome helicase WRN or the component of the base excision repair (BER) pathway, apurinic/apyrimidinic endonuclease 1 (APE1). Both these proteins have specific functions in the nucleolus. For example, APE1 is recruited by NPM1 and is thought to control rRNA quality and WRN interacts with RNA Pol I (Shiratori *et al.,* 2009). Many of these proteins interact with nucleolar protein nucleolin (NCL) which modulates their function (Indig *et al.,* 2012). Unexpectedly, Poly (ADP-ribose) polymerase 1 (PARP1) was demonstrated to facilitate DNA damage-induced nucleolar-nucleoplasmic shuttling of genome maintenance factors, namely WRN or XRCC1 (Veith *et al.,* 2019).

Some proteins are considered to be stored in the nucleolus and kept inactive, such as tumour suppressor protein VHL (Mekhail *et al.,* 2004), adenosine deaminase ADAR2 (Sansam *et al.,* 2003) or telomerase reverse transcriptase hTERT (Yang *et al.,* 2002). Nonetheless, the prime example of protein inactivation by nucleolar sequestration is considered MDM2. Ubiquitin ligase MDM2 is sequestrated by tumour suppressor protein ARF into the nucleolus. As a result, p53 is activated because its inhibitor MDM2 is inactive (Weber *et al.,* 1999). This finding was based on the observation that ARF and MDM2 co-localize in the nucleolus when proliferative transcription factor Myc is activated. Later, the model was extended by the discovery of the interaction between NPM1 and ARF (Korgaonkar *et al.,* 2005).

Remarkably, ARF binds NPM1 through the same domains that facilitate nucleolar localization and MDM2 binding. It means that NPM1 could regulate ARF localization and compete with MDM2 for ARF association at the same time. If NPM1 is depleted, ARF-MDM2 association is enhanced, p53 is activated and cellular growth decreases.

3.4. Nucleolar DNA damage response

In past years, the n-DDR processes have been intensively studied. Concerning the current knowledge, the immediate repair of nucleolar DSBs is mostly mediated by the NHEJ pathway in the nucleolar interior. This strategy does not lead to nucleolar reorganization and affects rRNA transcription only minimally (Harding *et al.,* 2015). However, if DSBs are not efficiently repaired by NHEJ, the transcription is silenced, and rDNA is moved to the NCs, large foci at the periphery which are enriched by HR factors. At this moment, the repair strategy is switched from NHEJ to HR.

3.4.1.Immediate strategy of rDNA breaks repair

The question of which repair strategy, HR or NHEJ, is chosen for the direct repair of nucleolar DSBs, was discussed by several authors. In one of the first attempts (Berkovich *et al.,* 2007), the inefficiency of nucleolar DNA damage repair in cells depleted of NHEJ factors was demonstrated using comet assay. It is worth to remind that comet assay is a technique based on DNA electrophoresis taking place on the slide covered by the agarose layer, the outcome is a large smear of fragmented DNA in cells undergoing DNA damage and, oppositely, rounded nucleoids in case of repaired or intact DNA (Olive *et al.,* 2006). In addition, the authors identified ATM-dependent nucleolar recruitment of XRCC4 in response to I-PpoI induced DSBs in rDNA. In cells treated with a pharmacological NHEJ inhibitor specifically targeting DNA-PK, the nucleolar transcriptional silencing was prolonged as a result of inefficient rDNA repair. Depletion of NHEJ results in increased fraction of cells with NCs, depletion of HR not (Harding *et al.,* 2015). Besides the nucleolar repair of DSBs, NHEJ is involved in the meiotic shield against undesirable homologous recombination in the nucleolus (Sims *et al.,* 2019), as was demonstrated in *Arabidopsis thaliana*. All these studies support the fact that the NHEJ pathway is the first-choice strategy for rDNA DSBs repair.

The predominant strategy of rDNA DSBs repair, NHEJ, allows transcription to recommence, as it is usual elsewhere in the genome. Additionally, nucleolar NHEJ does not demand nucleolar restructuralization. Whether nucleolar NHEJ processes differ in some points from the canonical NHEJ pathway is not known and further research is demanded. Nucleolar NHEJ depends on DNA-PK, XRCC4 and XRCC5 factors, and takes place in the interior of nucleoli without requirement of sustained ATM activity. One recent study (Sims *et al.,* 2019) indicates that rDNA arrays are repaired independently on RAD51, the protein required for the repair is LIG4.

3.4.2. RNA polymerase I inhibition

The mechanism of RNA Pol I transient shutdown and its importance for genomic stability have been addressed by several authors. Transcriptional discontinuation is widely accepted to be ATM-dependent, and the resumption of RNA Pol activity relies on successful repair processes (Kruhlak *et al.,* 2007 and Harding *et al.,* 2015). Combination of live-cell imaging, micro-irradiation and photobleaching demonstrated an ATM-dependent premature displacement of elongating holoenzymes from rDNA in response to rDNA damage. Phosphoproteomics revealed that RNA Pol I subunits RPA34 and TAF1C belong to putative ATM substrates together with several RNA Pol I transcription factors, such as Pol I transcription factors TTF1, UBF (Matsuoka *et al.,*2007; Stokes *et al.,*2007), TCOF1 (Isaac *et al.,* 2000) or a compound of the early pre-rRNA processing machinery, UTP14A (Hu *et al.,* 2011). Nucleolar proteome analysis identified an ATM pool constantly occurring in nucleoli (Andersen *et al.,* 2002) and cell pre-extraction enabled the detection of nucleolar ATM in untreated cells (van Sluis & McStay, 2015). It is likely that this pre-existing pool mediates the transcriptional shutdown in response to rDNA damage.

Additionally, ATM may inhibit transcription through the cohesin complex. ATM phosphorylates the cohesin subunits SMC1 and SMC3 (Kim *et al.,* 2002, Meisenburg et al., 2019). rRNA transcriptional silencing could be regulated by the cohesin complex recruiting the Human Silencing Hub (HUSH) complex. The HUSH complex comprises Suv39H1/2 methyltransferase to introduce H3K9me3 repressive chromatin mark leading to the transcriptional shutdown (Marnef *et al.,* 2019). If some of the subunits of the HUSH complex is depleted, NCs are not formed, and the transcription is not stopped upon rDNA DSBs induction.

Some authors also suggested DNA-PK contribution in transcriptional silencing after I-PpoI-induced DNA damage while ATM orchestrated large-scale nucleolar architecture changes (Pankotai *et al.,* 2012). Afterwards, the most recent study indicates that ATR is engaged in RNA Pol I inhibition through its interacting partner TOPBP1 as well (Mooser *et al.,* 2020). Inhibition of ATM or ATR leads to unstopped rRNA transcription.

The assumed range of inhibition varies depending on the author and selected experimental model. For instance, data obtained from mice cells indicate that the transcription in the whole nucleolus is affected upon rDNA damage (Kruhlak *et al.,* 2007), and in contrast, recent studies using human cells demonstrated that the transcription inhibition could be even more restrictive, affecting only a part of the nucleolus (Korsholm *et al.,* 2019, Marnef *et al.,* 2019 and Siebenwirth *et al.,* 2019). The question is how the inhibitory signal spreads, how the range of inhibition is regulated by the topology of

chromatin (Ochs *et al.,* 2019) and whether it is restricted to rDNA units or single NORs. In this field of research, super-resolution microscopy techniques might give some answers.

3.4.3.Nucleolar segregation

One of the n-DDR processes is directed movement of the nucleolar chromatin to the periphery of the nucleolus. The segregation is a consequence of transcriptional inhibition (van Sluis & McStay, 2015). Translocated rDNA is more accessible for the DNA repair machinery, so the repair of these critical genes is more efficient. The isolation of rDNA breaks can prevent inter-chromosomal exchanges and disruption of genomic integrity (Floutsakou *et al.,* 2013). The heterochromatic environment at the nucleolar periphery may also decrease the mobility of the rDNA break-ends and as a consequence, limit inter-chromosomal recombination (Lemaître *et al.,* 2015). Also, the proximity of undamaged repeats within one NOR in the NCs could facilitate the repair.

The dynamics of n-DDR during segregation has been documented: first, there are few smaller foci of n-DDR factors in the interior which merge into large foci and move to the nucleolar periphery where the NCs are formed (Korsholm *et al.,* 2019 and Marnef *et al.,* 2019). rDNA is translocated with UBF which extensively binds rDNA throughout the cell cycle and creates a specific form of chromatin typical for active NORs (Kruhlak *et al.,* 2007). There was an attempt to put into context the amount of UBF in the NCs and number of translocated rDNA repeats. The conclusion was that some of rDNA repeats were lost during the segregation (Warmerdam *et al.,* 2016). Nevertheless, it is not likely that the relationship between the amount of UBF and number of rDNA repeats is applicable for evaluating the copy number and possible loss of rDNA repeats because the reduced amount of the transcription factor UBF can simply mean fewer transcriptionally active rDNA repeats.

rDNA mobilization is suggested to be driven by ATM- and ATR-dependent transcriptional inhibition (Mooser *et al.,* 2020). rDNA is segregated in response to rDNA DSBs and also ActD treatment (van Sluis & McStay, 2015). In addition, the role of DSB ends processing is emerging from recent data from DIvA cell line (Marnef *et al.,* 2019). Both total RPA and RPA2 phospho-serine 33 are accumulated in the nucleoli prior to rDNA segregation. It was independently confirmed that the first steps of rDNA damage repair were linked to the increase of RPA2 phospho-serine 33 (Mooser *et al.,* 2020). RPA2 is involved in response to rDNA damage induced by AsiSI-facilitated cleavage, but not after ActD treatment which inhibits RNA Pol I. siRNA-mediated knockdown of RPA2 prevents nucleolar segregation after rDNA breaks induction (Marnef *et al.,* 2019). Based on these observations, the current view gives emphasis to the priority of resection, which is related to transcriptional inhibition in response to rDNA DSBs, before rDNA segregation. Interestingly, the pattern of nucleolar RPA2 phosphorylation in rDNA differs from RPA2 phosphorylation elsewhere in the genome (Syed *et al.,*

2018). Genomic DNA processed by HR was shown to have up to 1000 bp long overhangs coated by phosphorylated RPA2 while in case of rDNA, the entire 13 kb of transcribed region is occupied by phosphorylated RPA2 and co-localizes with RNA Pol I (Marnef *et al.,* 2019).

There is an opinion that the invaginations of the nuclear envelope are involved in nucleolar reorganization through the Linker of Nucleoskeleton and Cytoskeleton (LINC) complex, but the data were obtained from DIvA cells experiencing also non-nucleolar DSBs (Marnef *et al.,* 2019). The movement of nucleolar chromatin towards or away from the nucleolus may cause the silencing or activation of gene expression, indicating that the nucleolus is not only limited for ribosome biogenesis, but it can serve as a central hub controlling a number of nuclear and cellular processes (Allinne *et al.,* 2014). Subsequently, these chromatin movements were described to inactivate the whole chromatin domains (Matheson *et al.,* 2017). However, these suggestions should be closely investigated.

3.4.4. Double-strand break repair in the nucleolar caps

The typical sign of damaged nucleoli is the presence of the NCs, sites with condensed rDNA repeats and abundant HR proteins. These large foci are located in nucleolar heterochromatin at the periphery (Shav-Tal *et al.,* 2005). NCs are formed by merged FC/DFC foci translocated with rDNA (see **Fig. 6**).

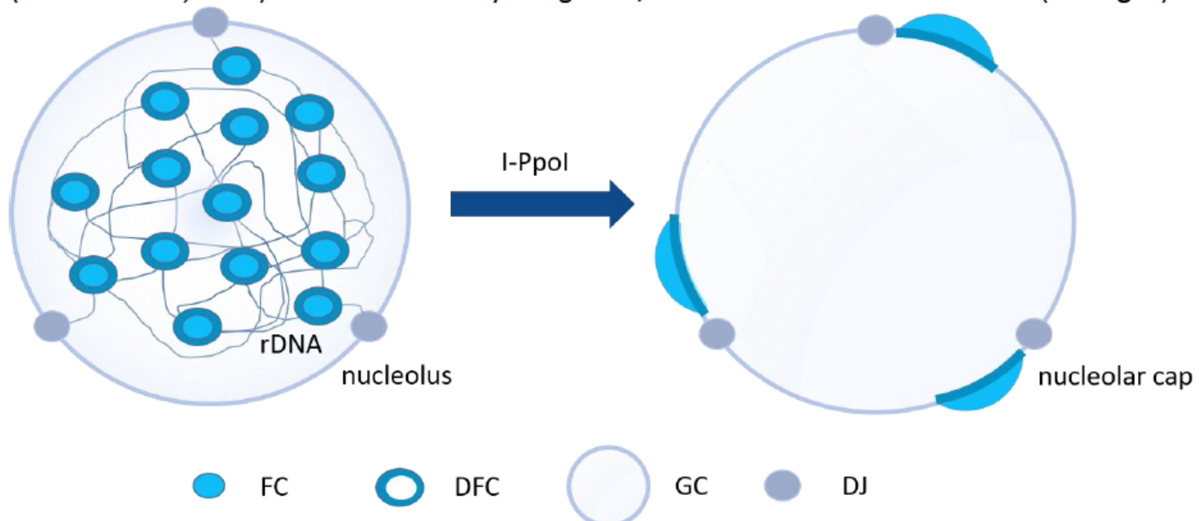

Figure 6*. Reorganization of the nucleolus upon I-PpoI cleavage in rDNA. In response to rDNA breaks, the nucleolus undergoes large reorganization triggered by RNA Pol I inhibition through liquid-liquid phase separation. rDNA is looped in the interior and upon rDNA breaks, it becomes condensed nearby DJs at the periphery. FC and DFC merge and form the NCs.*

To examine whether the NCs are the result only of transcriptional inhibition, not damage, cells were treated with ActD. NCs were formed in response to ActD treatment, but these caps were not enriched with γH2AX (van Sluis & McStay, 2015). ATM inhibition by the inhibitor KU-55933 lead to unstopped rRNA transcription and impaired formation of the NCs upon rDNA DSBs induction. These findings mean that the transcriptional silencing, not rDNA breaks themselves, trigger the reorganization which is consistent with previous studies (Kruhlak *et al.,* 2007).

NHEJ is the major DSBs repair strategy overall in the genome, in all stages of the cell cycle (Karanam et al. 2012). For that reason, a mechanism switching NHEJ to HR should exist in the NCs. The suggested candidates are DNA-PK and 53BP1 (Bunting *et al.,* 2010 and Neal *et al.,* 2011). It seems that the absence of DNA-PK and Ku in the NCs promotes HR because DSB ends are not blocked for resection. Another mechanism could be the acetylation of histone H4 on K16, which prevents recognition of monomethylated or dimethylated histone H4K20 by the Tudor domain of 53BP1 and promotes BRCA1 interaction with the ends of the DSBs (Tang *et al.,* 2013). Probably, the acetyltransferase responsible for these processes could be TIP60 which is known to interact with ATM and UBF (Halkidou et al. 2004; Sun et al. 2005). High-resolution microscopy techniques revealed that 53BP1 and BRCA1 foci formed in response to γ-irradiation do not overlap (Chapman *et al.,* 2012). Similarly, 53BP1 and BRCA1 signal does not overlap in the NCs induced by rDNA breaks. These findings indicate that the local chromatin modification could play an essential role in the switching from NHEJ to HR.

RAD51, RAD52, BRCA1 and RPA2 are the HR factors recruited to the nucleolar periphery. NCs are also enriched by activated ATM phospho-serine 1981. Activated ATM was not detected in the NCs induced by ActD treatment (van Sluis & McStay, 2015). The role of HR in the repair of nucleolar DSBs was supported by other authors and the content of the NCs was studied as well. It was independently confirmed that HR proteins, such as RPA, RAD51, BRCA1 and BRCA2 were accumulated in the NCs upon rDNA damage (Harding *et al.,* 2015). Apart from that, the caps are positive for damage markers γH2AX, 53BP1, MDC1 and UBF (van Sluis & McStay, 2015, Harding *et al.,* 2015, Warmerdam *et al.,* 2016, Franek *et al.,* 2016 and Korsholm *et al.,* 2019).

In contrast, it is interesting that depletion of HR factors BRCA1 and BRCA2 results in more efficient rDNA breaks repair (Warmerdam *et al.,* 2016). The authors suggested that the repair mediated by recombination could lead to the loss of repeat integrity. Additionally, it was demonstrated that rDNA instability was dependent on homology-directed repair (HDR) templated *in trans* and the loss is caused by recombination between sister chromatids or rDNA repeats on different chromosomes. Possible protection against undesirable inter-chromosomal recombination could be HDR templated by intact repeats within the same NOR. This idea is consistent with observations that the recruitment of the HR factors is not cell cycle-dependent and DNA synthesis in the NCs occurs during G1 phase. Additionally, there were detected NCs containing only one NOR (van Sluis & McStay, 2015). However, this is still associated with a high risk of crossovers within rDNA array leading to genomic instability. This threat could be prevented by the BLM helicase involved in BLM-dependent branch migration pathway because this strategy does not lead to crossover (Karow *et al.,* 2000). Cells with non-functional BLM have 10-fold higher frequency of sister chromatid exchange between rDNA array (Killen *et al.,* 2009).

4. Role of the MRN complex in the nucleolar DNA damage response

4.1. TCOF1 – nucleolar transporter of NBS1

In response to rDNA breaks, NBS1 is recruited into the nucleolus (Ciccia *et al.,* 2014 and Larsen *et al.,* 2014). The question how NBS1 is translocated into the nucleolus was intensively studied until the mechanism was identified. According to the nucleolar localization signal predictor NoD (Nucleolar localization signal Detector, http://www.compbio.Dun dee.ac.uk/www-nod/), NBS1 does not possess NoLS. The transporter of NBS1 is a nucleolar treacle ribosome biogenesis factor 1 (TCOF1) protein, also known as Treacle (Ciccia *et al.,* 2014 and Larsen *et al.,* 2014). The outcome from NoD is shown in **Fig. 7**. Mutated form of TCOF1 is responsible for Treacher Collins syndrome, a severe autosomal dominant disorder in 1 in 50,000 children characterized by hypoplasia of the facial bones, cleft palate, hearing loss and brain defects (Treacher Collins, 1900 and Phelps *et al.,* 1981).

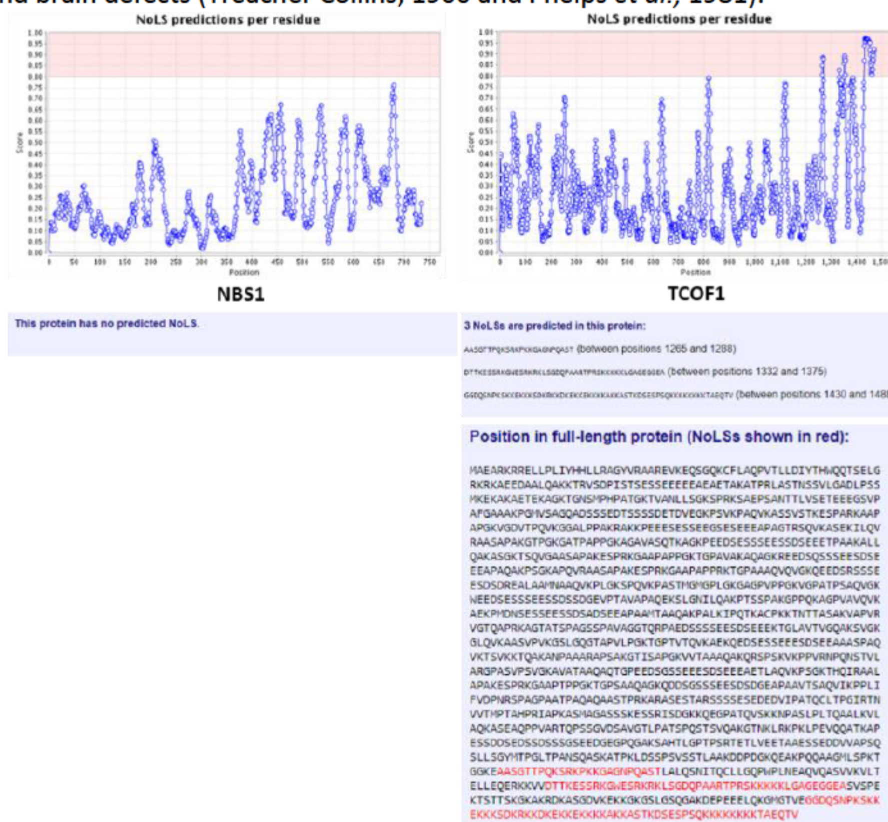

***Figure 7**. NoLS sequence in NBS1 and TCOF1 according to the NoD predictor. In contrast with NBS1 (left), TCOF1 possesses three NoLS sequences situated at the C-terminus which enable TCOF1 nucleolar localization. The graph shows the distribution of NoLS-associated amino acids within TCOF1 (right). The NoLS sequences are marked red in TCOF1 protein sequence.*

TCOF1 was identified as a DDR protein interacting with NBS1 and facilitating NBS1 damage-dependent recruitment into the nucleolus (Ciccia *et al.,* 2014). The latest studies suggested that besides NBS1, TCOF1 facilitated nucleolar recruitment of TOPBP1 in response to rDNA damage (Mooser *et al.,* 2020). TCOF1 is most abundant in the DFC components when rRNA transcription is in progress (Larsen *et al.,* 2014). The most recent study added that TCOF1 concentrated at the FC and DFC within the nucleoli

(Mooser *et al.,* 2020). TCOF1 interacts with RNA Pol I subunits (POLR1A, B and E) and the transcription factor UBF anf Fibrillarin to promote rRNA transcription in the nucleolus (Ciccia *et al.,* 2014). ChIP sequencing of the sequences occupied by TCOF1 revealed that TCOF1 is primarily situated at the promoter and coding regions of the rRNA gene arrays (Larsen *et al.,* 2014).

4.2. NBS1-TCOF1 interaction in response to nucleolar DNA damage

One of experimental approaches to identify protein interactors is mass spectrometry (MS). In the NBS1-TCOF1 context, this method was used to find NBS1 and TCOF1 interacting partners. Human embryonic kidney cell line HEK 293T-Rex carrying HA-tagged NBS1 was established. Following IR, anti-HA immunoprecipitation of NBS1 protein complexes was performed. Besides known NBS1 interactors, like ATR or MDC1, a potential NBS1-TCOF1 complex was identified by MS. This finding was then confirmed by immunoprecipitation of HA-tagged TCOF1 protein complexes after IR and the data from MS confirmed TCOF1 interaction with NBS1 (Ciccia *et al.,* 2014).

After micro-irradiation, NBS1-GFP nucleolar foci co-localized with TCOF1 and fibrillarin. NBS1 foci are formed independently on γH2AX and MDC1 which was demonstrated on cells with depleted H2AX or MDC1 (Korsholm *et al.,* 2019). The reason is that γH2AX is not present in the nucleolus, so MDC1 cannot be recruited. TCOF1 substitutes MDC1 in the nucleolus (Ciccia *et al.,* 2014 and Korsholm *et al.,* 2019). NBS1 is located in the nucleolus in the range of 5-30 minutes after irradiation while the nucleolar localization is only transient and dynamic (Ciccia *et al.,* 2014). These rDNA breaks-induced nucleolar NBS1-GFP foci did not form upon TCOF1 depletion (Ciccia *et al.,* 2014 and Larsen *et al.,* 2014, Korsholm *et al.,* 2019 and Mooser *et al.,* 2020). Apart from laser micro-irradiation, NBS1 nucleolar recruitment in response to rDNA DSBs was confirmed by the I-PpoI approach (Mooser *et al.,* 2020) or using Cas9 endonuclease with gRNA targeting rDNA repeats (Korsholm *et al.,* 2019). In these latest studies, different time points were studied. 3 hours after rDNA breaks induction by Cas9, there are many small foci in the interior which split into few larger NBS1 foci one hour later and at the time point 6 hours, NBS1 is enriched in the NCs (Korsholm *et al.,* 2019). The dynamics of TCOF1 after rDNA breaks is similar. Simultaneously, upon rDNA breaks induction, the irregular shape of nucleoli changed to round, and 2 hours after I-PpoI mRNA transfection, most of the nucleolar DNA was segregated (Mooser *et al.,* 2020).

TCOF1 depletion results in lower survival in response to rDNA damage (Ciccia *et al.,* 2014). GFP-labelled U2OS cells were treated with two independent TCOF1 siRNAs, mixed with non-labelled parental U2OS cells. 7 days after irradiation or treatment with cisplatin, the proportion of GFP/non-labelled cells was analysed by flow cytometry. Cells with depleted TCOF1 were more sensitive to DNA damage agents.

4.3. TCOF1 structure with respect to the interaction with NBS1

TCOF1 structure resembles the DDR protein MDC1, these proteins share homology in the N-terminal SDT phosphodomain. The structure of both TCOF1 and MDC1 is illustrated in **Fig. 8**. TCOF1 consists of three structurally distinct regions: an N-terminal region that contains SQ and TQ sites required for NBS1 interaction, a central region consisting of 10 clustered acidic and serine-rich sequence stretches called treacle repeats and a C-terminal region comprising nuclear and nucleolar localization signals (Wise *et al.,* 1997 and Winokur *et al.,* 1998). The N-terminal segment made up by of three motifs resembling SDT motifs in MDC1. The first motif, SETE, is made up of a serine residue accompanied by a glutamic acid and threonine, bordered by three consecutive glutamic acid residues. Secondly, SEDT and SDET motifs are characteristic by the presence of a serine residue followed by two acidic amino acids and a threonine residue (Larsen *et al.,* 2014). The N-terminal segment harbours NoLS and another NLS.

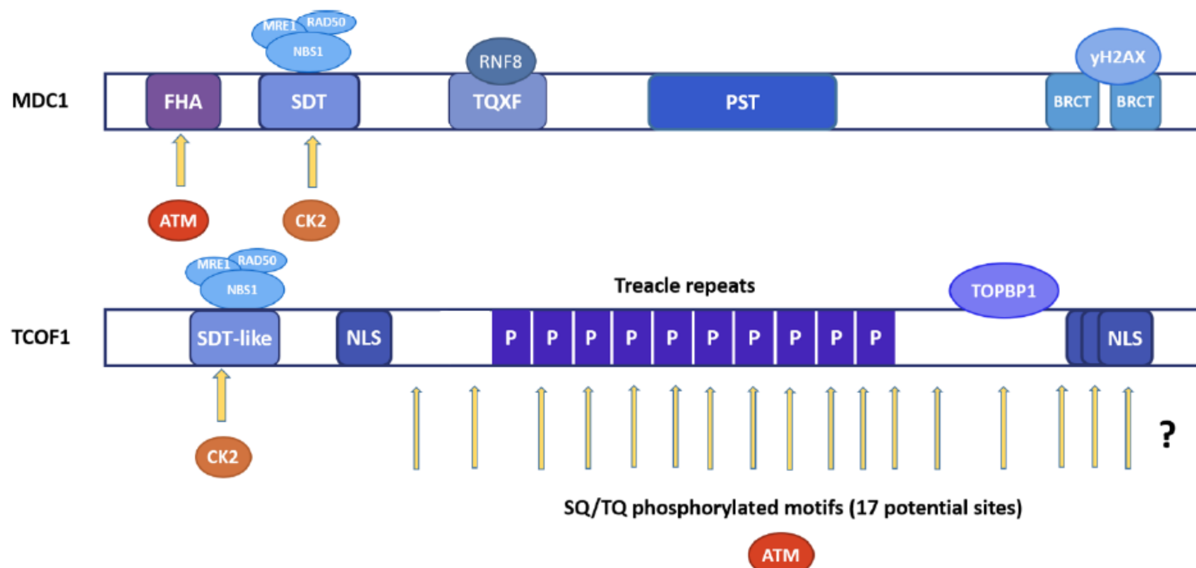

Figure 8*. Structural and functional comparison of MDC1 and TCOF1. Both proteins are constantly phosphorylated by CK2 and, in response to DSBs, ATM. The main difference is the localization of the proteins, MDC1 operates in the nucleus where it localizes to the site of DSBs through binding γH2AX. MDC1 interacts with the MRN complex via NBS1 FHA domain. Apart from that, it binds RNF8 through TQXF motifs. TCOF1 is a nucleolar protein binding NBS1 and TOPBP1 upon rDNA breaks (Mooser et al., 2020). TCOF1 comprises multiple NLS and NoLS sequences, NoLS sequences are located within C-terminal NLS. TCOF1 is phosphorylated by CK2 and ATM as well, but the key ATM site is not clear. MDC1 NLS situated within two BRCT domains is*

The structure of TCOF1 has been greatly studied in the context of NBS1 nucleolar recruitment. In one study, individual TCOF1 domains were gradually deleted which helped to analyse the key NBS1 interaction site. This important region is situated within the N-terminal 225 amino acids of TCOF1 with a potential minor contribution of the C-terminal region (Larsen *et al.*, 2014).

Concerning NBS1, the key regions for the interaction with TCOF1 are FHA and BRCT domains located at the N-terminus. These motifs form a supramodular phosphobinding surface (Williams *et al.*, 2009 and Lloyd *et al.*, 2009). Introducing point mutations in the N-terminal FHA and BRCT domains of NBS1 causes abrogated NBS1 nucleolar localization in response to rDNA breaks (Mooser *et al.*, 2020). This

observation confirms that the direct interaction between NBS1 FHA domain and TCOF1 is crucial for NBS1 nucleolar localization. The recruitment occurs not only upon nucleolar DSBs but also after damage elsewhere in the genome which transiently inhibits rRNA transcription (Larsen *et al.,* 2014).

4.4. The effect of phosphorylation on the interaction of TCOF1 with NBS1

The acidic region of MDC1 SDT domain is constitutively phosphorylated by CK2 and the phosphorylation is necessary for the interaction with NBS1 FHA domain (Ciccia *et al.,* 2014). Similarly, TCOF1 is phosphorylated by CK2 (Larsen *et al.,* 2014 and Ciccia *et al.,* 2014) and ATM (Isaac *et al.,* 2000). The CK2 phosphorylation primes TCOF1 for the phosphorylation by ATM activated in response to rDNA DSBs, the interaction between TCOF1 and NBS1 is CK2- and ATM-dependent (Ciccia *et al.,* 2014 and Larsen *et al.,* 2014).

TCOF1 protein sequence possesses many potential phosphorylation sites, especially 10 greatly repetitive regions called treacle repeats with sites recognized by CK2 (Jones *et al.,* 1999 and Wise *et al.,* 1997) and the interaction of TCOF1 with CK2 was confirmed (Isaac *et al.,* 2000). Bacterially purified TCOF1 fragments were *in vitro* massively phosphorylated by CK2, especially within the repeat region. To examine whether the interaction between TCOF1 and NBS1 is CK2-dependent, GST-tagged TCOF1 NBS1-interacting region was incubated with CK2 kinase and subsequently, added to untreated HeLa nuclear extracts. The fragment incubated with CK2 pulled down the MRN complex while the non-treated control fragment did not (Larsen *et al.,* 2014). On that account, it was deduced that CK2 pre-phosphorylation was essential for the interaction between TCOF1 and NBS1. On the other hand, it is interesting that only CK2 phosphorylation enabled the TCOF1-NBS1 interaction which, among other, relies on the phosphorylation by ATM (Isaac *et al.,* 2000). This observation originated from *in vitro* assay and it is known that proteins can interact less specifically *in vitro* than *in vivo* and sites which are not phosphorylated *in vivo* can be recognized by the kinase *in vitro*.

TCOF1 was validated as a CK2 substrate by another study. To give a further illustration on the impact of CK2 activity on TCOF1 function, CK2 subunits α and α′ were depleted. In these cells, NBS1 IR-induced translocation into the nucleolus was heavily impaired (Ciccia *et al.,* 2014).

In addition to CK2, the role of ATM was studied in detail as well because TCOF1 is an ATM substrate (Isaac *et al.,* 2000). Cas9-induced DSBs in rDNA lead to ATM activation (Korsholm *et al.,* 2019). ATM-dependent phosphorylation of TCOF1 is required for the MRN complex recruitment and subsequently, for rRNA transcription inhibition and translocation of rDNA breaks to the nucleolar periphery. U2OS cells treated with the ATM inhibitor KU-55933 showed abrogated NBS1 recruitment into the nucleolus after IR while TCOF1 formed typical nucleolar foci (Ciccia *et al.,* 2014). The same result was obtained after treating cells with KU-55933 and induction of rDNA damage by Cas9 cleavage in rDNA or I-PpoI

approach, the cells in both studies exhibited abrogated NBS1 foci formation (Korsholm *et al.,* 2019 and Mooser *et al.,* 2020). This means that ATM activity is important for NBS1 nucleolar recruitment via TCOF1 phosphorylation which does not affect the TCOF1 localization itself.

ATM and ATR kinases phosphorylate SQ or TQ motifs in their substrates (Ciccia *et al.,* 2010). TCOF1 comprises approximately 17 SQ/TQ sites, dispersed between 189 and 1473 amino acids (Korsholm *et al.,* 2019), the recognition of phosphorylated site could be mediated by NBS1 BRCT motifs which are known to bind ATM or ATR substrates (Ciccia *et al.,* 2014 and Manke *et al.,* 2003). However, it is not clear until now which one is the key residuum. Firstly, it was suggested that S1410 matched the SQ phosphorylation site recognized by ATM/ATR kinases (Matsuoka *et al.,* 2007). In another study, the serine in this sequence was mutated to alanine to confirm the ATM phosphorylation site. Nonetheless, when the experiment was independently repeated, proficient NBS1 translocation into the nucleolus upon nucleolar DSBs was observed (Ciccia *et al.,* 2014).

For this reason, other potential evolutionarily conserved SQ sites were examined, and the serines, specifically residues S1199 and S1216 were mutated to alanines as well. The first mutant cell line (S1199A) failed in NBS1 nucleolar recruitment in response to irradiation while the dynamics of NBS1 remained unchanged in the second mutant cell line (S1216A). This experiment suggested a possible role of ATM phosphorylation site S1199 and its effect on NBS1 recruitment to the rDNA damage sites (Ciccia *et al.,* 2014). However, mutation of this residue still did not fully abrogate NBS1 recruitment either in another study (Korsholm *et al.,* 2019). For that reason, 17 SQ/TQ sites were all mutated to alanines, establishing an ATM-null mutant, failing in NBS1 nucleolar recruitment. Unfortunately, the authors did not more specify the key residuum for the phosphorylation by ATM. Whether the previously selected sites (Matsuoka *et al.,* 2007, Ciccia *et al.,* 2014 and Korsholm *et al.,* 2019) are really responsible for ATM-dependent NBS1 recruitment or not, remains a question for further research. It is certain that mutation of all 17 sites could heavily impair TCOF1 functions, so this model may not be appropriate. Another point is that these sites could be redundant.

4.5. Nucleolar recruitment of MRE11 and RAD50

Whether the remaining components of the MRN complex, MRE11 and RAD50, are recruited after rDNA DSBs induction as well, was considered a question, and various authors stated conflicting views. First, there was an accepted opinion that MRE11 did not translocate into the nucleolus in response to irradiation (Ciccia *et al.,* 2014 and Larsen *et al.,* 2014). These findings originated from independent experiments with HA- or GFP-tagged exogenous MRE11. Following micro-irradiation, exogenous MRE11 was not recruited into the nucleolus. It was proposed that there was a possibility that the NBS1 role in the nucleolus was MRE11-independent (Larsen *et al.,* 2014). Another work analysed the

localization of the MRN complex and ATM and concluded that ATM was present both in the nucleoplasm and nucleolus while proteins of the MRN complex were excluded from the nucleolus. Nevertheless, the data were obtained from untreated samples of the human brain (Gorodetsky *et al.,* 2007). The most recent studies demonstrated the participation of MRE11 in rDNA DSBs resection (Korsholm *et al.,* 2019 and Mooser *et al.,* 2020).

The opposite observation of Ciccia *et al.,* 2014 and Larsen *et al.,* 2014 could be given by their experimental setup. The authors worked with HA or GFP-tagged MRE11 and did not provide exact information at which end the tag was located. It seems that the tags were situated at the N-terminus. Importantly, MRE11 binds NBS1 via its N-terminus, so the tag might have affected the interaction. Recently, the staining of endogenous MRE11 was repeated and MRE11 nucleolar localization upon rDNA damage was detected although MRE11 foci were less prominent than NBS1 foci (Korsholm *et al.,* 2019).

Despite some discrepancies in past years, the current knowledge emphasises the significant role of the entire MRN complex in the n-DDR processes. In early time points after rDNA breaks induction by Cas9 endonuclease, MRE11 foci are formed inside the nucleolus and co-localizes with NBS1. Later on, these foci are visible on the nucleolar periphery in the NCs (Korsholm *et al.,* 2019). RAD50 was confirmed to be recruited together with NBS1 and MRE11 into the nucleolus, but its specific nucleolar functions have not been elucidated. At least, RAD50 interacts with NCL (Goldstein *et al.,* 2013).

Interestingly, RAD50-interacting protein 1 (RINT-1) is recruited to rDNA loci upon rDNA damage induction and co-localizes with nucleolar transcription regulator MSP58 (Shimono *et al.,* 2005 and Lin *et al.,* 2002), RNA Pol I transcription factor UBF (Yang *et al.,* 2016) and nucleolar transporter NOPP140 (Campos-León *et al.,* 2017). Based on several observations, RINT-1 and MSP58 synergistically regulate rRNA transcription. If RINT-1 and MSP58 are depleted, rRNA transcription is increased while overexpression of both these two proteins leads to silenced transcription. Overexpression of either protein alone yields weaker transcription inhibition (Yang *et al.,* 2016). After ActD treatment, RINT-1, MSP58 and UBF form overlapping foci in the NCs. Whether the function of RINT-1 in the nucleolus is somehow connected with the nucleolar activity of RAD50 is not known and further research is needed.

4.6. Nucleolar phenotype of NBS1-deficient cells

There have been a number of attempts to generate NBS1 knockout cells. NBS1 regulates developmental processes, so NBS1 knockout mouse is not viable (Zhu *et al.,* 2001). One of the first NBS1-deficient cell models was established in hyper-recombinogenic chicken B-cell line DT40 by targeting exon 4 in all three alleles in these cells (Tauchi *et al.,* 2002). The knockout cells were viable,

but the cell growth was slowed down, and the cells were hypersensitive to irradiation which corresponds to the phenotype of cells from NBS patients.

For confirmation, the authors performed RT-PCR analysis and revealed NBS1 transcripts beginning from exon 7 in NBS1 knockout cells. Unfortunately, the information was just mentioned and not supported by data. However, subsequent immunoblot analysis detected no NBS1 truncated variants, so the authors considered the cells as NBS1 knockout cell line.

Their findings are quite surprising because transcription generally starts from the promotor. Furthermore, according to the current knowledge, the region surrounding exon 7 does not harbour an alternative site for RNA Pol II. The explanation could be alternative splicing, such as exon skipping. Alternative splicing was documented in NBS patients and results in milder phenotype of NBS syndrome (Varon *et al.,* 2006). For instance, patients with mutation in exon 7 of NBS1 express mRNA with excised exons 6 and 7 (see **Fig. 9C**). The authors did not describe the way how the transcript beginning from exon 7 was identified, whether using sequencing or specific primer pairs. If they analysed the transcripts using amplification of selected regions with specific primer pairs, they might have not detected mRNA rearrangements. Additionally, *NBS1* genomic sequence comprises many in-frame ATG codons. It means that besides translation of the protein with premature stop codon beginning from exon 1, ribosome can skip and start translation from different start codon (Maser et al., 2001 and Seemanová *et al.,* 2006). The absence of the C-terminal region, which means the absence of the whole MRN complex in the nucleus, seems to be for the cell so deleterious that the deficiency is partially rescued by expression of the truncated C-terminal fragment. The probability of this skipping seems to be low which corresponds to weaker expression of the truncated protein (Salewsky *et al.,* 2016). What is more, we are not able to detect this alternative translation start by mRNA analysis. The method can be used to examine alternative splicing or to confirm the genomic mutation in the transcript.

Expression of these truncated NBS1 variants was described in patients suffering from NBS syndrome. For example, the truncated NBS1 variant was detected in human fibroblasts carrying 657del5 hypomorphic mutation in exon 6 of NBS1 (NBS1; OMIM #2512609). 657del5, also known as Slavic mutation, is identified in 90% of patients and associated with the Central and Eastern Europe (Varon *et al.,* 2000). These cells express the p26 and p70 NBS1 fragments, corresponding to the molecular weight of the fragments which have 26 kDa and 70 kDa, respectively (Matsuura *et al.,* 1998 and Varon *et al.,* 1998), as is depicted in **Fig. 9B**.

On the contrary to RT-PCR, the truncated protein can be detected by immunoblot analysis. The authors did not observe NBS1 in cell lysates. For clarification, the antibody was raised against the tagged fragment comprising amino acids 607-753 of NBS1, referring to the C-terminal region of NBS1.

It is possible that the authors exposed the blot for a short time. It was documented (see **Fig. 9C**) that the longest possible exposure time is needed for the detection of the truncated variant in NBS1-deficient cells because the protein is less expressed than full-length NBS1 in parental cells (Varon *et al.*, 2006 and Salewsky *et al.*, 2016).

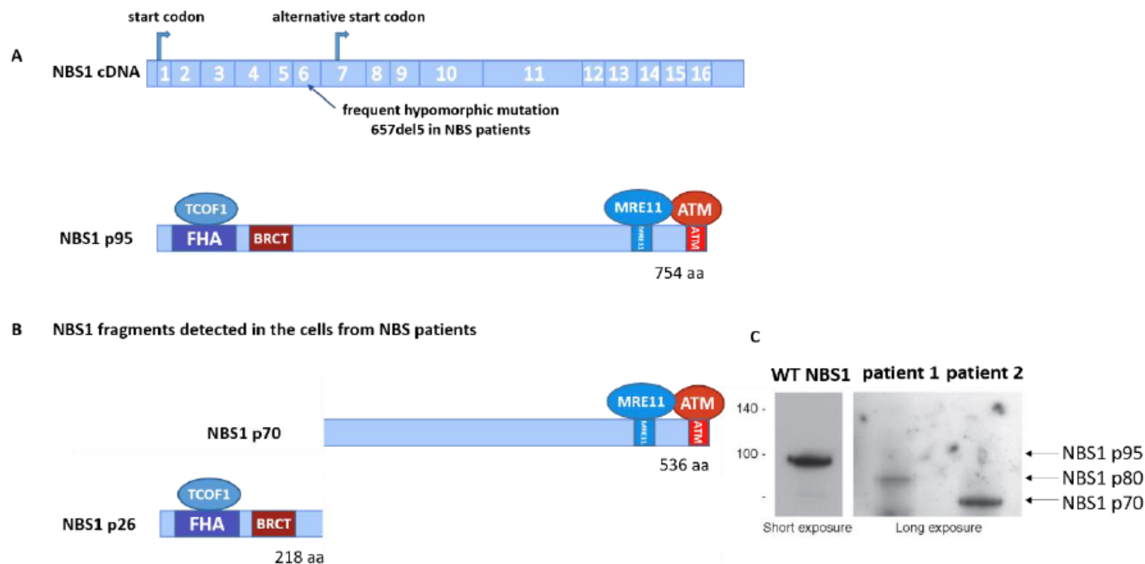

Figure 9*. NBS1 structure and expression in NBS patients. A – the main domains and binding motifs in the NBS1 protein together with its relevant n-DDR interactors and NBS1 cDNA with illustrated frequent mutation site in NBS and alternative translation start. B – NBS1 fragments detected in NBS patients. C – immunoblot of NBS1 in NBS patients in contrast with healthy control. After longer exposure time, NBS1 fragments, such as p70 or p80, are visible (Salewsky et al., 2016). WT NBS1 – parental SV40 immortalized cells (NBS1+/+), patient 1 – homozygous for NBS1 c.742-743insGG, patient 2 – homozygous for NBS1 c.657_661del5. NBS1 variant from patient 1 is an example of alternative splicing in NBS cells. In these cells, exons 6 and 7 are excised, resulting in the p80 fragment. Cells from patient 2 express p70 variant, p26 is not shown.*

For the examination of NBS1-dependent nucleolar phenotype, there was an attempt to establish NBS1 knockout cell line. Neither MRE11 nor RAD50 knockouts were used, mutations in MRE11 and RAD50 are lethal in vertebrates (Maser *et al.*, 2001). Exon 2 of NBS1 in U2OS cells was targeted using CRISPR/Cas9 technology. Similar to cells from NBS patients, this knockout cell line was more sensitive to IR, MRE11 was predominantly localized in the cytoplasm and upon DNA damage induction, and the cells had defects in ATR activation, G2/M checkpoint activation and DSBs resection (Mooser *et al.*, 2020).

Notwithstanding, as was illustrated in the very last paragraphs, the generation of the NBS1 knockout cell line can be really challenging. NBS1 C-terminus, necessary for cell viability, ATM activation, MRE11 binding and translocation of the whole MRN complex into the nucleus (Kim *et al.*, 2017), was suspected to be expressed in these generated NBS1 knockout cells. Subsequently, overexpression of NBS1 mRNA was detected in these cells. The cell line express low levels of a ~40 kDa protein that responses to NBS1 mRNA siRNA-mediated knockdown (Mooser *et al.*, 2020). The molecular weight does not correspond to the two fragments observed in cells from NBS patients, p26 and p70, probably because of alternative splicing. Regardless of the expression of NBS1 C-terminal fragment, the authors

decided to continue in the experimental work with this cell line (named as NBS1ΔN) because the N-terminal region harbouring phospho-binding domain FHA crucial for NBS1-TCOF1 interaction is presumably missing.

Concerning the nucleolar phenotype of cells lacking the N-terminal region of the protein, NBS1ΔN cells were hypersensitive to I-PpoI transfection in comparison to parental U2OS cells (Mooser *et al.,* 2020). Still, it is not possible to clearly state whether this decrease in cell survival is clearly caused by defective rDNA breaks repair because it is not ruled out that I-PpoI recognizes some non-nucleolar sites, the human genome contains 13 I-PpoI sites in non-nucleolar DNA (Muscarella *et al.,* 1990).

4.7. Role of the MRN complex in nucleolar DSBs repair

The MRN complex participation in the repair of rDNA breaks was a research question solved by few authors. Endo/exonuclease MRE11, involved in both HR and NHEJ, is necessary for rDNA breaks resection (Mooser *et al.,* 2020). In NBS1ΔN cells, no RPA foci were observed within the first 2 hours following I-PpoI mRNA transfection, and RPA phospho-serine 4/8 was also strongly reduced. The same phenotype came in U2OS cells treated with MRE11 inhibitor Mirin. Depletion of MRE11 results in declined accumulation of RPA2 phospho-serine 33, depletion of BLM or DNA2 yields in milder phenotype (Mooser *et al.,* 2020). MRE11-deficient cells have in response to rDNA DSBs more abnormalities in nuclear morphology, such as micronuclei, and the portion of apoptotic cells and dead cells is increased (Korsholm *et al.,* 2019). On the other hand, the presented data were not convincing, the difference between MRE11-depleted and parental cells was at the level of tenths of a percent.

MRE11-interacting partner CtIP was not identified in the nucleolar interior, but in the NCs where it co-localizes with NBS1 (Korsholm *et al.,* 2019). Following CtIP depletion, NBS1 forms foci in the nucleolar interior but it is not accumulated in the NCs, indicating that CtIP processing of rDNA DSBs following successful translocation of interior breaks to the nucleolar periphery promotes NBS1 association with the NCs. In addition, 53BP1 and BRCA1 are not recruited to the NCs in CtIP-depleted cells. The authors suggested that CtIP-facilitated resection predominantly occurred after formation of the NCs which allowed recognition of breaks mediated by HR factors, but the resection mediated by CtIP did not affect the initial steps preceding cap formation. Based on the presented data, it can mean that CtIP is recruited from the nucleoplasm to the NCs. The recruitment could be facilitated by the non-nucleolar MRN complex, in the NCs, there are rDNA breaks pre-resected by MRE11. If rDNA DSBs are then resected by CtIP, NBS1-dependent formation of the NCs takes place and the HR-associated factors accumulate in the NCs. However, this observation of CtIP in the NCs and its role in rDNA DSBs resection should be more elucidated. What is missing in the figures provided by the authors is co-staining of rDNA to show whether the rDNA breaks are segregated in CtIP-depleted cells.

4.8. Role of the MRN complex in RNA polymerase I inhibition

The MRN complex is indispensable for transcriptional silencing, but the exact mechanism is still unclear. Nucleolar transcriptional inhibition is usually quantified using qPCR or uridine analogue 5-ethynyluridine (5-EU). 5-EU is incorporated into nascent RNA within the cell nucleus, including mRNA, rRNA, tRNA, etc. Synthetized RNA with incorporated 5-EU is then visualized by Click-iT reaction. For specific gating of nucleolar transcription, cells are co-stained by some nucleolar markers, like fibrillarin (Jao *et al.,* 2008). In past years it was demonstrated that inhibition of rRNA synthesis is ATM- and NBS1-dependent. rRNA transcription in mouse fibroblast carrying NBS1 657del5 is radio-resistant (Kruhlak *et al.,* 2007). In response to rDNA breaks, the transcription inhibition in the nucleolus is only partial at the time when NBS1 foci are formed and comes with delayed kinetics in contrast to NBS1 accumulation. Inhibition of transcription is impaired in NBS1-depleted cells (Korsholm *et al.,* 2019). After rDNA breaks induction, MRE11-depleted cells exhibit only slightly reduced rRNA transcription in contrast with MRE11-proficient cells (Korsholm *et al.,* 2019 and Mooser *et al.,* 2020). Lastly, overexpression of NBS1-GFP construct with NoLS leads to the inhibition of rRNA synthesis even without DNA damage (Larsen *et al.,* 2014). Based on these observations, it is likely that the transcription inhibition relies on the formation of NBS1 foci upon rDNA breaks, and with it the recruitment of the entire MRN complex.

TCOF1 mutation or depletion lead to significant decrease in rRNA synthesis (Mooser *et al.,* 2020). There are no prominent differences between non-irradiated cells with depleted TCOF1, irradiated parental cell and irradiated cells after TCOF1 depletion, all conditions result in a decline by about 40% (Larsen *et al.,* 2014). This observation might emphasise the key role of TCOF1 which is considered as an important factor for RNA Pol I and at the same time, it recruits the MRN complex in response to rDNA DSBs.

One of the latest studies (Mooser *et al.,* 2020) suggests that RNA Pol I shutdown is both ATM- and ATR-dependent. Because of generating ssDNA coated by RPA, the MRN complex has been proposed to operate upstream of ATR activation in response to rDNA DSBs (Jazayeri *et al.,* 2006, Myers *et al.,* 2006 and Adams *et al.,* 2006). TCOF1 was observed to recruit ATR activator TOPBP1 into the nucleolus in ATM- and NBS1-dependent manner. Number of TOPBP1 foci was prominently reduced in NBS1ΔN cells (Mooser *et al.,* 2020). The phenotype was rescued by expression of the wild-type NBS1 but not FHA/BRCT mutated NBS1. The nucleolar recruitment of TOPBP1 is facilitated by direct phosphorylation-dependent interactions with the C-terminal region of TCOF1. The authors proposed that nucleolar-specific adapter TCOF1 orchestrated the nucleolar response to rDNA breaks through critical interactions with two DDR factors, NBS1 and TOPBP1 in the first place. Through these

interactions, ATM is activated, and ATR recruited into the nucleolus where both kinases inhibit RNA Pol I. To support this idea, the downregulation of TOPBP1 expression by siRNA results in unstopped rRNA synthesis upon I-PpoI induction. NBS1ΔN cells exhibit similar abrogation of transcriptional inhibition.

The view of ATR activation has been challenged by recent data showing that ATR acts upstream of nucleolar resection. Upon ATR inhibition, RPA2 foci are not formed. However, the resection-independent mechanism of ATR recruitment or activation was not explained (Mooser *et al.*, 2020). On the other hand, the authors observed abrogated MRN recruitment upon ATR inhibition and impaired resection in NBS1ΔN or MRE11-depleted cells. This could mean that both ATM and ATR may cooperate on MRN recruitment. The compounds of the MRN complex promote end resection which, in positive feedback, supports further ATR recruitment via TCOF1-TOPBP1.

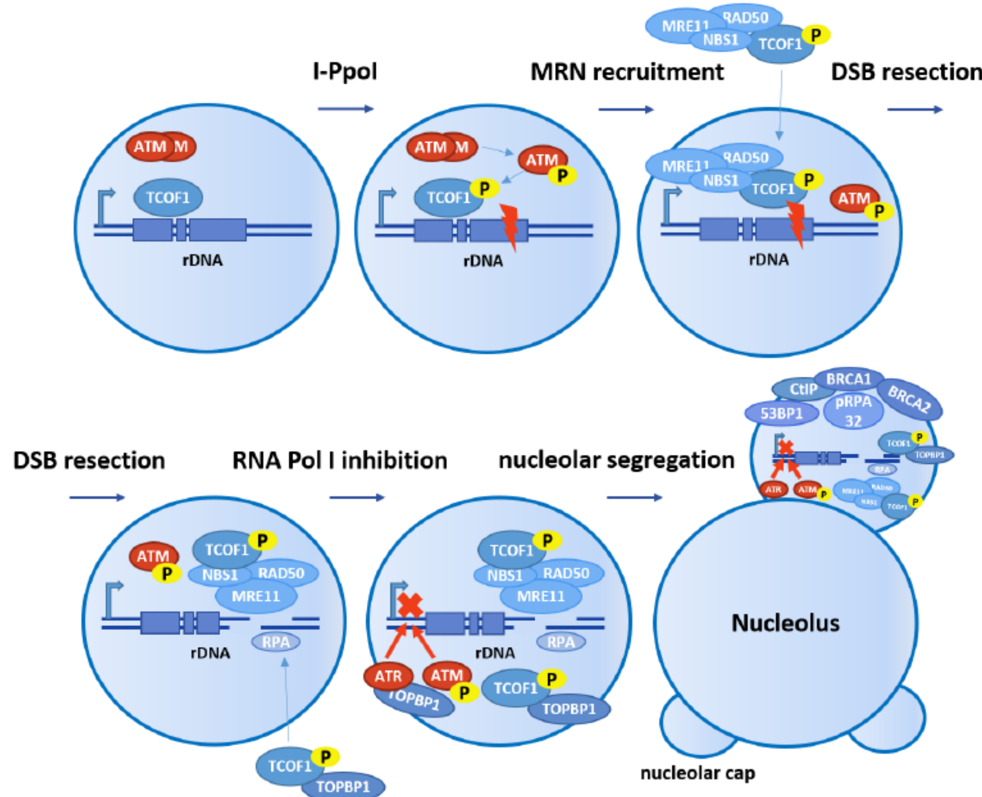

Figure 10. *Steps of the n-DDR. ATM serine/threonine kinase plays an indispensable role in DNA damage signalling and repair. After its dimerization and activation, ATM phosphorylates a number of its various substrates. One of these targets is TCOF1, an example of adaption of the n-DDR to a specific chromatin landscape and a physical environment excluding DDR proteins like MDC1. TCOF1 recruits the MRN complex promoting resection and is required for ATR recruitment facilitated by TOPBP1 recruitment. ATM and ATR orchestrate RNA Pol I shutdown and nucleolar segregation into the NCs.*

4.9. Role of the MRN complex in nucleolar segregation

NBS1 is involved in the DSBs recognition at the nucleolar periphery where it supports the formation of the NCs (Franek *et al.*, 2016). Here, NBS1 colocalizes with MDC1 and UBF. The formation of the NCs is MRN-dependent. In NBS1-depleted cells, the segregation of rDNA into the NCs is impaired and rDNA remains in the nucleolar interior, similarly in MRE11- (Korsholm *et al.*, 2019) and TCOF1-depleted cells

(Mooser *et al.,* 2020). Interestingly, NBS1 is more accumulated in the nucleolus in MRE11-depleted cells exposed to Cas9-induced rDNA breaks (Korsholm *et al.,* 2019).

Initially, NBS1 nucleolar localization into the NCs was studied by establishing cell lines stably expressing NBS1-GFP construct (Franek *et al.,* 2016). After transient transfection of plasmid carrying HA-tagged I-PpoI endonuclease, NBS1 recruitment to the NCs was clearly shown. On the other hand, some disadvantages of this expression system were manifested. The fusion protein localized to the nucleolus even without DNA damage which was really surprising. In this study, it was suggested that NBS1 could have another function in addition to its role in DNA damage sensing, repair and signalling. However, after revising the plasmid sequence, it seems that this observation could be caused by an artefact. There is a questionable basic KRRR amino acid sequence at the very C-terminus in NBS1 protein (see **Fig. 7**) and two additional LR basic residues in the linker sequence which might generate a NoLS. To support this explanation, it was evidenced that only four basic arginine tandem residues can result in a novel NoLS provided that the protein possesses NLS (Martin *et al.,* 2015). Thus, it is likely that the additional lysine and arginine residues in the linker sequence could have an impact on the protein localization.

Nevertheless, the fusion protein responded to the I-PoI treatment by accumulation in the NCs and then, it was documented that endogenous NBS1 is also recruited to the NCs 8 hours after transfection (Franek *et al.,* 2016). Overall, it would be reasonable to avoid using unnecessary linker sequences and study the localization of the endogenous variant of the protein as well to avoid some misinterpretations.

Another strategy for studying the impact of NBS1 on nucleolar segregation was the establishment of NBS1 N-terminal knockout cell line and transfection of I-PpoI mRNA (Mooser *et al.,* 2020). Following I-PpoI mRNA transfection, no NCs were detectable in NBS1ΔN cells within the first 2 hours after transfection and the decrease of RNA Pol I activity was only slightly reduced in comparison with the parental U2OS cells. In contrast with Franek *et al.,* 2016, earlier time points were used. The reason is that mRNA transfection is faster in rDNA DSBs induction because mRNA does not have be transcribed as plasmid sequence does. The phenotype was rescued by GFP-NBS1 transfection, the GFP tag was situated at the C-terminus in order not to impair NBS1-TCOF1 interaction but still, it is a question if this C-terminal GFP localization could affect MRE11 and ATM binding.

Nowadays, the attention is focused on the connection between the nucleolar activity of ATR and the MRN complex in context of nucleolar reorganization. The two-step kinase mechanism is indispensable for full rDNA segregation after DSBs (Korsholm *et al.,* 2019). ATM or ATR inhibition causes impaired segregation. Overexpression of TOPBP1 results in nucleolar segregation and ATR enrichment in the

NCs (Sokka *et al.,* 2015 and Mooser *et al.,* 2020). Depletion of either TCOF1 or TOPBP1 prior to rDNA damage induced noticeable decrease of γH2AX in the nucleolar periphery. These cells also failed to recruit RPA and HR factors like BRCA1 and RAD51. What is more, in TCOF1- or TOPBP1-depelted cells, there was a prominent decline in cell survival after I-PpoI mRNA transfection in comparison to parental U2OS cells (Mooser *et al.,* 2020).

4.10. Role of chromatin modifiers in the n-DDR

Many interesting interactions between nucleolar chromatin modifiers and the MRN complex or TCOF1 have been described. For instance, RAD50 interacts with NCL, nucleolar protein with histone chaperone activity, and recruits it to the sites of DNA damage where NCL removes histones H2A and H2B from the nucleosome (Goldstein *et al.,* 2013). The MRN complex interacts with cohesin and ATM-mediated phosphorylation of cohesin is NBS1-dependent (Yazdi *et al.,* 2002). Additionally, one of the latest studies indicate that the MRN recruitment by TCOF1 relies on histone demethylase JMJD6. The interaction between TCOF1 and JMJD6 upon rDNA damage was identified by MS and confirmed by co-immunoprecipitation and Proximity Ligation Assay (PLA) detecting co-localizing and interacting proteins at endogenous protein level. JMJD6 depletion leads to impaired NCs formation. (Fages *et al.,* 2020).

TCOF1 interacts with histone deacetylase 1, HDAC1, a potential critical factor in mediating rRNA transcription silencing and nucleolar DNA repair (Franek *et al.,* 2016). HDAC1 was identified as a compound of the nucleolar remodelling complex (Zhou *et al.,* 2002). Subsequently, it was evidenced that HDAC1 was important for transcriptional silencing at the rRNA gene locus (Santoro *et al.,* 2005). In response to I-PpoI induction, histone acetylation seems to be absent in the NCs. After micro-irradiation, HDAC1 was observed directly at the sites of rDNA damage (Miller *et al.,* 2010) and promoted NHEJ. Inhibition of histone deacetylase impairs aggregation of 53BP1 and BRCA1 at the site of DNA damage (Fukuda *et al.,* 2015).

Nucleolar chromatin modifications are different from the canonical modifications (Korsholm *et al.,* 2019 and Kutashev et al., 2020). Histone modifications are altered in the NCs and show different distributions relative to UBF (Franek *et al.,* 2016). Along with HDAC1, CARM1 is another factor involved in nucleolar chromatin remodelling which interacts with TCOF1 during the repair of rDNA DSBs (Franek *et al.,* 2016). Upon CARM1 inhibition by elagic acid, the size of the nucleoli becomes reduced. CARM1-mediated H3R17me2 was discovered co-localizing with UBF foci in the NCs. H3R17me2 interacts with RNA polymerase-associated factor 1 (Wu *et al.,* 2012). Interestingly, according to data obtained from ChIP, the level of H3R17me2 did not increase after DNA damage is common in the nucleolus, suggesting that H3R17me2 reorganizes with damaged rDNA repeats bound by UBF to the periphery.

TCOF1 possibly interacts with one of heterochromatin protein 1 (HP1) isoforms. HP1 is recruited to the site of DNA damage where it binds H3K9me2/3, a repressive histone mark (Ayoub *et al.,* 2007 and Luijsterburg *et al.,* 2009). MS analysis revealed TCOF1 interaction with HP1β (Zaidan *et al.,* 2018) which may explain the neuronal defects in the HP1β knockout mice because Treacher Collins syndrome affects neural crest development (Valdez *et al.,* 2004). Apart from HP1β foci at the nucleolar periphery, chromatin precipitation PCR revealed HP1β occupancy on rDNA genes (Horakova *et al.,* 2010).

There was not evidenced any recruitment of HP1 isoforms to UBF-positive NCs after I-PpoI induction (Franek *et al.,* 2016). Nevertheless, it is possible that this recruitment is triggered by different kind of DNA damage. HP1β and UBF protein are recruited to the nucleolar periphery in response to cyclobutane pyrimidine dimers (CPDs) induced by UV micro-irradiation (Stixova *et al.,* 2014). Recent studies documented that γ-radiation resulted in change of HP1 distribution pattern. A number of HP1 isoforms foci in the vicinity of the nucleolus is decreased in response to IR (Legartova *et al.,* 2019). Moreover, the level of HP1α in ribosomal genes was diminished after γ-radiation as well. Interestingly, nucleoli are decorated by all HP1 isoforms, but both HP1β and HP1γ foci at the nucleolar periphery do not co-localize with 53BP1 and the KRAB-associated protein (KAP1), a universal transcriptional co-repressor. These findings indicate specific functions of HP1 isoforms which need to be thoroughly investigated.

Another way proposed to achieve transcriptional silencing of nucleolar chromatin is the phosphorylation of histone H2B on serine 14 by MST2. The kinase is activated by the ATM-RASSF1A axis (Pefani *et al.,* 2018). The authors suggested that H2BS14p is a mark for nucleolar DNA damage whose specificity could be explained by high presence of MST2 kinase in the nucleolus. Nonetheless, an attentive reader could take notice of questionable specificity of anti-H2BS14p antibody. The signal of the antibody was localized throughout the cell, with some prominent foci co-localizing with NCL. Because histones are vital proteins for the cell, it is not possible to validate the antibody by siRNA/Cas9 targeting of the histone. Alternatively, it is possible to delete or deplete MST2 which facilitates the phosphorylation of H2B. After irradiation, the signal of pH2BS14 should not increase and this was shown in the study. Despite this validation, the experiments should be repeated, ideally with more convincing antibody, to consider pH2BS14 a marker of silenced nucleolar chromatin.

For the time being, these findings can only be considered as suggestions and need to be examined by other authors. Moreover, chromatin modifications are dynamic, and it is not trivial to study these processes. It has not been completely elucidated what have an impact on the state of chromatin and how the modifications are mediated. Despite everything, nucleolar chromatin modifications can be regarded as a fascinating issue.

5. Conclusion

The MRN complex, composed of MRE11, RAD50 and NBS1, has a pivotal role in DNA damage sensing, immediate repair, and signal transduction. In addition to its well-known functions, such as canonical DNA repair and DNA damage response, telomeres maintenance or regulation of the development (Syed *et al.,* 2018), it possesses some fascinating nucleolar activities which have not yet been completely covered by the research. These current findings open the floor for numerous questions.

It has been evidenced that the MRN complex localizes in the nucleolus upon rDNA damage induced by various agents, such as laser micro-irradiation and induction of I-PpoI-, AsiSI- or Cas9-mediated cleavage of rDNA repeats. For the reason that NBS1 and other members of the MRN complex do not have nucleolar localization signal, the translocation relies on a specific nucleolar transporter, the Treacher Collins syndrome protein TCOF1. Upon phosphorylation by ATM and CK2, TCOF1 interacts with the N-terminal FHA domain in NBS1 and recruits the MRN complex into the nucleolus.

In past years, there used to be an accepted opinion that upon rDNA damage, the nucleolar MRN complex fell apart and NBS1 alone was recruited by TCOF1 into the nucleolus while MRE11 with RAD50 remained in the nucleoplasm. Recently, this view has been challenged by several independent studies using different tools and experimental setups specifically inducing DSBs in rDNA. These studies demonstrate that the entire MRN complex is necessary for the n-DDR processes, including first steps of repair by NHEJ, RNA Pol I inhibition, nucleolar segregation and HR-mediated repair in the NCs, and all components of the MRN complex were detected in the nucleolus upon rDNA damage.

The research of the role of the MRN complex in the n-DDR provides a new insight into the process of rDNA breaks repair. As was thoroughly discussed, rDNA breaks are predominantly repaired by NHEJ in the nucleolar interior, if not efficient, rRNA transcription is abrogated, rDNA is segregated into the NCs where the repair strategy is switched to HR. The MRN complex is involved in canonical NHEJ and HR processes. It is not known whether the MRN complex participates in nucleolar NHEJ because it is very difficult to examine these processes, but it is obvious that the MRN complex is beneficial for NHEJ (Xie *et al.,* 2009)

According to the current view of nucleolar breaks repair, rDNA DSBs are processed by the MRN complex in the nucleolar interior. MRE11 produces ssDNA overhangs, the strands are coated with RPA which represents the activation signal for ATR. Through this resection, MRN and ATM induce TOPBP1 recruitment by TCOF1 and TOPBP1 recruits ATR in return. ATM and ATR then synergistically inhibit rRNA synthesis. The mechanism has not been completely elucidated yet, but it is known that factors of RNA Pol I belong to ATM substrates and are displaced from rDNA. Transcriptional inhibition induces

segregation of rDNA repeats into the NCs. The MRN complex is segregated with rDNA and nucleolar proteins as well. At the periphery, MRE11 interacts with CtIP in the NCs, and these proteins promote the critical steps of HR. The MRN complex is upstream of the NCs formation, if missing, the repair factors like BRCA1 or BRCA2 are not concentrated at the nucleolar periphery. These latest findings suggest that the MRN complex orchestrates all steps of the n-DDR. NBS1 is superior to all functions of the MRN complex in the nucleolus because the MRN complex is recruited through the interaction between NBS1 and TCOF1.

This is the current model of the n-DDR. However, the initial activation of ATM is still not clear. The MRN complex is indispensable for ATM activation. How is then nucleolar ATM activated provided that the recruitment of the MRN complex is ATM-dependent? Has the MRN complex its constant undetectable pool in the nucleolus? Or does CK2 participate in the initial activation of TCOF1? TCOF1 is constantly phosphorylated by CK2, but it comprises a lot of CK2 sites, many of them situated in SDT-like domain. Could be one of these residues the key for initial TCOF1 shuttling and the MRN complex recruitment? Only few MRN molecules can activate ATM. ATM might then in return phosphorylate TCOF1 and enhance the shuttling. This idea is consistent with several experimental findings. For instance, the N-terminal fragment of TCOF1 incubated with CK2 *in vitro* can pull down the MRN complex from cell lysates, even without ATM contribution. CK2 subunit CSNK2B was identified as a TCOF1 interactor by MS (Ciccia *et al.,* 2014). Furthermore, there is still no consensus about the crucial TCOF1 site phosphorylated by ATM on which the MRN recruitment relies. Finally, according to recent data, ATR might participate in MRN recruitment as well (Mooser *et al.,* 2020).

And there are another appearing questions. Is TCOF1 dephosphorylated when rDNA breaks are repaired? How is the MRN complex returned into the nucleoplasm? Does dephosphorylation of TCOF1 diminish the interaction with NBS1? Is the shuttling role of TCOF1 abrogated if the dephosphorylation is missing? There are some clues that the potential phosphatase dephosphorylating TCOF1 could be WIP1 which recognizes and dephosphorylates ATM substrates (Shreeram *et al.,* 2006). According to the NoLS predictor NoD (http://www.compbio.dundee.ac.uk/www-nod/), WIP1 possesses basic amino acid residues at its C-terminus which can create the NoLS. In one study (Kozakai *et al.,* 2016), the overexpression of WIP1 induces an increase in the number of nucleoli while WIP1 depletion reduces the number. The authors proposed that the nucleolar size relies on NPM1 through its activation by CDK1 and PLK1 which are upregulated by increased level of CDC25 via WIP1-dependent p53 downregulation. However, the explanation does not seem to be complete and there is open space for further research.

Many noteworthy roles of already known proteins in the n-DDR can be discovered, such as the surprising role of TOPBP1 and ATR in the RNA Pol I shutdown. Also, the role of CtIP in the n-DDR and rDNA DSBs resection needs to be investigated in more detail, especially the mechanism how CtIP is recruited to the NCs. The question remains how CtIP resection activity affects the formation of the NCs and recruitment of NBS1 or HR-associated repair factors into the NCs. In addition, there have been identified some interesting relationships between TCOF1 and the chromatin modifiers acting in the nucleolus, HDAC1, CARM1 or HP1. Whether these interactions rely on the presence of NBS1 has not been studied yet. In the nucleus, NBS1 has been demonstrated to be involved in the regulation of chromatin modifications. As an example, one of the NBS1 binding partners are the E3 ubiquitination ligase RNF20 modifying histone H2B and HP1 (Nakamura *et al.,* 2011 and Bosso *et al.,* 2019). Through these interactions, NBS1 can regulate the state of chromatin. Multiple studies have suggested that the relationship between the n-DDR and chromatin modifiers is relevant for rDNA breaks repair, but precise mechanistic description is missing.

Nucleolar defects, such as increased number and size of nucleoli, are related to poor prognosis in various types of tumours. However, the impact of defective nucleolar DDR on cellular physiology should be thoroughly investigated. The question is: what are the real consequences of nucleolar rDNA damage concerning cellular physiology? It should be pointed out on the fact that rDNA is only a fraction (almost 0.4%) of the genome, the amount of nucleolar damage is minimal in the genomic context. On the other hand, rRNA synthesis and ribosomal assembly are crucial for cell survival and human development as is demonstrated in case of Treacher Collins syndrome.

The recent attempts showing only slightly increased frequency of apoptotic cells or cells with micronuclei in response to rDNA damage and MRE11 depletion were not convincing. The same was one of the last observations that the n-DDR does not activate the cell cycle checkpoint through ATM-dependent activation of its substrates CHK2, CHK1 or KAP1. Neither these data were not conclusive because of comparison of cells treated two totally different DNA damage-inducing agents (Korsholm *et al.,* 2019).

Additionally, it would be really interesting to examine nucleolar phenotype in NBS patients. Does nucleolar instability affect the phenotype of NBS, or have only limited effects? Does alternative splicing of *NBS1* rescue nucleolar abnormalities?

To conclude, n-DDR processes, orchestrated by the MRN complex, are critical for the maintenance of genomic stability and ribosomal biogenesis with the potential to affect cellular physiology, and thus represent a fascinating field of research.

6. References

Image on the cover page https://cuni.cz/UK-6450-version1-znak_uk_cerna_barva.png

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